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Unique Mechanisms of Sheng Yu Decoction (聖愈湯 Shèng Yù Tang) on Ischemic Stroke Mice Revealed by an Integrated Neurofunctional and Transcriptome Analysis

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ABSTRACT

Sheng Yu Decoction (聖愈湯 Shèng Yù Tang; SYD) is a popular traditional Chinese medicine (TCM) remedy used in treating cardiovascular and brain-related dysfunction clinically; yet, its neuroprotective mechanisms are still unclear. Here, mice were subjected to an acute ischemic stroke to examine the efficacy and mechanisms of action of SYD by an integrated neurofunctional and transcriptome analysis. More than 80% of the mice died within 2 days after ischemic stroke with vehicle treatment. Treatments with SYD (1.0 g/kg, twice daily, orally or p.o.) and recombinant thrombolytic tissue plasminogen activator (rt-PA; 10 mg/kg, once daily, intravenously or i.v.) both significantly extended the lifespan as compared to that of the vehicle-treated stroke group. SYD successfully restored brain function, ameliorated cerebral infarction and oxidative stress, and significantly improved neurological deficits in mice with stroke. Molecular impact of SYD by a genome-wide transcriptome analysis using brains from stroke mice showed a total of 162 out of 2081 ischemia-induced probe sets were significantly influenced by SYD. Mining the functional modules and genetic networks of these 162 genes revealed a significant upregulation of neuroprotective genes in Wnt receptor signaling pathway (3 genes) and regulation of cell communication (7 genes) and downregulation of destructive genes in response to stress (13 genes) and in the induction of inflammation (5 genes), cytokine production (4 genes), angiogenesis (3 genes), vasculature (6 genes) and blood vessel (5 genes) development, wound healing (7 genes), defense response (7 genes), chemotaxis (4 genes), immune response (7 genes), antigen processing and presenting (3 genes), and leukocyte-mediated cytotoxicity (2 genes) by SYD. Our results suggest that SYD could protect mice against ischemic stroke primarily through significantly downregulating the damaging genes involved in stress, inflammation, angiogenesis, blood vessel formation, immune responses, and wound healing, as well as upregulating the genes mediating neurogenesis and cell communication, which make SYD beneficial for treating ischemic stroke.

Key words: Genome-wide transcriptome analysis, Ischemic stroke, Microarray, Positron emission tomography, Sheng Yu Decoction

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INTRODUCTION

Ischemic stroke is ranked as the leading common cause of death in developed countries, even though some drop in death rates has been reported.[1] Excitotoxicity is the major pathophysiological mechanism mediating ischemic stroke–induced brain injury by excessive glutamate accumulation in the extracellular space through which ionotropic *N*-methyl-D-aspartate (NMDA) receptors in the brain are overactivated under stroke onset.[2,3] Excessive amount of glutamate excites neurons to death by inducing robust oxidative stress by overproduction of reactive oxygen and nitrogen species, such as hydroxyl radicals (OH^-), superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($OONO^-$).[3,4] The main free radical producing enzyme systems involved include mitochondria, cyclooxygenase (COX), xanthine oxidase, $gp91^{phox}$ (NOX2), and inducible nitric oxide synthase (iNOS) in response to the activation of proinflammatory mediators (e.g., cytokines) produced by recruited leukocytes (e.g., neutrophils, macrophages, and T-cells), active microglial cells, damaged neurons, and astrocytes in ischemic stroke–damaged tissues.[3,5] A key role for inflammatory cell activation in the development of ischemic stroke in humans has been highlighted.[6]

An inflammatory cascade is consequently activated in damaged tissue by ischemic stroke, leading to leukocyte infiltration and release of proinflammatory cytokines for amplification of inflammatory responses that mediate early blood–brain barrier (BBB) dysfunction following stroke.[7] Moreover, activation of inflammation-related transcriptional factor(s) plays a pivotal role in mediating oxidative stress–induced cell injury and in regulating post-ischemic inflammation, possibly through upregulation of inflammatory genes and proteins that contribute to cell death in cerebral ischemia.[8,9] Although thousands of studies provide important evidence in understanding the pathophysiology of ischemic stroke at the cellular, molecular, and animal levels,[10,11] the recombinant thrombolytic tissue plasminogen activator (rt-PA) has continued to be the only US Food and Drug Administration (FDA)-approved drug. According to clinical experience, rt-PA is limited by its serious side effects, narrow therapeutic time window, and is suitable only for a limited group of patients with acute ischemic stroke.[12] Therefore, searching for drugs with more efficacy and lower toxicity from alternative and complementary medicine would be a practical and important therapeutic strategy for the treatment of ischemic stroke.

In traditional Chinese medicine (TCM), many formulas have clinically been used for treating stroke-induced disability for centuries. As a classic TCM formula, Sheng Yu Decoction (聖愈湯 Shèng Yù Tang; SYD) has been used for improving cardiovascular and neurological functional recovery in stroke-induced disability in China.[13] Recent clinical study reported that integrative medicine using SYD together with Western medicine showed promising neuroprotective effects against ischemic stroke in 42 patients,[14] and also the protective effect of SYD on the apoptosis of neural cells after traumatic brain injury in rats.[15] However, how SYD could protect and improve neurological functions in ischemic stroke animals and the potential molecular mechanisms of action based on a genome-wide view remain unclear.

In the present study, we investigated the protective effects and underlying molecular mechanisms of action of SYD, and compared it with a well-known drug for stroke (i.e., rt-PA) on animal survival rate, neurological functions, infarction volume, biochemical and the genome-wild expression profiling in the transient focal cerebral ischemic mice brains.

MATERIALS AND METHODS

Preparation of SYD

Sheng Yu Decoction (聖愈湯 Shèng Yù Tang; SYD) was prepared using the method as given in our previous report[16] (mentioned in supporting information) including microscopic examination of the property of the crude drug and decoction pieces, molecular sequence of internal transcribed spacer (ITS) region of the crude drug, and the high performance liquid chromatography (HPLC) fingerprint of each herb and whole remedy preparation. Briefly, the ingredients of SYD, i.e., *Radix Rehmanniae* (raw) (生地黃 Shēng Dì Huáng), *Radix Rehmanniae Preparata* (熟地黃 Shóu Dì Huáng), *Rhizoma Chuanxiong* (川芎 Chuān Xiōng), *Radix Ginseng* (人參 Rén Shēn), *Radix Angelicae Sinensis* (當歸 Dāng Guī), and *Radix Astragali membranaceus* (黃耆 Huáng Qí), were mixed in order in the ratio 20:20:20:20:15:15 (dry weight). SYD was prepared by boiling with distilled water at 100°C for 30 min twice, and the drug solution was vacuum cool-dried and made into drug powder and dissolved with distilled water to a final concentration of 2.0 g/ml (equivalent to the dry weight of the raw materials). The chemical fingerprint [Figure 1] was determined using chromatographic separation carried out on a Thermo Syncronis C18 column (2.1 mm × 100 mm i.d., 1.7 μm) in Waters Acquity

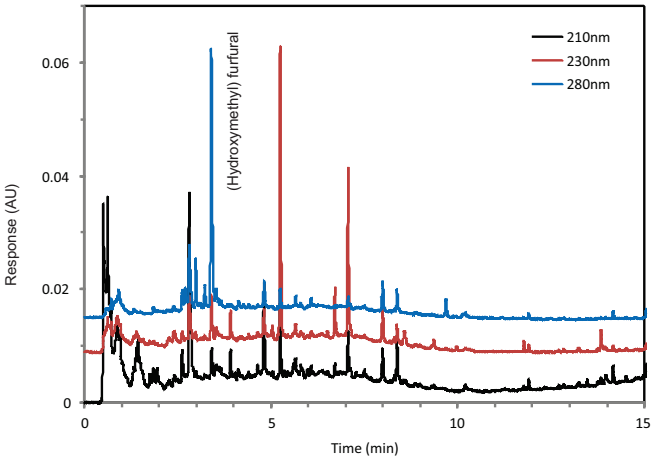

Figure 1. The representative chemical fingerprint of SYD product examined in this study. Chromatographic separation was carried out on a Thermo Syncronis C18 column (2.1 mm × 100 mm i.d., 1.7 μm) in Waters Acquity UPLC system with a diode array detector (DAD), monitored at 210, 230, and 280 nm. The peak [5-(hydroxymethyl) furfural] revealed at 3.4 min under 280 nm was used as a marker component. The mobile phase consisted of 0.1% phosphate water (A) and acetonitrile (B) using a gradient elution of 2% B at 0-1 min, 2-30% B at 1-10 min, and 30-70% B at 10-15 min. The flow rate was 0.4 ml/min, and the column temperature was maintained at 35°C

UPLC system equipped with a diode array detector (DAD), monitored at 230 nm. The mobile phase consisted of 0.1% phosphate water (A) and acetonitrile (B) using a gradient elution of 2% B at 0-1 min, 2-30% B at 1-10 min, and 30-70% B at 10-15 min. The flow rate was 0.4 ml/min, and the column temperature was maintained at 35°C.

Animals and induction of ischemic stroke injury

All animal procedures and protocols were performed in accordance with *The Guide for the Care and Use of Laboratory Animals* (NIH publication, 85-23, revised 1996) and were reviewed and approved by the Animal Research Committee at National Research Institute of Chinese Medicine. Ischemic stroke injury in mice was induced as reported previously,[17] with some modifications (NRICM-IACUC-P-99-011). In brief, male imprinting control region (ICR) mice weighing 28-32 g (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were anesthetized with a mixture of isoflurane (1.5-2%), oxygen, and nitrogen. A fiberoptic probe was attached to the parietal bone, 2 mm posterior and 5 mm lateral to bregma, and connected to a laser Doppler flowmeter (MBF3; Moor Instruments Ltd, Millwey, Axminster, UK) for continuous monitoring of cerebral blood flow (CBF). For the induction of ischemic stroke**,** right middle cerebral artery (RMCA) occlusion in mice was performed using a heat-blunted monofilament surgical suture (6-0, around 100 μm in diameter) which was inserted into the exposed external carotid artery, further advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the RMCA. The filament was left in place for 30 min and then withdrawn. Only animals that exhibited a reduction in CBF > 85% during RMCA occlusion and a CBF recovery by > 80% after 10 min of reperfusion were included in the study. This procedure leads to reproducible infarcts similar in size and distribution to those reported by others using transient RMCA occlusion of comparable duration.[17] Rectal temperature was monitored and kept constant (37.0 ± 0.5°C) during the surgical procedure and in the recovery period until the animals regained full consciousness. The experimental grouping was designed as described below. Additional animals (as indicated in each result) from the groups as described were used for other assays including analysis of survival rates, positron emission tomography (PET), free radical production in living mice, and transcriptome analysis.

Drug administration and animal grouping

The mice were randomly divided into the following groups (n = 20 in each group) including sham control, stroke, stroke + SYD (1.0 g/kg, p.o., twice daily), and stroke + rt-PA (10 mg/kg, intravenously or i.v., once daily; Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany). Two hours after ischemic stroke induction, the mice were treated with SYD (p.o.) or rt-PA (i.v.), or vehicle control distilled water (sham and stroke groups) daily. All animals were free to move and take food.

Assessment of neurological deficit scoring and analysis of survival rates

The neurological deficit of mice was determined just before the sacrifice on day 1 after stroke by analyzing their tracking distance and appearance of the typical behavior pattern (circling clockwise) related to ischemic stroke within 3 min in an observation box (60 × 60 × 60 cm^3) using a video-tracking system software (SMART v2.5.21 Barcelona; Panlab, Spain). For survival rate analysis, mice were kept in isolators (individually ventilated cage systems) after stroke induction, given food and water *ad libitum*, and kept at 22 ± 2°C with alternating 12 h periods of light and dark. Survival rates were calculated immediately (day 0) and within 7 days after stroke induction.

Evaluation of infarct volume

Twenty-four hours after ischemic reperfusion, the mice were sacrificed by rapid decapitation under deep anesthesia. The whole brain was rapidly removed. Immediately after being weighed, the brain was sliced into 2-mm-thick coronal sections and stained with 2% 2,3,5-triphenyltetrazoliumchloride (TTC, Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C in the dark, followed by fixation with 10% of formalin at room temperature (25°C) overnight. Brain slices lacking red staining defined the infarct area. The slices were photographed with a digital camera and analyzed by an image processing system (AlphaEaseFC 4.0; Alpha Innotech, San Leandro, CA, USA). Infarct volume was obtained according to the indirect method proposed by Swanson *et al*.[18] and corrected for edema by comparing the volume of ischemic and nonischemic hemispheres as described by Lin *et al*.[19] The infarct volume was expressed as mm^3 of the whole brain volume.

A PET evaluation of the brain function

Cerebral glucose metabolism was measured to evaluate the brain function after stroke. Animals were injected with 100 μCi of 2-deoxy-2-(F-18) fluoro-D-glucose [(F-18) FDG] and imaged on a small animal PET scanner (μPET; Concorde Microsystems, Knoxville, TN). Images were acquired for 10 min under inhalation anesthesia (isoflurane 2%). The level of radioactivity in brain tissue (percentage dose per gram) was estimated from the images according to the method published by Hsieh *et al*.[20]

Quantification of oxidative stress in living mice

Dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA; 2.0 mg in 200 μl normal saline) was given i.v. to mice just before the onset of reperfusion for *in vivo* determination of free radical production in living mice. Reactive oxygen species (ROS) production was monitored at 24 h after reperfusion using the IVIS® Imaging System 50 Series (Xenogen Corp., Alameda, CA, USA) under 488-nm excitation light. Living Image software (Xenogen Corp.) was used to analyze the bioluminescence in the brain. Data were expressed as photon flux in a region of interest (ROI). All the animals were imaged under inhalation anesthesia (isoflurane 2%). Signal intensities from ROIs were defined manually and expressed as photon flux (photons/sec/cm/sr), where steradian (sr) refers to the photons emitted from a unit solid angle of a sphere. Background photon flux was defined from an ROI of the same size drawn over the thorax of each animal, and these data were subtracted from the signal intensities measured the at sites of injury. Area under the curve (AUC) analyses were performed with AlphaEaseFC software (version 4.0; Alpha Innotech Corporation, San Leandro, CA, USA) and were expressed as the AUC of photon flux.

Array data sets, array probe preparation, and data processing

Twenty-four hours after induction of stroke, brains of sham-operated control mice, ischemic stroke mice, and SYD-treated mice were subjected to total RNA extraction and microarray hybridization. In each group, the RNAs from six different mice were hybridized onto two different chips to have biological replica. The Affymetrix™ Mouse Genome 430 2.0 chips were used. Robust multi-array analysis (RMA). log expression units were calculated from Affymetrix GeneChip array data using the "affy" package of the Bioconductor (http://www.bioconductor.org/) suite of software for the R statistical programming language (http://www.r-project.org/). The default RMA settings were used to background correct, normalize, and summarize all the expression values. Significant difference between sample groups was identified using the "limma" package of the Bioconductor. To control the multiple testing errors, a false discovery rate (FDR) algorithm was then applied to these *P* values to calculate a set of *q*-values: Thresholds of the expected proportion of false positives, or false rejections of the null hypothesis. Heatmap was created by the dChip software (http://biosun1.harvard.edu/complab/dchip/). Principal component analysis (PCA) was performed by the Partek Genomics Suite (http://www.partek.com/) to provide a visual impression of how the various sample groups were related. Gene annotation was performed by the ArrayFusion web tool (http://microarray.ym.edu.tw/tools/arrayfusion/). Gene Ontology (GO) database search were performed by the DAVID Bioinformatics Resources 6.7 interface (http://david.abcc.ncifcrf.gov/).

Statistical analysis

All values in the text and figures are presented as the mean ± SEM. Data, except indicated, were analyzed by one-way or two-way analysis of variance (ANOVA) depending on the number of parameters for comparison, followed by *post-hoc* Student–Newman–Keuls (SNK) *t*-test for multiple comparisons.

RESULTS

Effects of SYD on survival rate and cerebral infarction after ischemic stroke

Most of the mice (>80%) died within 2 days after stroke induction with vehicle (distilled water) treatment, but treatment with SYD (1.0 g/kg, twice daily, p.o.) and rt-PA (10 mg/kg, once daily, i.v.) both enhanced the survival rate as compared to vehicle (distilled water)-treated stroke group. This was especially true on day 1 after stroke [Figure 2] ($P < 0.05$). The infarct volume induced by ischemic stroke on day 1 after stroke (60 ± 11 mm^3, around 40% of the whole brain) was comparable with previous reports.[17] Treatment with SYD (1.0 g/kg) and rt-PA significantly decreased the stroke-induced cerebral infarction by 67% and 43%, respectively [Figure 3] ($P < 0.05$). The hemodynamic and arterial blood gas measurements showed no significant differences before, during, or after the experiments among these groups (data not shown). Neurological deficit scoring was measured on day 1 after stroke by determining the tracking distance within 3 min in a box; the distance (cm) of mice with ischemic stroke injury (510 ± 180) was significantly lower than those in the sham-operated group (1510 ± 120) [Figure 4] (one-way ANOVA,

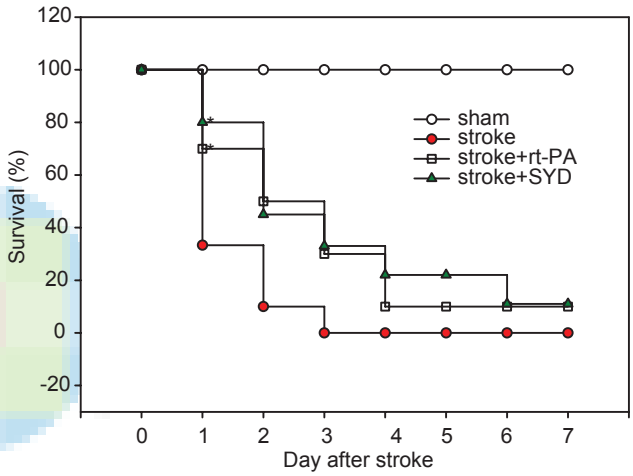

Figure 2. Protective effect of SYD on the survival rate in ischemic stroke-injured mice within 7 consecutive days. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o.; +SYD), and rt-PA treated (10 mg/kg, daily, i.v.; +rt-PA). Survival curves were computed using the Kaplan–Meier method. Differences in survival rates were assessed using the log-rank test followed by Holm–Sidak method for all pairwise multiple comparisons. *$P < 0.05$ as compared to stroke group analyzed by one-way ANOVA followed by SNK *t*-test ($n = 10$ for each group)

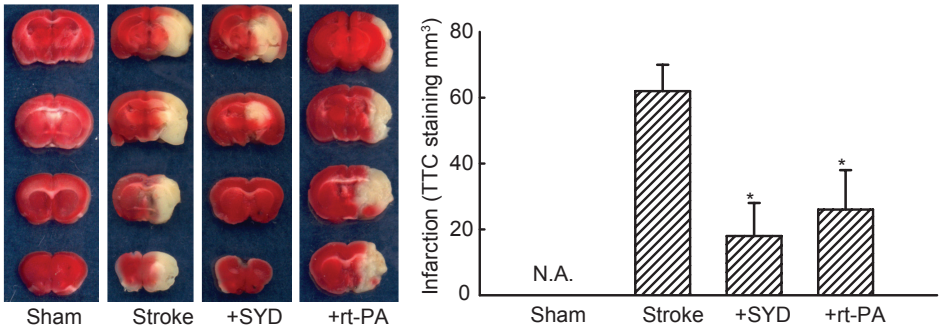

Figure 3. Protective effect of SYD on cerebral infarction at 24 h after ischemic stroke. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o., +SYD), and rt-PA treated (10 mg/kg, daily, i.v., +rt-PA). *$P < 0.05$ as compared to stroke group analyzed by one-way ANOVA followed by SNK *t*-test ($n = 10$ for each group). NA, data not available

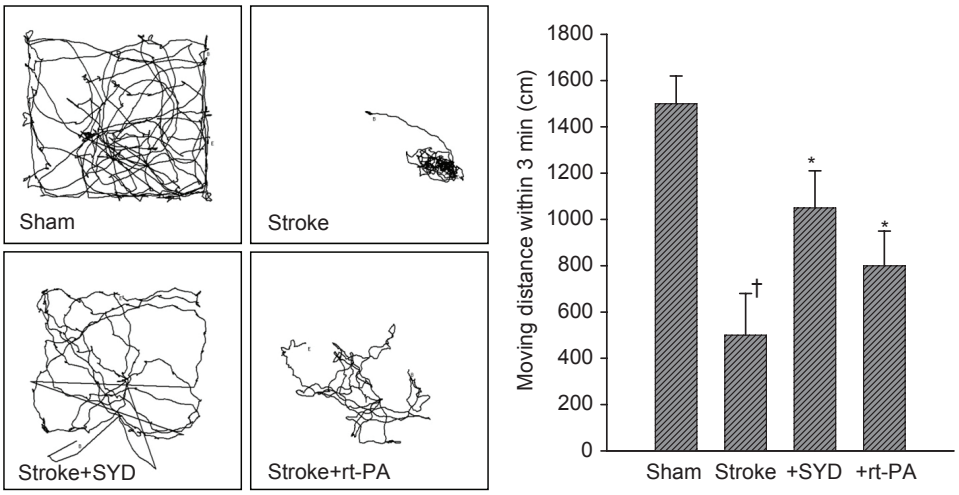

Figure 4. Protective effect of SYD on neurological deficit scoring at 24 h after ischemic stroke in live mice. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o.; +SYD), and rt-PA treated (10 mg/kg, daily, i.v.; +rt-PA). Left panel: Representative neurological deficit scoring by determining the tracking distance and profiling within 3 min in a box, measured on day 1 after ischemic stroke. Right panel: Statistical analysis. †,*$P < 0.05$ as compared to sham or stroke group, respectively, analyzed by one-way ANOVA followed by SNK *t*-test (n = 3-5 for each group)

$P < 0.05$). Treatment with SYD and rt-PA both significantly enhanced the tracking distance (cm) to 1050 ± 160 and 800 ± 150, respectively [Figure 4] (one-way ANOVA, $P < 0.05$). Besides, the typical neurological deficit behavior (circling clockwise) induced by ischemic stroke was significantly ameliorated by SYD and rt-PA [Figure 4].

Effects of SYD on brain function and oxidative stress of living mice after stroke

To determine the neurofunctional effect of SYD, the brain image after stroke was examined by measuring the glucose metabolism in the brain of living mice using μPET imaging system. To determine the brain oxidative stress generated from free radical production, oxidative stress was monitored by DHE fluorescence in the brain of living mice using IVIS imaging system. In this study, ischemic stroke injury dramatically impaired glucose metabolism [Figure 5] and produced a large amount of free radical (as assayed by IVIS imaging) [Figure 6] in living mice, which may create enormous oxidative stress to damage brain tissues after stroke. Treatment with SYD and rt-PA both significantly ameliorated brain function compromised by ischemic stroke [Figure 5]. SYD and rt-PA both significantly reduced free radical production on day 1 after stroke [Figure 6].

Molecular impacts of SYD on ischemic mice brain

To provide more insight into the *in vivo* influences of SYD on ischemic mice brain, a genome-wide transcriptome analysis was performed. A total of 162 out of 2081 ischemia-influenced probe sets were found significantly reversed by the SYD using transcriptome analysis [Figure 7a]. A PCA plot using these 162 probe sets illustrated the differential gene expression patterns between different mice groups. The gene expression pattern of SYD-treated mice was closer to that of sham mice [Figure 7b]. On measuring the average linkage distances between mice transcriptomes and

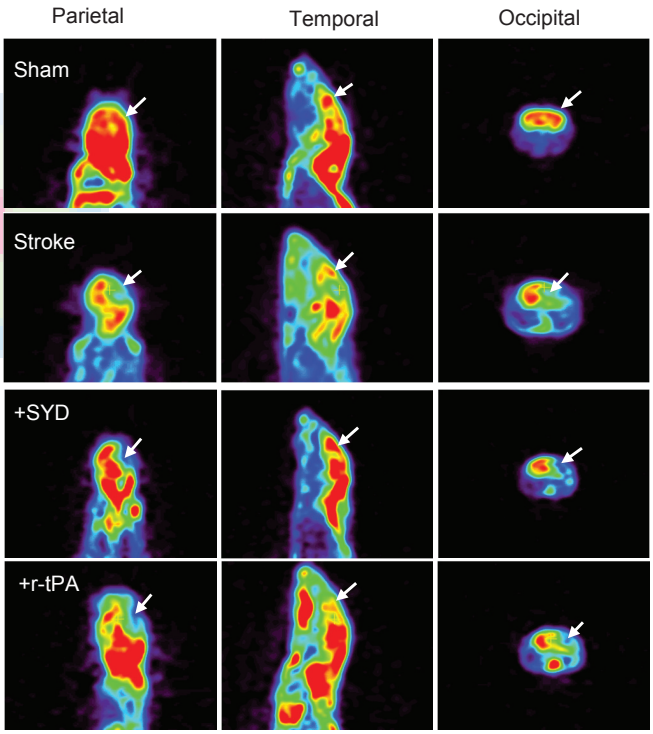

Figure 5. A representative μPET analysis of brain function (glucose metabolism) in live mice at 24 h after stroke. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o., +SYD), and rt-PA treated (10 mg/kg, daily, i.v., +rt-PA). Arrows indicate ischemic brain areas for comparison

comparing with ischemic mice, SYD-treated ones were found to be closer to sham controls [Figure 7c]. A heat map shows 64 probe sets were commonly more abundant in SYD-treated and sham mice, while another 98 ischemia-induced probe sets were suppressed by SYD [Figure 7d]. The top 50 up- and downregulated genes are listed in Tables 1 and 2, respectively.

Table 1. The top 50 up-regulated known genes in ischemic stroke mice treated with remedy SYD

Probe Set ID	UniGene ID	Gene title	Gene symbol	Location
1427371_at	Mm.439858	ATP-binding cassette, sub-family A (ABC1), member 8a	Abca8a	chr11 E1
1421024_at	Mm.8684	1-acylglycerol-3-phosphate O-acyltransferase 1 expressed	Agpat1	chr17 B1|17 18.7 cM
1435417_at	Mm.27054	sequence AI464131	AI464131	chr4 A5
1436258_at	Mm.44841	Cdc42 guanine nucleotide exchange factor (GEF) 9	Arhgef9	chrX C3
1429190_at	Mm.300178	arylsulfatase B	Arsb	chr13 C3-D1|13 50.0
1426336_at	Mm.214994	calcium channel, voltage-dependent, gamma subunit 7	Cacng7	cM
1442707_at	Mm.131530	calcium/calmodulin-dependent protein kinase II alpha	Camk2a	chr7 A1|7 2.0 cM
1452453_a_at	Mm.131530	calcium/calmodulin-dependent protein kinase II alpha	Camk2a	chr18 E1|18 33.0 cM
1441572_at	Mm.167882	deleted in colorectal carcinoma	Dcc	chr18 E1|18 33.0 cM
1451751_at	Mm.250841	DNA-damage-inducible transcript 4-like	Ddit4l	chr18 E2|18 45.0 cM
1419581_at	Mm.27256	discs, large homolog 4 (Drosophila) distal-less homeobox 1,	Dlg4	chr3 G3
1419845_at	Mm.4543	antisense desmoplakin	Dlx1as	chr11 B4
1435494_s_at	Mm.355327	Enhancer of polycomb homolog 1 (Drosophila) Fibronectin	Dsp	chr2 C2|2 44.0 cM
1442279_at	Mm.312133	leucine rich transmembrane protein 2 frizzled-related	Epc1	chr13 A3.3
1447204_at	Mm.341948	protein	Flrt2	chr18 A1 chr12 E
1416658_at	Mm.427436	frizzled homolog 2 (Drosophila)	Frzb	chr2 C3|2 49.75 cM
1418533_s_at	Mm.36416	GC-rich promoter binding protein 1-like 1	Fzd2	chr11 E1 chr4 C7
1449113_at	Mm.41715	G protein-coupled receptor 34	Gpbp1l1	chrX
1422542_at	Mm.391232	Glutaminase	Gpr34	A1.3
1435708_at	Mm.398608	inhibitor of DNA binding 4	Gls	chr1 C1.1|1 25.9 cM
1423259_at	Mm.458006	imprinted gene in the Prader-Willi syndrome region	Id4	chr13 B|13 31.0 cM
1431229_at	Mm.335715	kalirin, RhoGEF kinase	Ipw	chr7|7 28.0 cM
1442341_at	Mm.441119	Kv channel-interacting protein 2	Kalrn	chr16 B3
1425870_a_at	Mm.213204	lin-7 homolog A (C. elegans)	Kcnip2	chr19 D1|19 45.2 cM
1438450_at	Mm.268025	membrane-associated ring finger (C3HC4) 6	Lin7a	chr10 D1
1445928_at	Mm.272185	RAB3C, member RAS oncogene family	Mar6	chr15 B2 chr13 D2.2
1432432_a_at	Mm.390643	RAB3C, member RAS oncogene	Rab3c	chr13 D2.2
1449494_at	Mm.390643	poliovirus receptor-related 3	Rab3c	chr16 B5 chr6 C3
1423331_a_at	Mm.328072	leucine rich repeat transmembrane neuronal 4	Pvrl3	chr6
1437214_at	Mm.94135	LUC7-like 2 (S. cerevisiae)	Lrrtm4	B1
1436767_at	Mm.276133	mitogen-activated protein kinase kinase kinase 5	Luc7l2	chr10 A3
1439830_at	Mm.6595	Mitochondrial ribosomal protein S18A	Map3k5	chr17 C
1457813_at	Mm.287443	nuclear factor I/A	Mrps18a	chr4 C4-C6|4 45.8
1421163_a_at	Mm.31274	netrin G1	Nfia	cM
1441634_at	Mm.39262	O-linked N-acetylglucosamine (GlcNAc) transferase	Ntng1	chr3 G1
1436780_at	Mm.259191	oxysterol binding protein-like 3	Ogt	chrX D
1428484_at	Mm.44153	p53-associated parkin-like cytoplasmic protein	Osbpl3	chr6 B3
1427620_at	Mm.329076	protocadherin 20	Parc	chr17 C
1424701_at	Mm.128861	phosphatidylinositol transfer protein, cytoplasmic 1	Pcdh20	chr14 E1
1431074_a_at	Mm.439910	poliovirus receptor-related 3	Pitpnc1	chr11 E1
1448673_at	Mm.328072	R3H domain 1 (binds single-stranded nucleic acids)	Pvrl3	chr16 B5
1458539_at	Mm.221041	solute carrier organic anion transporter family, member 1c1	R3hdm1	chr1 E4
1423343_at	Mm.284495	transducin-like enhancer of split 1, homolog of Drosophila	Slco1c1	chr6 G1
1422751_at	Mm.278444	E(spl)	Tle1	chr4 C3
1432130_a_at	Mm.275710	tetratricopeptide repeat domain 14	Ttc14	chr3 B
1415694_at	Mm.38433	tryptophanyl-tRNA synthetase	Wars	chr12 F2
1436485_s_at	Mm.300397	whirlin	Whrn	chr4 C1|4 31.4 cM

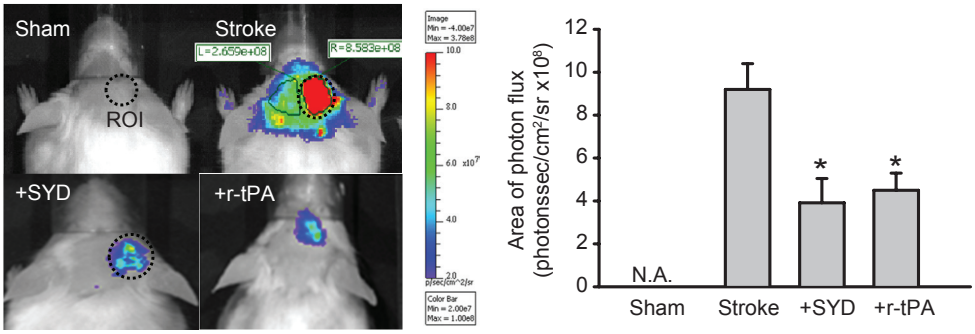

Figure 6. IVIS imaging of free radical production in live mice at 24 h after stroke. Animal groups include sham, vehicle treated (stroke), SYD treated (1.0 g/kg, twice daily, p.o., +SYD), and rt-PA treated (10 mg/kg, daily, i.v., +rt-PA). Left panel: Representative IVIS imaging for free radical production; ROI, region of interest. Right panel: Statistical analysis. *$P < 0.05$ as compared to stroke group analyzed by one-way ANOVA followed by SNK *t*-test ($n = 10$ for each group). NA, data not available

Table 2. The top 50 down-regulated genes in ischemic stroke mice treated with remedy SYD.

Probe Set ID	UniGene ID	Gene title	Gene symbol	Location
1419706_a_at	Mm.27481	A kinase (PRKA) anchor protein (gravin) 12	Akap12	chr10 A1
1451675_a_at	Mm.302724	aminolevulinic acid synthase 2, erythroid	Alas2	chrX F3|X 63.0 cM
1448789_at	Mm.140988	aldehyde dehydrogenase family 1, subfamily A3	Aldh1a3	chr7 C
1417130_s_at	Mm.196189	angiopoietin-like 4	Angptl4	chr17 B1
1460330_at	Mm.7214	annexin A3	Anxa3	chr5 E3|5 54.0 cM
1457827_at	Mm.317021	arylsulfatase J	Arsj	chr3 G1
1425658_at	Mm.32955	CD109 antigen	Cd109	chr9 E1
1419144_at	Mm.37426	CD163 antigen like family member F	Cd163	chr6 F2
1449130_at	Mm.1894	CD1d1 antigen	Cd1d1	chr3 F1|3 48.0 cM
1427994_at	Mm.277387	cyclin-dependent kinase inhibitor 1A (P21)	Cd300lf	chr11 E2
1424638_at	Mm.195663	CEA-related cell adhesion molecule 1	Cdkn1a	chr17 A3.3|17 15.23 cM
1460682_s_at	Mm.439731	chloride channel calcium activated 1	Ceacam1	chr7 A3|7 5.5 cM
1417852_x_at	Mm.454553	claudin 5	Clca1	chr3 H2-H3|3 72.5 cM
1417839_at	Mm.22768	cytotoxic T lymphocyte-associated protein 2 alpha	Cldn5	chr16 A3|16 11.65 cM
1448471_a_at	Mm.30144	cytotoxic T lymphocyte-associated protein 2 beta	Ctla2a	chr13 B2|13 36.0 cM
1452352_at	Mm.439734	chemokine (C-X-C motif) ligand 1	Ctla2b	chr12 C1|13
1419209_at	Mm.21013	endomucin	Cxcl1	chr5 E-F|5 51.0 cM
1425582_a_at	Mm.27343	epithelial membrane protein 3	Emcn	chr3 G3
1417104_at	Mm.20829	Fc receptor, IgG, low affinity III	Emp3	chr7 B4|7 24.5 cM
1448620_at	Mm.22119	Fc receptor, IgG, low affinity IV	Fcgr3	chr1 H3|1 92.3 cM chr1
1425225_at	Mm.251254	G protein-coupled receptor 151	Fcgr4	H3|1 92.29 cM chr18
1457555_at	Mm.186779	G protein-coupled receptor 84	Gpr151	B3
1420591_at	Mm.160369	histocompatibility 2, class II antigen A, alpha	Gpr84	chr15 F3
1435290_x_at	Mm.235338	hemoglobin alpha, adult chain 1	H2-Aa	chr17 B1|17 18.65 cM
1417714_x_at	Mm.196110	high mobility group AT-hook 2	Hba-a1	chr11 A4|11 16.0 cM
1422851_at	Mm.157190	inhibitor of DNA binding 1	Hmga2	chr10 D2|10 67.5 cM
1425895_a_at	Mm.444	interferon-induced protein with tetratricopeptide repeats 2	Id1	chr2 H1|2 84.0 cM
1418293_at	Mm.2036	interferon induced transmembrane protein 6	Ifit2	chr19 C1
1440865_at	Mm.276440	lipocalin 2	Ifitm6	chr7 F5
1427747_a_at	Mm.9537	lymphocyte antigen 6 complex, locus C1	Lcn2	chr2 A3|2 27.0 cM
1421571_a_at	Mm.458089	mitogen-activated protein kinase 12	Ly6c1	chr15 D3
1449283_a_at	Mm.38343	membrane-spanning 4-domains, subfamily A, member 6B	Mapk12	chr15 E3 chr19 B chr14
1418826_at	Mm.436639	PDZ and LIM domain 2	Ms4a6b	D2
1423946_at	Mm.283968	proviral integration site 1	Pdlim2	chr17 A3.3|17 16.4 cM
1435458_at	Mm.328931	plasminogen activator, urokinase receptor	Pim1	chr7 A3
1452521_a_at	Mm.1359	proline-serine-threonine phosphatase-interacting protein 1	Plaur	chr9 C
1424560_at	Mm.2534	RNA binding motif protein 3	Pstpip1	chrX A1.1|X 2.0 cM
1422660_at	Mm.128512	ras homolog gene family, member J	Rbm3	chr12 C3
1418892_at	Mm.27467	ribosomal protein L7-like 1	Rhoj	chr17 C
1439780_at	Mm.218533	S100 calcium binding protein A4	Rpl7l1	chr3 F1-F2|3 43.6 cM
1424542_at	Mm.3925	S100 calcium binding protein A6 (calcyclin)	S100a4	chr3 F1-F2|3 43.6 cM
1421375_a_at	Mm.100144	S100 calcium binding protein A8 (calgranulin A)	S100a6	chr3 F1-F2|3 43.6 cM
1419394_s_at	Mm.21567	S100 calcium binding protein A9 (calgranulin B)	S100a8	chr3 F1-F2|3 43.6 cM
1448756_at	Mm.2128	solute carrier family 10, member 6	S100a9	chr3 F1-F2|3 43.6 cM
1428776_at	Mm.7446	sphingomyelin phosphodiesterase, acid-like 3B	Slc10a6	chr5 E5
1417300_at	Mm.287187	SET and MYND domain containing 4	Smpdl3b	chr4 D2.3
1442308_at	Mm.340377	serglycin	Smyd4	chr11 B5 chr10 B4
1417426_at	Mm.338790	transmembrane protein 40	Srgn	chr6 E3
1424966_at	Mm.29739	xanthine dehydrogenase	Tmem40	chr17 E2|17 45.3 cM
1451006_at	Mm.11223		Xdh	

Coordinated changes of functional modules in SYD-treated mice

We next organized SYD-affected genes into functional groups for having better insight into the biological consequences of gene expression changes. According to the GO database, SYD treatment significantly enriched genes involved in the regulation of cell communication and Wnt receptor signaling pathway ($P = 0.01$ and 0.04, respectively) [Table 3]. Seven genes related to the regulation of cell communication and three Wnt receptor signaling pathway genes were specifically induced in the brains of ischemic mice by SYD [Table 3]. Among the SYD-downregulated genes, genes' response to stress (13 genes, enrichment P value = 0.0015), wounding (7 genes, enrichment P value = 0.0021), inflammation (5 genes, $P = 0.0110$), development of vasculature (6 genes, $P = 0.0027$) and blood vessel (5 genes, $P = 0.0145$), angiogenesis (3 genes, $P = 0.0130$), defense response (7 genes, $P = 0.0074$), chemotaxis (4 genes, $P = 0.0086$), immune response (7 genes, $P = 0.0094$), cytokine production (4 genes, $P = 0.0165$), antigen processing and presentation (3 genes, $P = 0.0445$), and leukocyte-mediated cytotoxicity (2 genes, $P = 0.0454$) was reverted by SYD [Table 3]. The details of enriched GO categories, as well as the genes involved can be found in Tables 1-3.

Table 3. Functional module analysis for SYD-induced neuroprotection in ischemic stroke mice.

SYD-induced GO terms	Count	%	*P* value
Regulation of cell communication	7	12.96296	0.010505
Wnt receptor signaling pathway	3	5.555556	0.040555
SYD-repressed GO terms			
Response to stress	13	16.88312	0.001596
Response to wounding	7	9.090909	0.002142
Vasculature development	6	7.792208	0.002744
Defense response	7	9.090909	0.007438
Chemotaxis	4	5.194805	0.00861
Immune response	7	9.090909	0.009402
Inflammatory response	5	6.493506	0.011088
Regulation of angiogenesis	3	3.896104	0.013076
Blood vessel development	5	6.493506	0.014564
Regulation of cytokine production	4	5.194805	0.016565
Antigen processing and presentation	3	3.896104	0.044586
Leukocyte mediated cytotoxicity	2	2.597403	0.045459

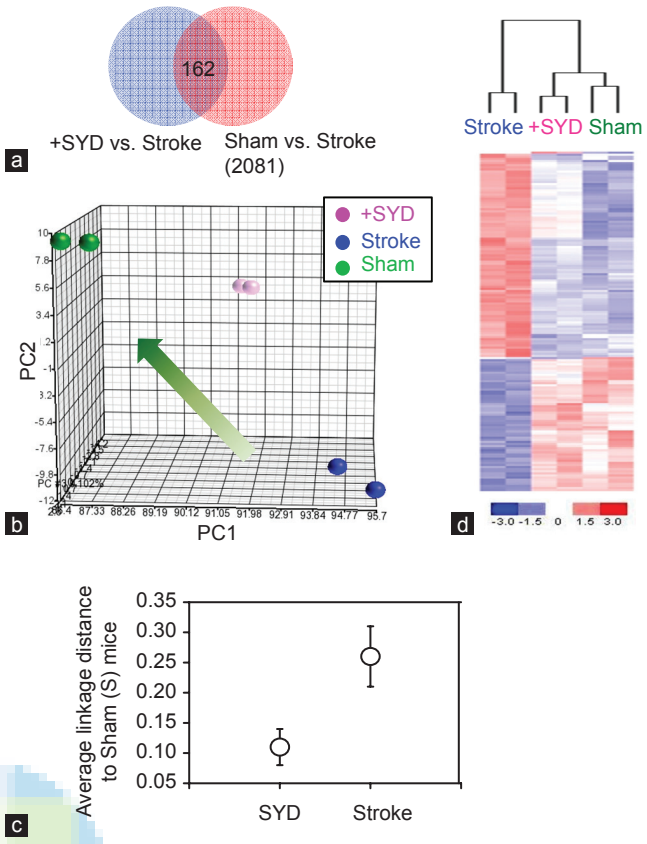

Figure 7. Transcriptome analyses on ischemic stroke and SYD-treated mice. (a) A Venn diagram illustrates there were 162 probe sets deregulated in ischemic stroke mice, but were rescued by the SYD treatment. (b) A PCA plot using the 162 probe sets. (c) Average linkage analysis using the above 162 probe sets shows the relationships between sham, stroke-induced, and SYD-treated mice. (d) A heat map shows the upregulation (in red) and downregulation (in blue) patterns of the 162 probe sets

DISCUSSION

Although SYD has been reported to be neuroprotective against brain injury in clinical and animal study,[13-15] its mechanism of action based on a brain functional and a genome-wide transcriptome analysis has not been revealed. Our results demonstrate for the first time that treatment with SYD (1.0 g/kg, p.o., twice daily) shows a protective effect against ischemic stroke in mice and significantly extends their survival rate and lifespan, as compared with vehicle-treated ischemic stroke mice. The neuroprotective effect of SYD is effective up to at less 3 days after ischemic injury (around 40% survival), and is more potent than rt-PA in the improvement of neurological function, indicating that novel mechanism(s) or targets could be involved in the neuroprotective effects of SYD on ischemia-induced brain injury. Here, we reveal the brain protective effect of SYD in living mice that parallels with significant improvement in brain function (by PET imaging) and neurological deficits, as well as reduction of inflammation and oxidative stress (by IVIS imaging) without significant modulation of the hemodynamic, arterial blood gas, or physiological conditions.

The major pathological mechanisms leading to ischemic brain injury include ionic imbalance, oxidative/nitrosative stress, inflammatory responses, and apoptotic cell death, and all these mechanisms have been linked to excitotoxicity, an inappropriate activation of ionotropic NMDA receptors.[2,21] In this study, our results showed that ischemia induced a dramatic production of free radicals, which could be measured by oxidized DHE in living mice (by IVIS). Treatment with SYD dramatically reduced the oxidative stress and spared the tissue against ischemia-induced brain infarction. An inflammatory cascade is initiated in the tissue damaged by free radicals, which leads to complement activation (e.g.,C5a), firm adhesion molecule upregulation, microglial activation, leukocyte infiltration, chemotaxis, and release of pro-inflammatory cytokines that may amplify inflammation through activation of inflammatory signals (e.g., transcription factors) for induction of brain infarction.[4,22]

A pattern recognition analysis illustrated that SYD treatment reversed ischemia-induced brain damage at a molecular level (162 genes reversed) [Figure 7]. Among these genes modulated by SYD, proinflammatory cytokine/chemokine receptor, cytotoxic T lymphocyte–associated proteins and genes promoting inflammation, such as *CxCl1, Fcgr3, Fcgr4, Ctla2a, Ctla2b, Ifit2, Ifitm6, CD109, CD163, CD1d1, and CD3001f*, were suppressed by SYD. We showed for the first time by GO database-based gene set enrichment analysis that SYD also upregulated a neuroprotective gene, *camk2a*, which is involved in the calcium signaling pathway. The gene product CaMKII is a member of calcium/calmodulin-dependent kinase (CaMK) cascade, which is well-established for its effects on modulating neuronal synaptic plasticity and learning and memory.[23] SYD also significantly potentiated the expression of a protective factor in the damaged area [e.g.,frizzled-related protein (Frzb, alias Sfrp3), a secreted protein activating the Wnt survival and proliferating pathway, was induced by SYD] [Tables 1 and 3]. On the other hand, genes involved in wounding, stress, and acute inflammation were reverted by SYD [Table 3]. Leukocyte-mediated cytotoxicity, immune response, and chemotaxis genes were all reduced after SYD treatment, implying the prevention of leukocyte infiltration in damaged regions, as well as

the rescue of inflammation response and prevention of neuron cells from inflammation-associated cell death. Furthermore, S100 calcium-binding proteins (e.g.,S100a9) have been reported to display many proinflammatory functions and act as damage-associated molecular pattern molecules.[24] Here, we observed that S100a4, S100a6, S100a8, and S100a9 were all significantly downregulated [Table 2], indicating that damaged BBB and inflammation could be compromised by SYD. Similar observation using DNA microarray chips containing 512 cDNA probe also identified S100a9 as one of the six potential targets downregulated by an effective TCM remedy.[25]

Angiogenesis genes such as vascular endothelial growth factor (VEGF) and its receptor Flk1 have been reported to be upregulated by an effective TCM remedy for stroke protection in rats on days 7-14 after stroke.[26] In our array analysis, however, blood vessel and vasculature development as well as angiogenesis genes were less active in SYD-treated mice [Tables 1 and 2]. This could be due to the upregulation of VEGF signaling, mediating increased BBB permeability,[27] possibly by enhancing VEGF-mediated activation of Rac-1 caused free radical generation,[28] and anti-VEGF receptor antagonist (VGA1155) has been demonstrated to reduce infarction in rat permanent focal brain ischemia.[29] According to these observations, we propose that suppression of angiogenesis-related pathways by SYD may be due to the fact that at the time point (day 1 after stroke) we harvested brain RNA for transcriptome analysis, ischemia-induced inflammatory responses, especially BBB leakage, may have been ameliorated by SYD *in vivo*, so blood vessel formation is no longer active *in vivo*.

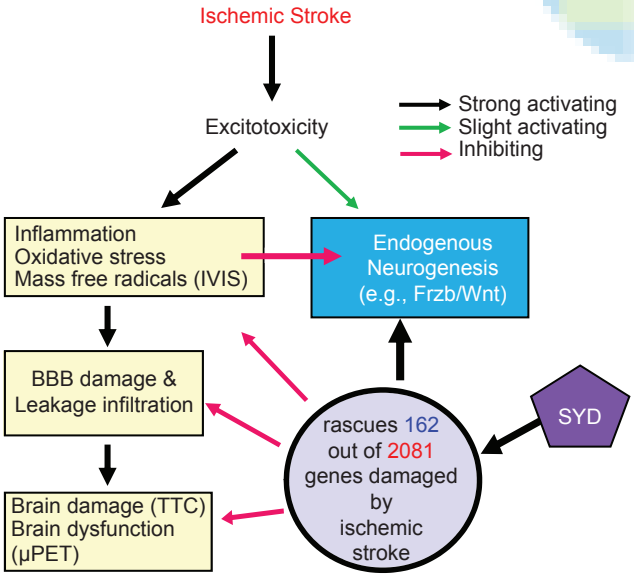

Figure 8. SYD reverses 162 out of 2081 genes impaired by ischemic stroke to protect mice against ischemic stroke. Ischemic stroke damages neurons by triggering excitotoxicity through inducing strong inflammation and oxidative stress to damage BBB and neurons. SYD could protect mice against ischemic stroke primarily by significantly downregulating the genes involved in oxidative stress and strong inflammation, as well as by upregulating the genes mediating neurogenesis (Frzb/Wnt), which make SYD beneficial for ischemic stroke

In conclusion, our results reveal for the first time that the neuroprotective effect of SYD on ischemic stroke–induced brain injury in mice may depend on modulation of multiple molecular targets (162 genes) and pathways involved in the downregulation of stress, inflammation, immune response, and angiogenesis, as well as upregulation of neurogenesis-related signals (Frzb/Wnt) and regulation of cell communication [Figure 8]. Our results provide a possible explanation based on a genome-wide transcriptome analysis integrated with neurofunctional assay, and the opportunity for the evaluation of SYD, a typical traditional Chinese medicine formula, in the treatment or combination therapy of cerebral ischemia–associated diseases.

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