Proteomic Analysis of Mouse Cortex Postsynaptic Density following Neonatal Brain Hypoxia-Ischemia

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Keywords

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Abstract

Proteomics of the synapses and postsynaptic densities (PSDs) have provided a deep understanding of protein composition and signal networks in the adult brain, which underlie neuronal plasticity and neurodegenerative or psychiatric disorders. However, there is a paucity of knowledge about the architecture and organization of PSDs in the immature brain, and how it is modified by brain injury in an early developing stage. Mass spectrometry (MS)-based proteomic analysis was performed on PSDs prepared from cortices of postnatal day 9 naïve mice or pups which had suffered hypoxic-ischemic (HI) brain injury. 512 proteins of different functional groups were identified from PSDs collected 1 h after HI injury, among which 60 have not been reported previously. Seven newly identified proteins involved in neural development were highlighted. HI injury increased the yield of PSDs at early time points upon reperfusion, and multiple proteins were recruited into PSDs following the insult. Quan-

titative analysis was performed using spectral counting, and proteins whose relative expression was more than 50% up- or downregulated compared to the sham animals 1 h after HI insult were reported. Validation with Western blotting demonstrated changes in expression and phosphorylation of the N-methyl-D-aspartate receptor, activation of a series of postsynaptic protein kinases and dysregulation of scaffold and adaptor proteins in response to neonatal HI insult. This work, along with other recent studies of synaptic protein profiling in the immature brain, builds a foundation for future investigation on the molecular mechanisms underlying developing plasticity. Furthermore, it provides insights into the biochemical changes of PSDs following early brain hypoxia-ischemia, which is helpful for understanding not only the injury mechanisms, but also the process of repair or replenishment of neuronal circuits during recovery from brain damage.

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Introduction

Synaptogenesis and experience-based synaptic changes after birth and during critical periods are important components of brain development. One distinct marker

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of synapse maturation is postsynaptic specializations and the appearance of postsynaptic densities (PSDs) at excitatory synapses. Neurotransmitter receptors are enriched and linked to cytoskeleton and signaling molecules at PSDs, which enables information transmission between neurons. Activity-dependent dynamic changes in the protein components of the PSDs are the molecular basis of synaptic plasticity and cognitive function. Brain injury in early postnatal life, including hypoxia-ischemia, could affect PSD/synapse maturation and thereby disrupt brain development.

The structure and protein composition of the PSDs have been studied in great detail in the adult rodent (mouse and rat) brain during the past 15 years. The advances in mass spectrometry (MS)-based large-scale proteomics and phosphoproteomics have enabled the identification and quantification of hundreds to more than 2,000 PSD proteins and their posttranslational modifications [1–7]. These studies unraveled not only the postsynaptic organization of synapses, but also the signaling networks driven by neurotransmitter receptor activation [8, 9]. They greatly enhanced our understanding of synaptic plasticity and related neurodegenerative or psychiatric disorders. However, quantitative proteomic analysis of the highly malleable developing brain and the knowledge about temporal changes in PSD profiles during brain development, as well as its alterations in response to neonatal encephalopathy, including neonatal hypoxic-ischemic (HI) injury, is very limited.

Age-related proteomic studies revealed a list of proteins that are differentially expressed or phosphorylated in the neonatal and adult mouse brain [10–15]. The higher levels of proteins in the developing brain are mostly involved in neurogenesis, neuronal migration or neurite outgrowth. To date, there are less than 5 MS-based proteomic studies that were performed in a neonatal (postnatal day 3 or 7) HI model, all of which used proteins extracted from the entire brain or cortex or hippocampus without further fractionation [16–19]; a comprehensive proteomic profiling of PSD proteins in the developing brain is still lacking.

The present study provides a global characterization of PSD proteins purified from the neonatal C57BL/6 mouse cortex at postnatal day 9 (P9), a developing stage that is equivalent to term human infants. A total of 512 proteins were identified, which are classified into different functional categories, including 60 proteins that have not been reported in previous synapse proteomic studies with adult and developing brain. In addition, we listed proteins that were newly recruited to PSD or were more than

50% up-or downregulated 1 h after HI injury compared to their sham-operated litter mates. HI-induced molecular modifications of PSDs may be associated with signaling pathways leading to neuronal death/survival and early disturbance of synaptic plasticity and brain development in term babies with HI encephalopathy.

Materials and Methods

All animal experiments were approved by the University of California San Francisco (UCSF) institutional animal care and use committee. C57BL/6 mice (Simonsen) with litters were allowed food and water ad libitum. Both sexes were used for these studies at P9.

Neonatal Brain HI Injury

Neonatal HI injury was performed as previously described with the Vannucci procedure [20]. At P9, pups were anesthetized with isoflurane (2–3% isoflurane/balance oxygen), and the left common carotid artery was electrocauterized. Animals were allowed to recover for 1 h with their dam and then exposed to 60 min of hypoxia in a humidified chamber at 37°C with 10% oxygen/balance nitrogen. Sham-operated control animals received isoflurane anesthesia and exposure of the left common carotid artery without electrocauterization or hypoxia. HI and sham animals were returned to their dams until they were euthanized.

PSD Sample Preparation

Purification of PSD-associated synaptic proteins was carried out as described using a subcellular fractionation approach followed by extraction with Triton X-100 [21, 22]. Cortical tissue was homogenized in ice-cold sucrose buffer containing 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitors (Complete mini and Phospho-Stop cocktail tablets, Roche, Indianapolis, IN, USA). A low-speed (1,000 g) centrifugation was performed to remove the nuclear fraction and tissue debris. The resulting supernatant (S1) was spun at 10,000 g for 15 min to yield a crude membrane fraction (P2). The supernatant (S2) was then centrifuged at 100,000 g for 60 min to separate cytoplasmic protein (S3) and intracellular light membrane fraction (P3). The P2 was subsequently resuspended in 120 μ l sucrose buffer and mixed with 8 vol of 0.5% Triton X-100 buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitors. The mixture was homogenized again with 30 pulses of a glass pestle and rotated at 4°C for 30 min followed by centrifugation at 32,000 g for 30 min in a TL-100 tabletop ultracentrifuge (Beckman). The resultant pellet containing Triton X-insoluble PSD proteins was dissolved in TE buffer (100 mM Tris-HCl, 10 mM EDTA) with 0.5% sodium dodecyl sulfate (SDS) and stored at –80°C until use. The protein concentration was determined by the bicinchoninic acid method (Pierce). The purity of PSD was verified by Western blotting as described [21].

LC-MS/MS Analysis and Protein Identification

Fifteen micrograms of PSD protein mixture were precipitated overnight at –20°C with addition of a 4-fold volume of ice-cold

acetone. After centrifugation, the pellet was redissolved and reduced in 9 μ l of 50 mM ammonium bicarbonate solution containing 8 M urea and 10 mM Tris (2-carboxyethyl) phosphine at 37°C followed by alkylation with 20 mM chloroacetamide in the dark at room temperature. The sample was diluted with 50 mM ammonium bicarbonate solution at least 4 times to make the final concentration of urea less than 2 M and then proceeded to in-solution digestion with combined trypsin (sequencing grade, Promega) and lysyl endopeptidase C (MS grade, Wako) at the ratio of enzyme:protein of 1:25 in mass. After incubation at 37°C for 16 h, the digested peptides were extracted and desalted with a C₁₈ solid-phase extraction tip (10- μ l bed C₁₈ ZipTip, Pierce) and then eluted with 2 sequential 10- μ l aliquots of 50% acetonitrile in 0.1% trifluoroacetic acid. The combined elute was dried in Speed Vac and resuspended in 0.1% formic acid/2% acetonitrile for MS analysis.

The peptides were analyzed by liquid chromatography (LC)-MS/MS on an LTQ Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) equipped with a Waters NanoAcquity LC system (Milford, MA, USA) at the UCSF Mass Spectrometry Facility. Peptides were separated on an Easy-Spray column (Thermo, PepMap, C₁₈, 3 μ m, 100 Å, 75 μ m $\times$ 15 cm) using a linear gradient from 2% solvent A (0.1% formic acid in water) to 25% solvent B (0.1% formic acid in acetonitrile) at 300 nL/min over 47 min. MS precursor spectra were measured in the Orbitrap from 300 to 2,000 m/z at 30,000 resolving power; the top 6 precursor ions were selected and dissociated by collision-induced dissociation for MS/MS. The MS/MS data were searched against mouse protein sequences in the SwissProt database (downloaded from Uniprot on October 11, 2015) using the MSGF+ search engine [23] with a concatenated database consisting of normal and randomized decoy databases. The false discovery rates for peptide identification were estimated to be 1.2%, corresponding to a maximum expectation value of 0.102. Parent/precursor mass tolerance and fragment mass tolerance were 20 ppm and 0.6 Da, respectively. Constant modification of carbamidomethylation on cysteine and variable modifications of deamidation of N-terminal glutamine, initial protein methionine loss with or without N-terminal acetylation were employed.

Semiquantification of PSD Proteins Using Spectral Counting

A semiquantitative analysis was performed using spectral counting, which relies on the number of peptides (peptide count) identified from a given protein. This simple label-free quantification technique is based on the observation that the more abundant a protein is, the more peptides can be identified from it. It provides a tool for rapid screening and broad estimation of the relative protein amount between samples [24]. For each protein, the mean peptide counts and standard deviations (SDs) from triplicate analysis (3 repeated LC-MS/MS analyses of the same sample) of the naïve, sham and HI groups were determined, and their 95% confidence intervals were calculated with the formula: $\text{mean} \pm 1.96 \times (\text{SD}/\sqrt{n})$, in which SD and n ($= 3$) are the standard deviation and the number of replicates, respectively. The mean ratio (n -fold change) of HI/sham was used to represent differential expression of each protein after HI insult, and the 95% confidence interval of the ratio was reported to indicate reliability of the results.

Western Blotting of PSD Samples

For Western blot analysis, an equal amount of PSD protein samples (5 μ g) was applied to 4–12% Bis-Tris SDS polyacrylamide

gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blots were probed with the primary antibodies (listed in online suppl. Table 2; for all online suppl. material, see www.karger.com/doi/10.1159/000456030) overnight at 4°C. Appropriate secondary horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology Inc.) were used, and the signal was visualized with enhanced chemiluminescence (Amersham, GE Healthcare). Image J software was used to measure the mean optical densities (OD) and areas of protein signal on radiographic film after scanning.

Statistical Analysis

Proteins detected by MS experiments were semiquantified using spectral counting from a triplicate MS run of 2 sets of samples of each group. The differences of relative protein expression are presented as mean ratio (n -fold change) of HI/sham counts and the mean confidence interval of the ratio. Data of ODs of immunoblots are presented as means $\pm$ SD and were evaluated statistically using the SAS Wilcoxon-Mann-Whitney test. Differences were considered significant at $p < 0.05$.

Results

Identification of PSD Proteins in the Neonatal Mouse Brain

To study the protein composition of PSDs in the immature brain, PSD fractions were purified from naïve P9 C57BL/6 mouse cortices. A short protocol published previously [21, 22] was utilized instead of the original method of Carlin et al. [25], which employed a long 2-step sucrose density gradient centrifugation procedure. The PSD-associated proteins prepared with this protocol had similar migration profiles in Coomassie blue-stained SDS polyacrylamide gel electrophoresis gel (Fig. 1b) to those isolated from adult brain, and the purity was comparable to that extracted with the original Carlin method [26]. After in-solution digestion and LC-MS/MS analysis/database search, a total of 512 proteins was identified, all of which were categorized in different groups based on their published biological functions (complete list in online suppl. Table 1). The false-positive rate for the database search was estimated to be $\approx 1.2\%$ with the maximal peptide expectation value (e -value) of 0.102. The e -value threshold was determined based on the distributions of e -values of spectra matched against the normal SwissProt database and matched against the randomized version of the normal database (Fig. 1c). The identified proteins were reported with their name, UniPro accession number, the number of unique peptides used to identify the protein, the percent sequence coverage for each protein, and the best expectation values for the most confident peptide identification for each protein. Similar to reports

Fig. 1. a Schematic representation of the experimental workflow. **b** Coomassie blue-stained SDS-polyacrylamide gel lane containing purified P9 mouse cortex PSD proteins. A rainbow protein molecular weight (MW) ladder is shown on the left, and a representative PSD sample (input) gel is shown on the right. **c** The true (red curve) and false (blue curve) distributions of expectation values (e-values) of spectra matched against the SwissProt database. The false match (or random match) distribution was obtained using matches against the randomized version of the normal database.

The true match distribution is the subtraction of matches against the normal database from the random matches. The false-positive rate for the MS database search was estimated to be $\approx 1.2\%$ with the maximal peptide e-value of 0.102. **d** Yield of PSD, intracellular light membrane (P3), and cytoplasmic proteins (S3) in sham and HI-injured mice at 15 min and 1 h after HI injury. Data are expressed as means $\pm$ SD and normalized to the sham values (*n*-fold of sham). There is a significant increase in protein yield of PSD at 15 min compared to sham animals (* $p < 0.05$, $n = 8-9$).

from adult PSD proteomic studies, the proteins fell into the following major categories: receptors/channels/transporters; scaffold/adaptors/trafficking proteins; kinases/phosphatases and regulators; cytoskeleton/motor and modulators; small G proteins/GTPases/ATPases; adhesion/chaperon proteins [1, 2, 4-6, 27]. It is not surprising that a large amount of ribosomal proteins, elongation factor 1 and heterogeneous nuclear ribonucleoproteins were detected indicating active local protein translation/synthesis at or proximal to PSDs [1, 4, 5]. Proteins from pre-synaptic sites (protein bassoon, piccolo, synapsin, etc.) from mitochondria and nuclei have been frequently identified in postsynaptic proteomic experiments [4, 5, 27]. Interestingly, a previous study showed that RNA binding protein heterogeneous nuclear ribonucleoprotein A3 that

was also found in this study was localized in both nucleus and spines [2]. Other identified heterogeneous nuclear ribonucleoproteins A1, A2/B1, A3, L, and M were demonstrated to accumulate at PSDs with synaptic activity [28]. Some of the known PSD proteins that were not detected in the MS experiments are likely to be low in abundance.

Newly Identified PSD Proteins That Regulate Neural Development

To discover the novel proteins that have not been reported in previous synapse proteomic studies, proteins identified in this work were compared to published data from 6 other proteomic experiments with the PSD or synaptic membrane fractions [1, 4, 5, 7, 13, 27]. Of the 512

Table 1. Newly identified PSD proteins that regulate neural development

Acc. No.	Protein name	Number unique	Percent coverage	Expect. value	Function in the brain
P97929	Breast cancer type 2 susceptibility protein homolog (BRCA2)	1	0.2	7.60E-3	Regulates apoptosis and neurogenesis during embryonic and postnatal neural development, deficiency leads to microcephaly
Q921W0	Charged multivesicular body protein 1a (CHMP1A)	1	4.6	7.79E-2	Regulates neural progenitor cell proliferation, deficiency leads to pontocerebellar hypoplasia and microcephaly
Q9WVI9	C-Jun amino-terminal kinase-interacting protein 1 (JIP1)	1	1.6	7.70E-2	Collaborates with AKT to promote axon growth Required for neuronal polarization and migration
Q8VHJ5	Serine/threonine-protein kinase MARK1	1	2.6	6.21E-3	Polarity protein involved in axon-dendrite specification, neurite outgrowth and neuronal migration Regulates dendrite and spine development and functioning Overexpressed in a prefrontal region in autism patients
Q6PGB8	Probable global transcription activator SNF2L1	1	1.1	4.5E-2	Potentiates neurite outgrowth Inhibits progenitor cell expansion Promotes neuronal differentiation
Q80UK0	Sec 14 domain and spectrin repeat-containing protein 1 (SESTD1)	1	1.1	3.52E-2	Negatively regulates dendritic spine density Reduces excitatory synaptic transmission in hippocampal neurons
Q99MB1	Toll-like receptor 3 (TLR3)	1	1.1	2.10E-2	Constrains neural progenitor cell proliferation and adult hippocampal neurogenesis Negative regulator of axonal growth in DRG neurons Inhibits hippocampus-dependent learning and memory

The identified proteins are reported with their UniPro accession (acc.) number, name, number of unique peptides used to identify the protein (number unique), percent sequence coverage for the protein, the best expectation (expect.) values for the most confident peptide identification for each protein, and function in the brain. DRG, dorsal root ganglion.

proteins identified with high confidence, 452 were detected in mature or immature synapses by MS, while 60 have not been reported in previous PSD or synapse proteomes (underlined in online suppl. Table 1). Seven novel proteins with functions in neural development are highlighted (Table 1). Breast cancer type 2 susceptibility protein homolog (BRCA2) is known to be a tumor suppressor involved in the repair of DNA double-strand breaks. It is required for neurogenesis during embryonic and postnatal neural development whose deficiency leads to increased apoptosis and microcephaly [29]. Charged multivesicular body protein 1a (CHMP1A) was originally identified as a member of the ESCRT-III endosomal sorting complex that assists in the trafficking of ubiquitinated cargo proteins to the lysosome for degradation [30]. It also localizes to the nuclear matrix and regulates chromatin structure [31]. A recent study demonstrated its in-

volvement in neural progenitor cell proliferation and cerebellar development [32]. C-Jun-amino-terminal kinase-interacting protein 1 (JIP1) is a scaffold protein that activates JNK cascade pathways, and an adaptor linking cargo to kinesin-1, a major motor protein for axonal transport. It is highly expressed in neurons and, through interplay with AKT, promotes axonal growth in cortical cultures [33, 34]. In vivo, it is important for polarization and migration of cortical neurons [35]. Serine/threonine protein kinase MARK1 (microtubule affinity-regulating kinase 1) encodes a kinase-regulating microtubule-dependent transport in axons and dendrites [36]. It is a polarity protein implicated in axon-dendrite specification and neurite outgrowth [37, 38]. MARK1 is involved in neuronal migration by phosphorylating doublecortin, MAP2 and tau [39]. Through phosphorylating PSD95 [40] or DIX domain containing 1 (DIXDC1) [41], MARK1

Table 2. Proteins recruited to PSDs early (1 h) after neonatal HI injury

Acc. No.	Protein name	Number unique	Percent coverage	Expect. value
<i>Calcium-regulated proteins</i>				
Q8CBW3	Abl interactor 1	1	3.33	1.44E-7
P62484	Abl interactor 2	1	3.59	1.13E-6
P08414	Calcium/calmodulin-dependent protein kinase type IV	2	7.25	2.70E-8
O08688	Calpain-5	1	2.81	1.43E-3
Q80YA9	Connector enhancer of kinase suppressor of Ras 2 (CNKSR2)	2	2.81	1.24E-6
Q9QYB5	γ -Adducin	2	4.25	1.95E-10
P84075	Neuron-specific calcium-binding protein hippocalcin	1	4.10	2.00E-3
O35465	Peptidyl-prolyl <i>cis-trans</i> -isomerase FKBP8	1	3.98	2.34E-3
P20444	Protein kinase C α -type	6	10.30	2.50E-5
P68404	Protein kinase C β -type	2	4.92	2.91E-2
P63318	Protein kinase C γ -type	16	35.90	2.60E-9
Q9EQZ6	Rap guanine nucleotide exchange factor 4	1	1.58	1.13E-3
Q9QUG9	Ras guanyl-releasing protein 2 (RASGRP2)	3	9.38	4.65E-7
Q9R0N7	Synaptotagmin-7	1	4.47	3.19E-2
<i>Others</i>				
Q3UHD9	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 2 (PIKE-L)	1	1.35	2.95E-7
Q8VHH5	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 3	1	2.09	1.60E-4
Q9Z2H5	Band 4.1-like protein 1	3	6.37	2.52E-5
P39053	Dynamin-1	1	1.20	2.70E-5
Q61735	Leukocyte surface antigen CD47	1	4.62	2.45E-3
P22005	Proenkephalin-A precursor	1	2.61	2.07E-2
Q6NZL0	Protein SOGA3	2	2.65	1.65E-7
A2A690	Protein TANC2	1	0.40	7.45E-3
Q9JKN6	RNA-binding protein Nova-1	1	2.76	9.81E-4
Q9QZX7	Serine racemase	1	7.96	4.89E-3
Q7TT50	Serine/threonine protein kinase MRCK β	2	2.63	6.17E-5
Q8R570	Synaptosomal-associated protein 47	6	21.55	4.32E-9
Q921F2	TAR DNA-binding protein 43 (TDP-43)	1	3.14	1.36E-6
Q9WTS5	Teneurin-2	2	1.37	1.66E-3

The identified proteins are reported with their UniPro accession (acc.) number, name, number of unique peptides used to identify the protein (number unique), percent sequence coverage for the protein, and the best expectation (expect.) values for the most confident peptide identification for each protein.

plays essential roles in dendritic spine morphogenesis. It is overexpressed in the prefrontal cortex of autistic patients and proposed as a susceptibility gene for autism [37]. Two other proteins regulate neurite outgrowth with opposite effects. Probable global transcription activator SNF2L1, a chromatin-remodeling protein, is enriched in the brain and potentiates neurite outgrowth [42], while Toll-like receptor 3 (TLR3), an innate immunity receptor usually found in glial cells, acts as a negative regulator of axonal growth [43]. SNF2L is also found to inhibit progenitor cell expansion and promote neuronal differentiation in the developing brain [44]. TLR3 is strongly expressed in the brain during early embryogenesis and decreases in the postnatal period [45]. It constrains the

proliferation of embryonic neural progenitor cells and adult hippocampal neurogenesis [45, 46]. In the adult brain, TLR3 signaling reduces hippocampus-dependent learning and memory and AMPA receptor expression in CA1 neurons [46]. Sec 14 domain and spectrin repeat-containing protein 1 (SESTD1) is a binding partner of group 4 calcium-permeable transient receptor potential channels TRPC4 and TRPC5 [47]. In vitro studies suggested that SESTD1 negatively regulates dendritic spine density and reduces excitatory synaptic transmission in cultured hippocampal neurons [48]. It may play a role in synapse formation or maturation given its residence at PSD and being more prevalent during embryogenesis and the early postnatal weeks [48].

Increase in PSD Yield and Recruitment of Proteins into PSD in Response to Neonatal HI

To investigate the early responses of postsynaptic protein machinery to hypoxia-ischemia, PSD fractions were purified from sham-operated control mice and mice subjected to 60 min of hypoxia-ischemia, followed by 15 min and 1 h of reperfusion. There was an increase in PSD yield at these early time points (by 51.23% at 15 min and 14.94% at 1 h, $n = 8-9$, Fig. 1d). The yield of PSDs was 1.218 ± 0.16 $\mu\text{g}/\text{mg}$ cortical tissue in sham brains, 1.842 ± 0.173 $\mu\text{g}/\text{mg}$ at 15 min after HI insult ($p < 0.05$), and 1.4 $\mu\text{g}/\text{mg}$ at 1 h after HI insult. There were no differences in the yield of intracellular light membrane and cytoplasmic protein fractions following HI injury (Fig. 1d).

The MS-based proteomic experiments with PSDs from sham and HI-injured animals provided an overall picture of the alterations in PSD protein composition. Similar to naïve animals, 544 proteins were detected in sham animals and 576 proteins at 1 h after HI insult. To take into consideration that sham-operated animals received isoflurane anesthesia, which might modify the neurotransmitter receptors at PSD, proteins from naïve, sham and HI-injured animals were compared and the ones that were only present under the HI condition were selected. These were defined as proteins that are newly recruited into the PSD in response to neonatal HI injury (Table 2). Proteins that are regulated by calcium were separated because elevated intracellular calcium in postsynaptic neurons from both external influx and internal release following hypoxia-ischemia is an important regulator of neurotransmitter receptor function and signaling pathways leading to cell death/survival. It mostly involves activation of protein kinases/phosphatases, Ras family GTPases and their coplayers, for example, conventional protein kinase C (PKC α , β , and γ), CaMKIV and 2 proteins implicated in Ras signaling pathways (CNKSR2 and RASGRP2). Calpain-5 is a nonclassical member of the calpain family that is highly expressed in the CNS [49], but whether it functions the same way as the other members in synaptic activity and neurotoxicity is unknown. Some proteins are associated with neurite extension/synapse maturation or neuronal viability during neural development (Abl interactor [50, 51], γ -adducin [52, 53], FKBP8 [54, 55], hippocalcin [56, 57], CD47 [58], Nova-1 [59, 60], TANC2 [61], and teneurin [62, 63]). And several listed proteins have been reported to participate in cell death mechanisms in adult brain ischemia (dynamin-1 [64, 65], FKBP8 [66], CD47 [67, 68] and TDP-43 [69, 70]). PIKE-L [71] and Nova-1 [72] have been shown

to play roles in neuroprotection and repair after brain ischemia.

Identification of Proteins with Differential Expression Early after Neonatal HI Injury

To reveal HI-induced changes in protein expression, a comparison was made for the peptide counts from triplicate LC-MS/MS runs for each PSD sample from naïve, sham and post-HI brains. After calculating the mean count ratio of each protein in HI over sham group (HI/sham), proteins whose relative expression (peptide counts) in HI samples were at least 50% higher (ratio ≥ 1.5 , Table 3) or 50% lower (ratio ≤ 0.5 , Table 4) than those in sham samples were identified. 95% confidence intervals were also included to show reliability of the results. These differentially expressed proteins are mainly involved in kinases/phosphatases, scaffold/adaptor and membrane trafficking proteins, cytoskeleton and modulators and cell adhesion proteins. HI injury enhanced the expression of CaMKII, CaMKV, PKA, DCLK2, Fyn, Src kinase signaling inhibitor 1 (SNIP), and protein phosphatase PP1 (Table 3). On the other hand, receptor-type tyrosine-protein phosphatase ζ (phosphacan), which is restricted to the CNS [73] and primarily expressed in neural progenitors and induced in remyelinating oligodendrocytes [74], was downregulated after HI insult (Table 4). Expression of Blk, another Src family kinase that was not previously found in the developing brain, was decreased (Table 4). These changes, along with alterations in other proteins, suggest rewiring of cellular signaling networks and reconstruction of PSD cytoskeleton after neonatal HI, which might be associated with vulnerability to injury or impairment of synaptic plasticity following the insult.

Neonatal HI Affects Phosphorylation of NMDA Receptor Subunit NR2B

Validation of some proteins detected in MS experiments with Western blotting was carried out and focused firstly on the NMDA receptors because NMDA receptor-mediated excitotoxicity is an early event after hypoxia-ischemia and an important contributor of neuronal cell death. It was surprising that the NR2A subunit was not detected in the MS runs, possibly due to its significant lower abundance than NR2B in the developing brain. The protein level of PSD NR1 was not affected by HI insult within the first 24 h of reperfusion, while expression of NR2A and NR2B decreased over time. Phosphorylation of NR2B, including Fyn-mediated phosphorylation at tyrosine (Y) 1472 and CK2 α -mediated phosphorylation at

Table 3. Identification of upregulated proteins at PSDs early (1 h) after neonatal HI injury

Acc. No.	Protein name	Mean peptide counts			<i>n</i> -fold change (HI/sham)	95% confidence interval (±)
		naïve	sham	HI		
<i>Receptors, channels, and transporters</i>						
P55096	ATP-binding cassette subfamily D member 3	1.00	1.67	3.67	2.20	2.12
Q14BI2	Metabotropic glutamate receptor 2	0.67	0.67	1.00	1.50	1.47
Q9QYS2	Metabotropic glutamate receptor 3	0.00	0.67	1.00	1.50	3.17
<i>Scaffold/adaptor proteins</i>						
P17427	AP-2 complex subunit α ₂	5.67	5.33	9.33	1.75	0.54
P84091	AP-2 complex subunit μ	4.33	4.00	8.00	2.00	1.41
<i>Kinase/phosphatase and regulators</i>						
P11798	Calcium/calmodulin-dependent protein kinase type II subunit α	22.67	21.67	38.67	1.78	0.16
P28652	Calcium/calmodulin-dependent protein kinase type II subunit β	26.00	23.00	36.67	1.59	0.47
Q6PHZ2	Calcium/calmodulin-dependent protein kinase type II subunit δ	3.00	2.33	5.67	2.43	0.96
Q923T9	Calcium/calmodulin-dependent protein kinase type II subunit γ	2.00	3.67	8.33	2.27	0.76
Q3UHL1	CaM kinase-like vesicle-associated protein (CaMKV)	0.33	1.00	11.33	11.33	0.65
P05132	cAMP-dependent protein kinase catalytic subunit α	1.00	0.33	1.00	3.00	1.87
P68181	cAMP-dependent protein kinase catalytic subunit β	0.00	1.00	1.67	1.67	0.65
P12367	cAMP-dependent protein kinase type II-α regulatory subunit	0.00	0.33	1.00	3.00	5.88
P31324	cAMP-dependent protein kinase type II-β regulatory subunit	0.33	1.33	3.67	2.75	2.33
Q6PGN3	Serine/threonine protein kinase DCLK2	0.67	1.00	2.00	2.00	0.00
P62137	Serine/threonine protein phosphatase PP1-α catalytic subunit	1.67	2.33	6.33	2.71	2.75
Q9QWI6	SRC kinase signaling inhibitor 1	6.00	3.00	14.67	4.89	2.28
P39688	Tyrosine protein kinase Fyn	1.00	1.00	2.67	2.67	0.65
<i>Small G protein, GTPase, ATPase, and regulators</i>						
F8VPU2	FERM, RhoGEF and pleckstrin domain-containing protein 1 (FARP1)	1.33	2.66	8.67	3.25	1.44
P46460	Vesicle-fusing ATPase (NSF)	0.67	1.00	11.33	11.33	15.18
<i>Cytoskeleton and modulators</i>						
Q9QYC0	α-Adducin	2.67	1.67	6.00	3.60	4.41
Q8R5C5	β-Centractin	2.33	1.67	4.33	2.60	1.80
Q7TMB8	Cytoplasmic FMR1-interacting protein 1	1.67	1.67	3.00	1.80	1.38
Q5SQX6	Cytoplasmic FMR1-interacting protein 2	1.67	1.67	3.33	2.00	1.25
Q9R0P5	Destrin	0.33	0.33	1.00	3.00	5.88
Q9QXZ0	Microtubule-actin cross-linking factor 1	2.33	2.66	4.67	1.75	1.38
P10637	Microtubule-associated protein tau	5.33	5.33	8.00	1.50	0.85
Q9CQV6	Microtubule-associated proteins 1A/1B light chain 3B	0.33	0.66	1.33	2.00	2.94
O88809	Neuronal migration protein doublecortin	2.00	1.66	3.33	2.00	1.82
Q9CWF2	Tubulin β _{2B} chain	1.00	1.00	2.33	2.33	0.65
Q9D6F9	Tubulin β _{4A} chain	4.67	10.00	20.33	2.03	0.51
P99024	Tubulin β ₅ chain	4.00	12.67	25.67	2.03	0.64
P20152	Vimentin	0.33	0.33	2.67	8.00	17.64
<i>Cell adhesion</i>						
Q80TS3	Adhesion G protein-coupled receptor L3 precursor	0.00	0.33	1.67	5.00	11.76
P15116	Cadherin-2 precursor	0.00	0.33	2.00	6.00	15.15
Q61301	Catenin α ₂	1.33	1.67	12.33	7.40	5.73
Q02248	Catenin β ₁	3.33	3.33	8.00	2.40	0.47
O35927	Catenin δ ₂	0.00	1.00	2.33	2.33	0.65
Q68FD5	Clathrin heavy chain 1	6.33	4.33	31.33	7.23	1.89
P13595	Neural cell adhesion molecule 1	4.33	6.67	12.67	1.90	0.97
P11627	Neural cell adhesion molecule L1	2.00	3.00	5.00	1.67	0.00
Q99PJ0	Neurotrimin	7.00	7.66	13.33	1.74	0.87
<i>Chaperone/protein folding</i>						
P63017	Heat shock cognate 71-kDa protein	29.67	26.00	52.33	2.01	0.28
P17156	Heat shock-related 70-kDa protein 2	5.00	4.33	11.33	2.62	1.59

Table 3 (continued)

Acc. No.	Protein name	Mean peptide counts			<i>n</i> -fold change (HI/sham)	95% confidence interval (±)
		naïve	sham	HI		
<i>DNA/nucleus</i>						
Q60899	ELAV-like protein 2	0.67	1.00	2.00	2.00	1.13
P49312	Heterogeneous nuclear ribonucleoprotein A1	2.66	2.66	4.00	1.50	0.79
Q8R081	Heterogeneous nuclear ribonucleoprotein L	1.33	1.33	2.00	1.50	1.58
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	2.67	2.67	5.00	1.88	2.08
Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	1.33	3.00	4.67	1.56	0.22
O88569	Heterogeneous nuclear ribonucleoproteins A2/B1	3.00	4.33	8.67	2.00	0.75
P09405	Nucleolin	1.33	0.67	2.67	4.00	8.82
Q99JF8	PC4 and SFRS1-interacting protein	0.00	0.67	1.00	1.50	1.47
<i>Others</i>						
Q99P72	Reticulon-4 (Nogo protein, neurite outgrowth inhibitor)	2.33	0.67	7.33	11.00	11.76
P62984	Ubiquitin-60S ribosomal protein L40 precursor	3.67	4.67	9.67	2.07	0.72

The identified proteins are reported with their UniPro accession (acc.) number, name, the mean peptide counts in naïve, sham, and HI samples, the mean *n*-fold change (ratio) of peptide counts of HI over sham (HI/sham), and the range ($\pm$) for the 95% confidence interval.

serine (S) 1480, was significantly changed. Y1472 phosphorylation increased at 15 min and 1 h after HI insult (Fig. 2a, b, $p < 0.05$, $n = 4$), while S1480 phosphorylation gradually decreased at 1, 6, and 24 h after hypoxia-ischemia (Fig. 2a, b, $p < 0.05$, $n = 4$). Phosphorylation of Y1336, another Fyn target site on NR2B, remained the same. Src kinases, the core glutamate receptor kinases at PSD [75], were profoundly activated as indicated by the increase in their active form pY416 Src (Fig. 2a, c, $n = 5$). Fyn and Src were detected in proteomic analysis, with their protein levels unaffected by HI injury (Fig. 2a), which is consistent with our previous study [26]. Fyn was found increased in MS analysis (Table 3). Src kinase signaling inhibitor 1 (SNIP or SNAP-25-interacting protein, p130Cas-associated protein), a negative regulator of Src, was upregulated at 1 h (from both MS and Western blotting results), and then declined at 6 and 24 h after HI insult (Fig. 2a, c). Casein kinase 1 (CK1) and 2 (CK2) are highly conserved serine/threonine kinases that have a wide variety of substrates, including NR2B. CK1 was identified in the proteomic analysis, but not CK2. The expression of CK1 α increased early after HI injury, while CK2 α expression was unchanged (Fig. 2a, d).

Neonatal HI Activates Multiple Signaling Kinases at PSD

PSD is considered as a postsynaptic signaling hub where stimulation of neurotransmitter receptors activates multiple and overlapping signaling pathways lead-

ing to biochemical and morphological changes of the PSD itself and the overall neuronal viability. To investigate how HI injury would modify these complex networks, the expression of major stress-evoked protein kinases and their phosphorylation status after hypoxia-ischemia were determined. CaMKII α was markedly activated early after HI insult (15 min, 1 and 6 h) as presented as the ratio of (phospho-)pCaMKII α /total CaMKII α (Fig. 3a, b, $p < 0.05$, $n = 5$). CaMKII β , also neuron-specific and highly enriched in PSD, was upregulated up to 6 h following HI injury (Fig. 3a, b). The MS analysis detected translocation of PKC α , - β , and - γ to PSD after HI injury, which was validated by Western blotting with a robust induction for up to 6 h as shown in Figure 3a and d ($p < 0.05$, $n = 3$). Expression of neuronal nitric oxide synthase, an important downstream effector of the NR2B-PSD95 complex but undetectable in all postsynaptic proteomics, was increased for at least 24 h after HI injury (Fig. 3a, b, $p < 0.05$, $n = 5$). Phosphatidylinositol-4,5-bisphosphate 3-kinase is also implicated in long-term potentiation and multitudinous cellular function. Its regulatory subunit p85 was found to be persistently overexpressed at PSD after HI insult (Fig. 3a, b, $p < 0.05$, $n = 4$). HI insult induces mitogen-activated protein kinases including ERK1/2, p38 and JNK kinases. Mitogen-activated protein kinases are involved in a variety of fundamental cellular processes and have been studied intensively in neonatal brain HI models. It is interesting that ERK and its phosphorylated form (pERK), but not p38, are localized at synapses in sham

Table 4. Identification of downregulated proteins at PSDs early (1 h) after neonatal HI injury

Acc. No.	Protein name	Mean peptide counts			Percent of sham (HI/sham)	95% confidence interval (±)
		naïve	sham	HI		
<i>Receptors, channels, and transporters</i>						
Q01097	Glutamate receptor ionotropic, NMDA 2B	5.67	9.33	1.00	0.11	0.15
Q8VDN2	Sodium/potassium-transporting ATPase subunit α ₁	5.67	6.33	2.00	0.32	0.24
Q6PIE5	Sodium/potassium-transporting ATPase subunit α ₂	1.66	2.00	1.00	0.50	0.00
P14094	Sodium/potassium-transporting ATPase subunit β ₁	2.00	1.67	0.67	0.40	0.94
<i>Kinase/phosphatase and regulators</i>						
B9EKR1	Receptor-type tyrosine protein phosphatase ζ	2.00	3.00	1.33	0.44	0.39
P16277	Tyrosine protein kinase Blk	1.00	1.00	0.33	0.33	0.65
<i>Scaffold/adaptor proteins</i>						
Q8BKX1	Brain-specific angiogenesis inhibitor 1-associated protein 2	10.67	9.00	2.33	0.26	0.19
Q811D0	Disks large homolog 1 (SAP97)	1.33	2.67	1.00	0.38	0.09
Q91XM9	Disks large homolog 2 (PSD93)	0.67	0.33	0.00	0.00	0.00
P70175	Disks large homolog 3 (SAP102)	6.00	4.33	0.33	0.08	0.18
Q62108	Disks large homolog 4 (PSD95)	6.33	5.33	0.67	0.13	0.19
Q6PH08	ERC protein 2	5.33	1.67	0.67	0.40	0.96
Q9Z2Y3	Homer protein homolog 1	9.33	9.66	4.00	0.41	0.07
Q80TE7	Leucine-rich repeat-containing protein 7 (densin-180)	1.33	2.00	0.67	0.33	0.16
O88737	Protein bassoon	3.67	3.00	1.33	0.44	0.77
Q9QYX7	Protein piccolo	2.67	4.00	1.67	0.42	0.44
D3YZU1	SH3 and multiple ankyrin repeat domains protein 1(shank 1)	0.67	1.67	0.00	0.00	0.00
Q80Z38	SH3 and multiple ankyrin repeat domains protein 2 (shank 2)	5.67	8.67	1.67	0.19	0.17
<i>Small G protein, GTPase, ATPase, and regulators</i>						
P60766	Cell division control protein 42 homolog	1.67	1.00	0.00	0.00	0.00
P50518	V-type proton ATPase subunit E 1	2.00	1.00	0.00	0.00	0.00
<i>Cytoskeleton and modulators</i>						
P59999	Actin-related protein 2/3 complex subunit 4	1.67	2.67	1.00	0.38	0.09
Q80VC9	Calmodulin-regulated spectrin-associated protein 3	1.00	0.33	0.00	0.00	0.00
Q8BMK4	Cytoskeleton-associated protein 4	11.67	11.67	5.67	0.49	0.26
Q9WV69	Dematin	1.67	1.67	0.67	0.40	0.55
Q5DU25	IQ motif and SEC7 domain-containing protein 2	1.33	0.67	0.33	0.50	0.06
<i>Motor proteins</i>						
Q61595	Kinectin	4.67	5.33	0.00	0.00	0.00
P28740	Kinesin-like protein KIF2A	4.33	2.67	1.00	0.37	0.55
<i>DNA/nucleus</i>						
O70251	Elongation factor 1β	5.67	6.00	2.67	0.44	0.19
P57776	Elongation factor 1δ	2.67	2.67	0.33	0.13	0.28
Q9D8N0	Elongation factor 1γ	5.67	10.33	3.67	0.35	0.15
Q9D0E1	Heterogeneous nuclear ribonucleoprotein M	4.00	3.33	1.67	0.50	0.28
<i>Mitochondrial</i>						
O35129	Prohibitin-2	2.33	3.67	1.00	0.27	0.63
<i>Others</i>						
P04370	Myelin basic protein	2.67	1.50	0.00	0.00	0.00
Q91V61	Sideroflexin-3	1.00	1.33	0.00	0.00	0.00

The identified proteins are reported with their UniPro accession (acc.) number, name, the mean peptide counts in naïve, sham, and HI samples, the mean ratio (percent of sham) of peptide counts of HI over sham (HI/sham), and the range ($\pm$) for the 95% confidence interval.

Fig. 2. a–d Western blotting of NMDA receptor, phosphorylation of NR2B subunit, Src family kinase (Fyn, Src, and activated form pY416 Src), Src kinase signaling inhibitor 1 (SNIP), and casein kinase (CK1α and CK2α) at different time points after HI injury. The levels of protein expression are presented as means $\pm$ SD of the OD values of the blots and normalized to the sham values (n-fold of sham). * $p < 0.05$ compared to sham ($n = 4–5$).

animals (Fig. 3a, $n = 4$). p38 was translocated into PSD after HI injury and stayed there for at least 24 h. ERK2 was activated at PSD early after HI injury (15 min and 1 h) while ERK1 phosphorylation seemed unchanged (Fig. 3a, $n = 4$).

Neonatal HI Injury Modifies PSD Scaffold and Adaptor Proteins

The neurotransmitter receptors are anchored on the postsynaptic membrane through their interactions with PSD scaffold/adaptor proteins and cytoskeleton proteins. The MS data showed a downregulation of many scaffold proteins (Table 4) including both presynaptic and post-

synaptic proteins that are important for synapse formation and function. The disks large subfamily of membrane-associated guanylate kinase (MAGUK) scaffold proteins (PSD95, PSD93, SAP102, and SAP97) links the receptors to intracellular signaling networks. All 4 MAGUK members were identified and showed reduced expression after hypoxia-ischemia in proteomic analysis (Table 4). Western blotting validated the decreased post-HI levels of PSD95, PSD93, and SAP102 (Fig. 4, $p < 0.05$, $n = 4$). The expression of SynGAP, a Ras-GTPase-activating protein that interacts with the PDZ domains of PSD95 and SAP102, was decreased at 24 h after HI insult ($p < 0.05$, $n = 4$).

Fig. 3. a–d Neonatal HI injury activates multiple signaling kinases at PSDs. The antibodies used are listed on the left side of the blots. The levels of protein expression are presented as means $\pm$ SD of the OD values of the blots and normalized to the sham values (*n*-fold of sham). The protein levels of pCaMKIIα/CaMKIIα, p38, and PKCα, β, and γ were normalized to the values of 15 min. * $p < 0.05$ compared to sham ($n = 4–5$).

Discussion

Recent synaptic proteomic studies in the postnatal brain have provided valuable findings for understanding the enhanced plasticity during critical periods and possible synaptic deficits underlying developmental brain disorders [13–15]. The present work focused on profiling PSD proteins from the P9 mouse cortex. A total of 512 PSD proteins was identified, among which 60 proteins have not been reported in previous synaptic proteomic studies. Seven of them are unique for their involvement in neurite maturation and neural development. An episode of hypoxia-ischemia increased the yield of PSD early after the insult and triggered translocation of many pro-

teins, including Ca^{2+} -regulated proteins into PSDs. Differentially expressed proteins 1 h after hypoxia-ischemia were tabulated. Validation demonstrated the expression and modification of the NMDA receptor, as one example of neurotransmitter receptors, in response to hypoxia-ischemia. Activation of a series of protein kinases that are involved in synaptic transmission or pathways leading to cell death/survival in a neonatal model of hypoxia-ischemia was also seen. Finally, impaired expression of PSD scaffold and adaptor components may suggest a local disassembly or disruption of the postsynaptic apparatus predicting defect plasticity following early brain injury.

Previous postsynaptic proteome studies have identified several hundreds to more than 2,000 proteins from

Fig. 4. a, b Neonatal HI injury modifies MAGUK family scaffold and adaptor proteins. The antibodies used are listed on the left side of the blots. The levels of protein expression are presented as means $\pm$ SD of the OD values of the blots and normalized to the sham values (*n*-fold of sham). * $p < 0.05$ compared to sham ($n = 4-5$).

the mouse brain, mostly from the whole brain or forebrains [1, 4, 5, 27], while only cortical tissue was used in this study. Instead of the routine in-gel digestion after 1- or 2-dimensional gel electrophoresis for protein identification with MS, an in-solution digestion procedure was employed to avoid loss of proteins during gel extraction; however, it may also reduce the chances of detecting low abundant proteins, which is a common issue of MS-based proteomic study. The false-positive rate for peptide identification was estimated to be 1.2% indicating the reliability of protein identification. The protein profile of PSD from the P9 mouse is similar to that of the adult brain, consisting of proteins classified in the same functional categories except those with unknown biological functions. This suggests that synapses at this developing stage may have already assembled the majority of proteins as when they mature, although the synapses are still scarce on spines but increase steadily within the first few weeks of rodent life. This is in line with a recent report showing that the same number of synaptic membrane proteins was identified across the ages from P9 to P280 with differences in abundance of many of them over time [13]. Core presynaptic and postsynaptic proteins are revealed at low levels at P9 with an overall increase in expression levels or subunit shift during development [13]. By comparison with existing PSD or synaptic proteomic data, we detected 60 novel proteins, including 7 that are key regulators of brain development in addition to their recognized roles beyond this stage. These proteins are involved in highly active biological processes in the immature brain, which

include neural progenitor proliferation and neurogenesis (BRCA2, CHMP1A, SNF2L1, and TLR3), neuronal migration (JIP1 and MARK1), neurite extension (JIP1, MARK1, SNF2L1, and TLR3), or dendrite/synapse development or functioning (MARK1 and SESTD1). Among these proteins, MARK1 and SESTD1 were proven to be localized at PSDs [41, 48], whereas the presence and specific roles for the other 5 proteins at the postsynaptic site require further clarification.

Early brain hypoxia-ischemia led to increased yield and changes in molecular composition of neonatal PSDs by recruiting proteins into PSDs and modifying the expression of proteins that belong to diverse functional categories. As expected, the increase in intracellular Ca^{2+} from extra- and intracellular sources following hypoxia-ischemia activates a variety of kinases/phosphatases and small G proteins, as shown in Tables 2 and 3 and Figures 2 and 3, which are essential for signal transduction downstream of postsynaptic neurotransmitter receptors or for modification of other intracellular signaling pathways. For example, Src family kinase Fyn and Src, PKC, CaMKII, casein kinases, phosphatidylinositol-4,5-bisphosphate 3-kinase and mitogen-activated protein kinases are all able to regulate the function of NMDA receptors by direct phosphorylation and/or modifying their interactions with intracellular binding partners. Activation or translocation of these kinases into PSDs immediately after hypoxia-ischemia may suggest their involvement in NMDA receptor-mediated glutamate excitotoxicity or enhanced synaptic transmission. The NR2B subunit is

known to be substrate for CaMKII α , Src kinase, PKC and CKII. Coordination of these kinases (synergistically or antagonistically) and with synaptic phosphatases is critical for synaptic plasticity. For instance, S1480 phosphorylation by CKII decreases Y1472 phosphorylation by Fyn, which underlies the switch from NR2B to NR2A at synapses during development [76]. Phosphorylation of S1480 was suppressed, while a concomitant increase in Y1472 phosphorylation of NR2B was observed after hypoxia-ischemia. This may be associated with post-HI brain damage as we demonstrated previously that sustained enhancement of NR2B phosphorylation at Y1472 is detrimental in neonatal HI injury [77]. Therefore, dysregulation of well-balanced postsynaptic signaling networks by hypoxia-ischemia could not only impair the normal synapse functionality and developmental plasticity, but also result in neuronal death and brain damage.

HI-induced changes in protein expression of postsynaptic scaffolds and cytoskeleton-associated proteins are another finding from this proteomic study. MAGUK protein PSD95, PSD93, and SAP102 are downregulated as proved by both MS and Western blotting. This is consistent with an earlier protein sequencing study in an adult rat model of transient cerebral ischemia [78]. Among the proteins that are recruited to PSD early after hypoxia-ischemia, Abl interactor, γ -adducin, band 4.1-like protein 1 [79], and dynamin-1 are all involved in the assembly of actin or the spectrin-actin network, the major cytoskeletal components in dendritic spines. These proteins play important roles in maintaining physical integrity and stability of the synapses. As summarized in Tables 3 and 4, hypoxia-ischemia triggered alterations in the abundance of numerous cytoskeleton molecules and their modulators. These changes in scaffold and cytoskeleton proteins may be indicative of synapse disassem-

bly/degeneration and dysfunction following neonatal HI injury.

In conclusion, proteomic profiling of PSD proteins of the neonatal mouse brain in this study offers useful information on postsynaptic molecular organization in the developing brain. Early brain injury reflecting on the modifications of PSD includes recruitment of proteins into the apparatus, regulation of neurotransmitter receptor phosphorylation, activation of overlapping postsynaptic kinase signaling molecules and dysregulation of scaffold/adaptors and cytoskeleton proteins. Although these early biochemical changes precede the delayed neuronal death, the contribution of an individual protein to injury or defense mechanisms after HI injury remains to be determined. This work provided additional information on the recent efforts of understanding the molecular mechanisms of synaptic development and plasticity during normal brain maturation and following early brain injury. With the recent proteomic studies of human neocortex PSDs [80], a comparative proteomic study of human and mouse PSDs [81], and human brain after ischemic stroke [82], it would be feasible to characterize PSDs in the developing human brain and in patients suffering from HI encephalopathy, to aid early diagnosis and timely intervention to reduce HI encephalopathy mortality and long-term adverse consequences.

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