

i.p., respectively) 24 hours post-injury. Brain tissue sections (2-3 mm thick) were dissected from the core lesion area (including frontal cortex and striatal brain regions) of each animal for Western Blot or HTPI analysis.

#### 1. CSF (Cerebral Spinal Fluid)

**[0033]** The midline incision in the occipital region was made from 0.5 cm anterior to interauricular line of 4 cm in length. Exposing the atlanto-occipital dura mater by separating the nuchal muscles, the CSF was collected through a small hole made by an 18 G syringe needle. Usually 0.05-0.10 ml of the CSF could be obtained, if the injured brain was not significantly swollen. CSF samples were centrifuged at 2000 rpm at 4° C. for 10 min. The supernatant was collected into 1.5 ml tube and then stored in -80° C. for Western Blot and proteomics analysis.

#### 2. Brain Tissue

**[0034]** A 3-mm coronal section of brain tissue was dissected out from each rat brain (to include cortical and striatal tissue) starting at 5 mm posterior to the frontal pole. Immediately, the brain tissues were separated from ipsilateral (injured) and contralateral (uninjured) hemispheres and then stored in -80° C. for HTPI, proteomics and Western blot analysis.

#### High-Throughput Immunoblotting

**[0035]** The HTPI procedure was performed on injured brain tissue as a custom service by BD Biosciences Transduction Laboratories (Bringamham, Ky.). Briefly, placed 200 mg pooled protein in 5 ml lysis buffer (10 mM Tris pH 7.4, 1 mM Sodium Orthovanadate and, 1% SDS) on ice for 30 minutes, then sonicated and centrifuged at 3000 rpm 4° C. for 15 minutes. Added 2x sample buffer (25 mM Tris pH 6.8, 4% SDS, 10% Glycerol, 0.006% Bromphenol Blue, 2% b-mercaptoethanol) in to the samples and then loaded the samples into a 13x10 cm, 0.5 mm thick, 4-15% gradient SDS-polyacrylamide gel (Bio-Rad Criterion IPG well comb) and run for 1.5 hours at 150 volts. The protein samples were then transferred to Immobilon-P membrane for 2 hours at 200 mA. After transfer, the membrane was dried, re-wetted in methanol and blocked for one hour with blocking buffer. The membrane was next clamped in a Western blotting manifold to isolate the 40 channels. A complex antibody cocktail was added to each channel and allowed to hybridize for one hour at 37° C. The blot was removed from the manifold, washed and hybridized for 30 min at 37° C. with secondary goat anti-mouse conjugated to Alexa 680 fluorescent dye. Fluorescence detection was performed at a wavelength of 700 nm using an Odyssey infrared imaging system.

**[0036]** Each experimental group consisted of two pooled samples and was run in triplicate using a 3x3 matrix comparison of each of the five HTPI templates (A-E). The HTPI templates included 39 lanes with 4-7 monoclonal antibodies probed in each. A 40<sup>th</sup> lane was probed with molecular weight marker-antibodies. Raw and normalized signal intensity of HTPI data was performed for each blot using BD Biosciences proprietary software. In total, each sample group was probed with 998 well-characterized monoclonal antibodies to produce a protein profile of injured to non-injured brain for

comparison of PBBI and MCAo injuries. Data is reported as a fold-change increase or decrease between sham and injured samples for each injury type.

#### Western Blotting

**[0037]** Western blotting was performed on brain lysate and CSF samples (n=3-6 per group) as described previously (Chen et al., 1999) with individual protein antibodies taken from the HTPI panel. 100 ul of plasma with 200 ul lyses buffer or 100 mg brain tissue with 500 ul lyses buffer (20 mM Tris, pH7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% TritonX-100 and 1 mM DTT and 1x Protease inhibitor) were well mixed on ice for 30 mins, then sonicated 10 to 20 seconds. Protein lysate were centrifuged at 3000 rpm at 4° C. for 15 mins. Concentrations of the proteins in the preparations were determined using the BCA™ Protein Assay Kit (PIERCE, Rockford, Ill.). Twenty micrograms of each sample were loaded and separated by 4-20% SDS-polyacrylamide gradient gel electrophoresis and then transferred to an Immobilon-P membrane (Chen et al., 2003). After blocking for one hour in PBST (10 mM sodium phosphate, 0.9% NaCl and 0.1% Tween 20) containing 5% nonfat dry milk, blots were incubated overnight at 4° C. with the primary antibodies STAT3 (Cat# 610189), Tau (Cat# 610672), PKA<sub>R1B</sub> (Cat# 610625), 14-3-3H(Cat#610542), p43/EMAP-II precursor (Cat# 611884) (BD Bioscience Pharmingen, CA), respectively, in PBST containing 3% nonfat milk. Blots were washed four times in PBST (40 min) and incubated for one hour with horseradish to peroxidase-conjugated secondary antibody in PBST containing 3% nonfat dry milk. Immunoreactivities of the protein bands were detected by enhanced chemiluminescent autoradiography (ECL kit, Amersham Pharmacia Biotech, Piscataway, N.J.) as instructed by the manufacturer. A molecular weight standard (Bio-Rad Laboratories, Hercules, Calif.) was loaded in the last lane of each gel to assess relative molecular mass of detected bands.

#### Immunohistochemistry

**[0038]** Upon removal from the skull, rat brains were rapidly frozen in -70° C. isopentane pre-cooled with dry ice. Two cryostat sections (20 μm) were cut coronally through the cerebral cortex containing the striatum (approximately 5 mm from the frontal pole cf. the Rat Brain in Stereotaxic Coordinates by Paxinos & Watson, 1986). Every first and second section of each rat brain was mounted directly on Superfrost plus slides. The sections of the first set were stained with hematoxylin & eosin. The sections of the second set were processed for p43/EMAP-II-immunohistochemistry. Thus, sections were fixed in 0.1M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 30 minutes at 4° C., followed by washes in 0.01 M phosphate-buffered saline (PBS; pH 7.4). After inactivating the endogenous peroxidase activity with hydrogen peroxidase, sections were incubated separately with avidin and biotin solutions (Vector Lab, Burlingame, Calif.) for blocking nonspecific binding of endogenous biotin, biotin-binding protein and lectins. Sections were then incubated for overnight at 4° C. in 0.01 M PBS containing 1% normal goat serum, 4% BSA, 0.1% Triton X-100 (Sigma, St. Louis, Mo.) and a mouse monoclonal EMAP-II precursor antibody (BD Bioscience Pharmingen, CA). Subsequently, the immunoreaction product will be visualized according to the avidin-biotin complex method of Hsu et al. (1981) with the Vectastin elite ABC Peroxidase kit