Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 121 | 680–692

doi: 10.1111/j.1471-4159.2012.07721.x

ORIGINAL ARTICLE

Sex differences in brain proteomes of neuron-specific STAT3-null mice after cerebral ischemia/reperfusion

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Abstract

Signal transduction and activator of transcription-3 (STAT3) plays an important role in neuronal survival, regeneration and repair after brain injury. We previously demonstrated that STAT3 is activated in brain after cerebral ischemia specifically in neurons. The effect was sex-specific and modulated by sex steroids, with higher activation in females than males. In the current study, we used a proteomics approach to identify downstream proteins affected by ischemia in male and female wild-type (WT) and neuron-specific STAT3 knockout (KO) mice. We established four comparison groups based on the transgenic condition and the hemisphere analyzed, respectively. Moreover, the sexual variable was taken into account and male and female animals were analyzed independently. Results support a role for STAT3 in metabolic, synaptic, structural and transcriptional responses to cerebral ischemia, indeed the adaptive response to ischemia/reperfusion injury is delayed in neuronal-specific STAT3 KO mice. The differences observed between males and females emphasize the importance of sex-specific neuronal survival and repair mechanisms, especially those involving antioxidant and energy-related activities, often caused by sex hormones.

Keywords: middle cerebral artery occlusion, proteomics, sexual dimorphism, Signal transduction and activator of transcription-3, stroke.

J. Neurochem. (2012) **121**, 680–692.

Stroke is a leading cause of death and serious, long-term disabilities, and a major challenge to health care costs (Roger *et al.* 2011). Oxygen and glucose depletion following stroke induces major metabolic changes which result in increased extracellular potassium levels, depletion of ATP, suspension of protein synthesis, increased intracellular calcium levels, decreased pH, and accumulation of free radicals and lactic acid (Auriel and Bornstein 2010). Energy depletion, acidosis and loss of ionic homeostasis lead to cell swelling, membrane rupture and cell death. Other ionic, biochemical

Received December 23, 2011; revised manuscript received February 15, 2012; accepted March 1, 2012.

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Abbreviations used: Eno2, gamma enolase; Hint1, histidine triad nucleotide-binding protein; KO, knockout; MCAO, middle cerebral artery; Mdh1, malate dehydrogenase cytoplasmic; STAT3, signal transduction and activator of transcription-3; WT, wild-type.

and cellular mechanisms, including glutamate excitotoxicity, oxidative and nitrosative stress, calcium dysregulation, cortical spreading depolarization and inflammation exacerbate already compromised tissue and significantly contribute to ischemic cell death (Moskowitz *et al.* 2010).

Neuronal injury in ischemia is the result of a two-step process, where reperfusion is the cause of further damage being associated with 'oxidative burst' and inflammation. Consequently, ischemia treatments should act on both restoration of normal circulation and neuronal protection reducing or interfering with the events that trigger neuronal damage following the ischemic event. Currently, the effects of candidate neuroprotective agents are being investigated in animal models and in clinical trials, but the presence of numerous gaps between animal and human studies likely account for the negative reports of finding an effective drug (Auriel and Bornstein 2010). A better understanding of the molecular mechanisms behind the neuronal damage in ischemia is a necessary step towards the development of appropriate and more effective pharmacological approaches aimed to reduce the risk of long-term disabilities.

The signal transducer and activator of transcription-3 (STAT3) regulates immune response mediation, cell survival and differentiation. The wide series of functions attributed to STAT3 derive from its ability to control different gene sets depending on cell-type and stimulation. STAT3 involvement in brain and heart ischemia/reperfusion damage mediation has been demonstrated both *in vitro* and *in vivo*, but the mechanisms, the mediators and the final effects of STAT3 signaling have not yet been fully elucidated.

Stroke complexity originates from the number of variables that modulate both outcome and recurrence, and sex, other than life-style and age, is among them. It is in fact increasingly recognized that stroke is a sexually dimorphic condition both experimentally and clinically, although these sex differences are still poorly understood. The gender bias has been observed across cultures, ethnic groups and socio-economic status, suggesting that sex is an independent and strong predictor of stroke risk and outcome (Hurn and Macrae 2000).

The middle cerebral artery (MCAO) occlusion model in rodents provides a useful tool for stroke researchers, to explore the basis of gender-linked ischemic brain injury. The amount of brain tissue damage after experimental stroke was greater in male versus female rats (Alkayed *et al.* 1998), suggesting that sensitivity to cerebral ischemia, i.e. neural tissue damage once an ischemic event has occurred, is sex-specific.

We previously studied male and female neuronal-specific STAT3 KO mice demonstrating the presence of sex differences in the modulation of protein expression in response to the lack of STAT3 and confirming a prominent role of STAT3 in sexual dimorphism (Di Domenico *et al.* 2010). Here, we identified, by proteomics, altered proteins in the brains of male and female wild-type (WT) and neuronal-specific STAT3 KO mice that underwent transient MCAO to investigate the involvement of STAT3 in ischemic damage and recovery and its modulation by sex hormones.

Materials and methods

Animals

NFl-Cre and STAT3flox/flox mice were originally provided by Dr. Michael Sendtner, Institute for Clinical Neurobiology, University of Wurzburg, Wurzburg, Germany to Dr. Nabil Alkayed, Oregon Health & Science University, Portland, OR, USA who, upon appropriate Institutional Animal Care and Use Committee approval for animal research, performed crosses between these two lines to generate the neuronal STAT3 knockout (STAT3 KO) mice and control littermates used in these studies. Full characterization of transgenic lines was previously described (Akira 2000; Schweizer *et al.* 2002; Di Domenico *et al.* 2010).

Middle cerebral artery occlusion in mice

Adult (25–28 g) male and female NFl-Cre/negative STAT3 flox/+ (CTR) and neuron-specific STAT3 KO mice were subjected to 60-min MCAO under isoflurane anesthesia, as described previously, with modifications (Zhang *et al.* 2008b). Briefly, mice were anesthetized with isoflurane (induction 5%; maintenance 1.0%), and kept warm with water pads. A small laser-Doppler probe was affixed to the skull to monitor cerebral cortical perfusion and verify vascular occlusion and reperfusion. A silicone-coated 6-0 nylon monofilament was inserted into the right internal carotid artery via the external carotid artery until laser-Doppler signal dropped to less than 20% of baseline. After reperfusion, mice were allowed to recover and observed for 24 h.

Sample preparation

Mice, six for each group, were briefly anesthetized with isoflurane (2–3%) and decapitated at the age of 8 months. The brain was rapidly removed from skull, and placed on pre-chilled metal plate on ice for dissection. The olfactory bulbs and cerebellum were removed, the remaining brain tissue was bisected into two hemispheres, ipsilateral and contralateral and each hemisphere was dipped into 2-methylbutane on dry ice (−50°C). The whole procedure between decapitation and freezing took < 1 min. The two hemispheres were then manually and individually homogenized in ice- cold buffer (10 mM Tris pH 8, 0.32 M Sucrose, 0.1 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin) and sonicated for 10 s on ice. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA).

Two-dimensional electrophoresis

Proteins (150 μg) were precipitated in 15% final concentration of trichloroacetic acid for 10 min in ice. Each individual sample (six per group) was then spun down at 10 000 *g* for 5 min and precipitates were washed in ice-cold ethanol-ethyl acetate 1 : 1 solution four times. The final pellet was dissolved in 200 μL of 8 M urea, 2% CHAPS, 2 M thiourea, 20 mM dithiothreitol, 0.2% of ampholytes (Bio-Rad, Hercu-

les, CA, USA) and bromophenol blue, incubated at 20°C for 90 min and sonicated for 5 s. Samples were then loaded on 110-mm pH 3–10 immobilized pH gradients strips in a Bio-Rad Isoelectric focusing Cell system (Bio-Rad). Following 18 h of active rehydration (50 V) isoelectric focusing was performed as previously reported (Di Domenico *et al.* 2011). The focused isoelectric focusing strips were stored at −80°C until second dimension electrophoresis was performed. For the second dimension thawed strips were sequentially equilibrated for 15 min in the dark in 375 mM Tris pH 8.8, 6 M urea, 2% sodium dodecyl sulfate, 20% glycerol containing first 2% dithiothreitol and then 2.5% of iodoacetamide. sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in Criterion Tris-HCl Gels 8–16% (Bio-Rad) at 200 V for 1 h.

Gel staining and image analysis

Gels were fixed for 45 min in 10% methanol, 7% acetic acid and stained overnight on the rocker with SYPRO Ruby gel stain (Bio-Rad). After destaining in deionized water, gels were scanned with a STORM UV transilluminator (λex = 470 nm, λem = 618 nm; Molecular Dynamics, Sunnyvale, CA, USA). Images obtained were saved in Tagged Image File Format Gel imaging was software-aided using PD-Quest (Bio-Rad) imaging software. Briefly, a master gel was selected followed by normalization of all gels according to the total spot density. Gel to gel analysis was then initiated in two parts. First, manual matching of common spots that could be visualized among the differential 2D gels was performed. After obtaining a significant number of spots the automated matching of all spots was then initiated. Automated matching is based on user-defined parameters for spot detection. These parameters are based on the faintest spot, the largest spot, and the largest spot cluster that occur in the master gel and are defined by the user. This process generates a large pool of data, ~350 spots. Only proteins showing computer-determined significant differential levels between the two groups being analyzed were considered for identification. To determine significant differential levels of proteins, analysis sets were created using the analysis set manager software incorporated into the PD-Quest software. The numbers of pixels that occur in a protein spot, corresponding to an increase/decrease in protein level, were computed by the software. A quantitative analysis set was created that recognized matched spots with differences in the number of pixels that occur in each spot and a statistical analysis set was created that used a Student's *t*-test at 95% confidence to identify spots with *p*-values < 0.05. Spots with $p < 0.05$ were considered significant.

In-gel trypsin digestion

Protein spots statistically different than controls were digested in-gel by trypsin. Spots were taken from individual gels and pooled for mass spectrometric analysis. The amount of protein from one gel-spot is sufficient for identification. Briefly, spots of interest were excised using a clean blade and placed in Eppendorf tubes, which were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) 20°C for 15 min. Acetonitrile was then added to the gel pieces and incubated at 20°C for 15 min. This solvent mixture was then removed and gel pieces dried. The protein spots were then incubated with 20 μL of 20 mM dithiothreitol in 0.1 M NH_4HCO_3 at 56°C for 45 min. The dithiothreitol solution was removed and replaced with 20 μL of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at 20°C for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH_4HCO_3 and incubated at 20°C for 15 min. Acetonitrile (200 μL) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/μL-modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3 with the minimal volume enough to cover the gel pieces. The gel pieces were incubated overnight at 37°C in a shaking incubator.

Mass spectrometry

Protein spots of interest were excised, subjected to in-gel trypsin digestion, and resulting tryptic peptides were analyzed with an automated nanospray Nanomate Orbitrap XL MS platform. The Orbitrap MS was operated in a data-dependent mode whereby the eight most intense parent ions measured in the Fourier transform at 60 000 resolutions were selected for ion trap fragmentation with the following conditions: injection time 50 ms, 35% collision energy. MS/MS spectra were measured in the FT at 7500 resolution, and dynamic exclusion was set for 120 s. Each sample was acquired for a total of ~2.5 min. MS/MS spectra were searched against the ipi_Human Database using SEQUEST with the following criteria: Xcorr > 1.5, 2.0, 2.5, 3.0 for + 1, + 2, + 3, and + 4 charge states, respectively, *p* value (protein and peptide) < 0.01. peptide delta Cn > 0.1, RSp < 5 and preliminary score (Sp) > 500. IPI accession numbers were cross- correlated with SwissProt accession numbers for final protein identification (Table 1).

Western blot

Protein (50 μg) were added to sample buffer, denaturated for 5 min at 100°C, loaded on 12% precast Criterion gels (Bio-Rad) and separated by electrophoresis at 100 mA for 2 h. The gels were then transferred to nitrocellulose paper using the Transblot-BlotSD Semi-DryTransfer Cell at 20 mA for 2 h. Subsequently, the membranes were blocked at 4°C for 1 h with fresh blocking buffer made of 3% bovine serum albumine in phosphate-buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 phosphate buffered saline. The membranes were incubated with Pin-1 primary antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA). The membranes were then washed three times for 5 min with PBST followed by incubation with anti-mouse horseradish peroxidase conjugate secondary antibody (Sigma, St. Louis, MO, USA) in tween 20 phosphate buffered saline for 2 h at 20°C. Membranes were then washed three times in tween 20 phosphate buffered saline for 5 min and developed using enhanced chemiluminescence plus western Blotting detection reagents (Amersham Biosciences, Pittsburgh, PA, USA) for horseradish peroxidase conjugate secondary antibody. Blots were dried, scanned in Tagged Image File Format using STORM UV transilluminator (λex = 470 nm, λem = 618 nm, Molecular Dynamics) for chemiluminescence. The images were quantified with ImageQuant TL 1D version 7.0 software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Interactome maps

To build interactome maps Ingenuity Pathways Analysis (IPA) 8.6, (Ingenuity system, Redwood City, CA, USA) was used. The software use the Ingenuity Knowledge Base database, which includes findings and annotations from major NCBI databases, clinical biomarkers, gene ontology annotations, gene expression body atlas for over 30 tissues, GWAS databases, KEGG and LIGAND metabolic pathways and reactions. The interactome data

Table 1 Data from mass spectrometry analysis of the proteins identified

Protein	Accession number	pb	No of peptides matched/searched
Brain acid soluble protein 1 (BASP1)	Q91XV3	1e-016	6/7
Neuromodulin (GAP 43)	P06837	4e-011	2/3
14-3-3 protein zeta/delta	P63101	1e-030	14/60
		7e-014	8/12
14-3-3 protein beta/alpha	Q9CQV8	6e-009	2/2
Gamma enolase (ENO2)	P17183	2e-015	4/5
		2e-014	2/3
		4e-015	3/6
Alpha-enolase (ENO1)	P17182	1e-010	2/2
Histone H2A.J (H2A.J)	Q8R1M2	8e-012	2/5
Cytochrome c oxidase subunit 5A (COX5A)	P12787	1e-009	6/25
Profilin-2 (PFN2)	Q9JJV2	4e-013	2/6
SH3 domain-binding glutamic acid-rich-like protein (SH3BGRL3)	Q91VW3	5e-012	3/7
Myotrophin (Mtpn)	P62774	2e-007	2/6
Dihydrolipoyllysine-residue acetyltransferase (DLAT)	Q8BMF4	8e-016	7/13
Tubulin alpha-1A chain (TUBA1A)	P68369	1e-030	7/12
Dihydropyrimidinase-related protein 2 (CRMP-2)	O08553	9e-014	6/9
Transcriptional activator protein (PURA)	P42669	1e-015	10/39
Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1)	P00493	5e-012	3/5
Adaptin ear-binding coat-associated protein (NECAP1)	Q9CR95	1e-030	4/8
Mu-crystallin homolog (CRYM)	O54983	4e-015	7/13
Malate dehydrogenase, cytoplasmic (MDH1)	P14152	1e-030	4/7
Fructose-bisphosphate aldolase C (ALDOC)	P05063	6e-015	15/47
Dihydropteridine reductase (QDPR)	Q8BVI4	6e-015	4/8
Cytochrome b-c1 complex (UQCRFS1)	Q9CR68	2e-013	2/5
		5e-010	2/4
Actin-related protein 2/3 complex subunit 5 (ARPC5L)	Q9D898	2e-010	7/25
Nucleoside diphosphate kinase B (NME2)	Q01768	6e-011	2/4
Peptidyl-prolyl *cis*-trans isomerase (PIN-1)	P17742	4e-011	6/20
Isoform 1 of Ubiquitin-conjugating enzyme (UBE2V1)	Q9CZY3	3e-015	7/13
Histidine triad nucleotide-binding protein 1 (HINT1)	P70349	2e-010	3/13
		5e-010	3/5
Manganese superoxide dismutase (MnSOD)	P09671	7e-015	4/17
CB1 cannabinoid receptor-interacting protein (CNRIP1)	Q5M8N0	3e-013	3/8
Aconitate hydratase (ACO2)	Q03265	2e-011	18/30
Transketolase (TKT)	P40142	4e-015	15/25
Alpha synuclein (SNCA)	O55042-1	1e-011	3/7
Beta synuclein (SNCB)	Q91ZZ3	5e-009	3/8
Calreticulin (CALR)	P14211	7e-006	1/3
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P16858	2e-007	3/3
Isocitrate dehydrogenase (IDH)	O81796	2e-004	2/2
Peroxiredoxin 6 (PRDX6)	O08709	1e-006	1/3
Synapsin II (SYN2)	Q64332	7e-014	3/7

were filtered for mouse species, neuronal tissue and accounting for all direct and indirect interactions.

Statistical analysis

Statistical analysis of protein levels matched with spots on 2D-gels from Alzheimer's disease and mild cognitive impairment hippocampus and inferior parietal lobule compared to age-matched controls were carried out using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant. Only proteins that were considered significantly different by Student's *t*-test were subjected to in-gel trypsin digestion and subsequent proteomic analysis.

Results

In this study we used proteomics analysis to investigate the effects of neuron-specific STAT3 KO (NFl-Cre$^+$/

STAT3$^{flox/flox}$) and the influence of sex on the brain proteome for mice lines subjected to MCAO and 96 h reperfusion. We had at our disposal both the ipsilateral and the contralateral brain hemispheres of male and female Wild-type (WT) and STAT3 KO mice. All the samples were divided in four groups for comparison according to the transgenic status and the brain hemisphere. Each group was investigated for both male and female sex.

Hemispheres comparison in WT and neuronal STAT3 KO transgenic mice (Fig. 1)

WT ipsilateral versus WT contralateral

Males. In the comparison of samples from the contralateral and the ipsilateral brain area of male WT mice subjected to MCAO, we identified the altered expression of eight proteins. Seven of these are down-regulated in the brain area affected by ischemic damage: gamma enolase (Eno2) (62%), profilin-2 (Pfn-2) (51%), manganese superoxide dismutase (MnSOD) (72%), aconitase 2 mitochondrial (Aco2) (71%), malate dehydrogenase cytoplasmic (Mdh1) (55%), peroxiredoxin-6 (Prdx-6) (75%) and synapsin II (Syn II) (57%). The only protein up-regulated in the ischemic area is dihydrolipoyllysine-residue acetyltransferase (Dlat) (214%).

Females. In the comparison of the ipsilateral and contralateral brain area of female WT mice subjected to MCAO we identified changes in the expression of seven proteins. Five of these, namely SH3 domain-binding glutamic acid-rich-like protein (Sh3bgrl3) (56%), transcriptional activator protein pur-alpha (32%), dihydropteridine reductase (Qdpr) (71%), hypoxanthine-guanine phosphoribosyltransferase (Hprt1) (52%) and (Mdh1) (59%), are downregulated in the stroke-affected area. In contrast, two proteins identified in the same area with increased levels were: transketolase (Tkt) (202%) and Isoform 1 of Ubiquitin-conjugating enzyme (Ube2v1) (260%).

STAT3 KO ipsilateral versus STAT3 KO contralateral

Males. In the comparison of the ipsilateral and contralateral brain area of male neuronal-specific STAT3 KO mice subjected to MCAO we identified changes in the levels for eight proteins. Three of these are up-regulated in the ipsilateral brain area and are: neuromodulin (Gap43) (172%), Dlat (218%) and (MnSOD) (187%). In contrast, five proteins were identified with decreased levels in the

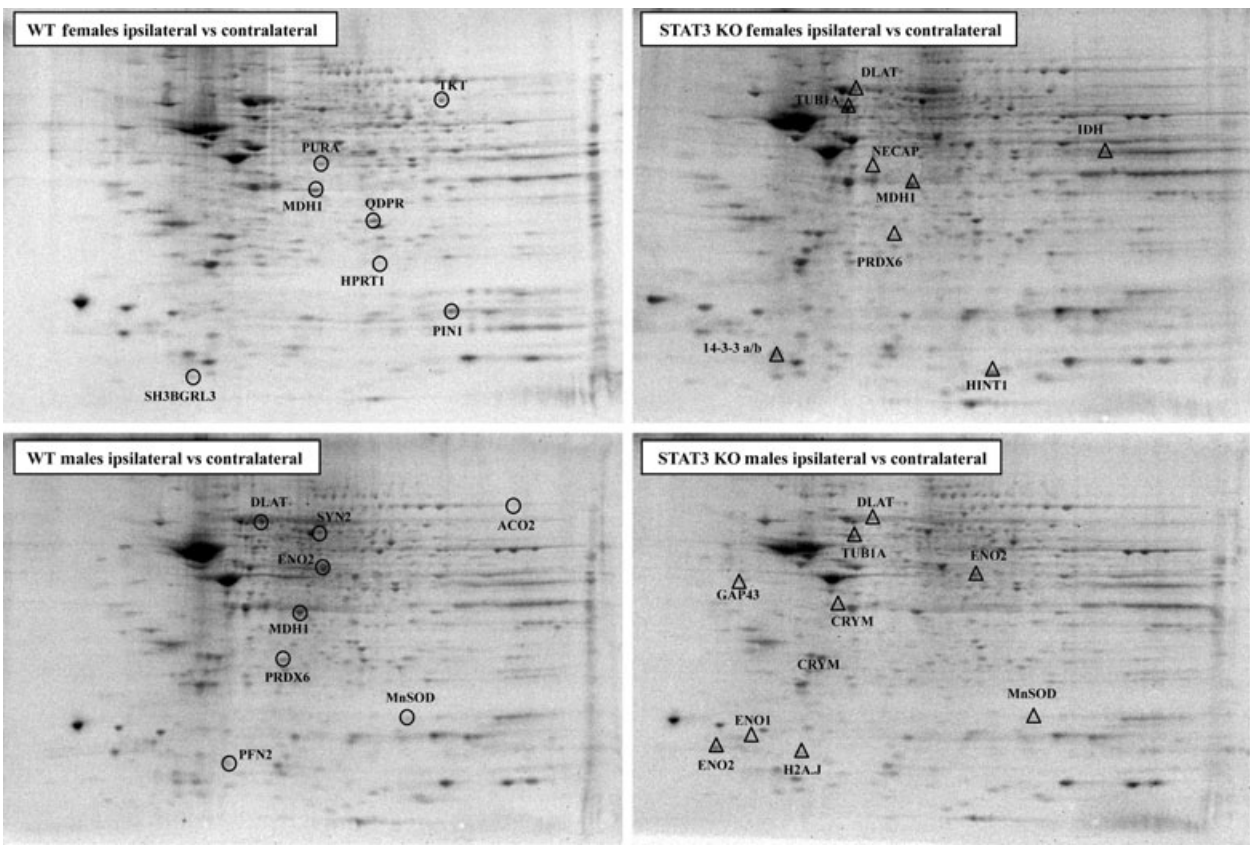

Fig. 1 Representative 2D gel of protein identified, by mass spectrometry, with altered levels in WT (left) and STAT3 KO (right) female (top) and male (bottom) ipsilateral versus contralateral proteomics comparison. O = altered proteins identified in WT animals; Δ = altered proteins identified in neuronal-specific STAT3 KO animals.

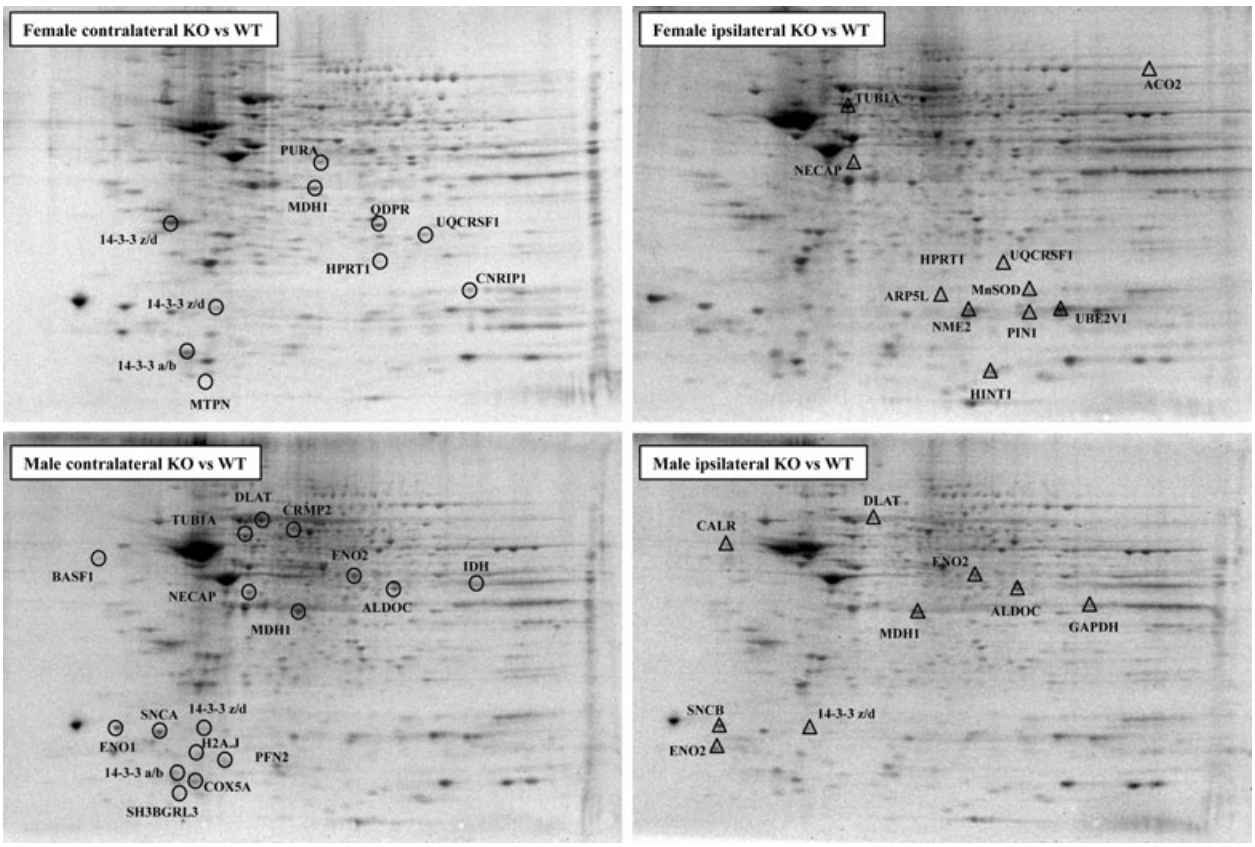

Fig. 2 Representative 2D gel of protein identified, by mass spectrometry, with altered levels in contralateral (left) and ipsilateral (right) female (top) and male (bottom) WT versus neuronal-specific STAT3 KO proteomics comparison. O = altered proteins identified in contralateral hemisphere; Δ = altered proteins identified in ipsilateral hemisphere.

ipsilateral brain area when compared to contralateral brain area: Eno2 (65 and 35%), alpha enolase (Eno1) (55%), histone H2A.J (H2afj) (30%), tubulin alpha-1A chain (Tuba1a) (16%) and Mu-crystallin homolog (Crym) (66%).

Females. In female neuronal-STAT3 KO mice subjected to MCAO the proteomics comparison of ipsilateral and contralateral areas identified changes in the levels for eight proteins. Four proteins were upregulated in the ipsilateral area: 14-3-3 protein beta/alpha (Ywhab) (241%), (Dlat) (589%), adaptin ear-binding coat-associated protein (Necap1) (588%) and peroxiredoxin-6 (Prdx-6) (179%). Four proteins had lower levels in ipsilateral area compared with the contralateral area:Tuba1a (15%), histidine triad nucleotide-binding protein (Hint1) (38 and 42%), (Mdh1) (15%) and isocitrate dehydrogenase (IDH) (5%).

Transgenic status comparison in ipsilateral or contralateral hemisphere (Fig. 2)

STAT3 KO ipsilateral versus WT ipsilateral

Males. The proteomics comparison between neuronal-specific STAT3 KO and WT ipsilateral hemispheres of male mice subjected to MCAO identified altered levels of eight proteins. Seven had higher levels in KO animals: Eno2 (308 and 228%), (Dlat) (283%), (Mdh1) (255%), beta synuclein

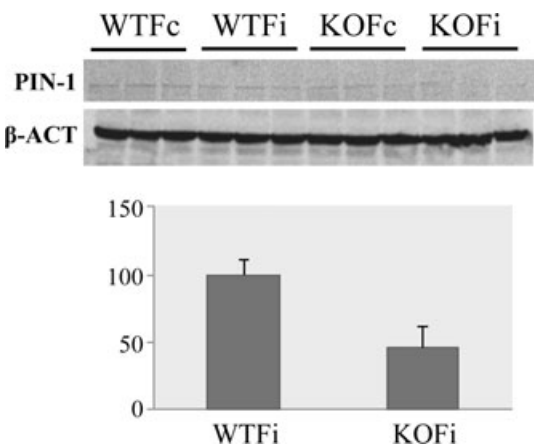

Fig. 3 Western blot image and relative graph bar of Pin-1 and β actin protein levels in WT and neuronal-specific STAT3 KO, ispsilateral and contralateral female samples; comparison between WT female ispilateral and neuronal-specific STAT3-KO female ipsilateral samples.

(bsyn) (250%), calreticulin (calr) (244%) and dihydropirimidase related protein 2 (CRMP2) (211%). The only protein with lower levels in KO compared to WT ipsilateral region was is 14-3-3 protein zeta/delta (Ywhzd) (41%).

Females. In female ipsilateral hemisphere of mice subjected to MCAO the proteomics comparison between neuronal-specific STAT3 KO and WT identified expression changes in total levels for ten proteins. Four out of the ten had higher levels in KO animals: (Tuba1a) (345%), (Necap1) (239%), actin-related protein 2/3 complex subunit 5 (Arpc5l) (299%) and Aco2 (144%). Six proteins had lower levels in KO mice: cytochrome b-c1 complex subunit (Uqcrfs1) (57%), nucleoside diphosphate kinase B (Nme2) (40%), peptidyl-prolyl cis/trans isomerase (Pin1) (39%), Hint1 (33%), (MnSOD) (63%) and isoform 1 of (Ube2v1).

STAT3 KO contralateral versus WT contralateral

Males. The proteomics comparison between neuronal-specific STAT3 KO and WT contralateral hemispheres of male mice subjected to MCAO identified altered levels of seventeen proteins. Thirteen proteins had higher levels in KO animals: brain acid soluble protein 1 (BASP1) (299%), 14-3-3 protein zeta/delta (Ywhzd) (351%), Eno2 (409%), Eno1 (687%), H2afj (240%), cytochrome c oxidase subunit 5A mitochondrial (Cox5a) (200%), Dlat (292%), (Tuba1a) (208%), (Necap1) (202%), (Mdh1) (250%), alpha synuclein (asyn) (206%), CRMP2 (221%) and (IDH) (348%). Four proteins are downregulated in KO animals and are: 14-3-3 protein alpha/beta (Ywhab) (62%), profilin-2 (Pfn2) (46%), Sh3bgrl3 (52%) and fructose-bisphosphase aldolase C (Aldoc) (64%).

Females. In female contralateral hemisphere of mice subjected to MCAO the proteomics comparison between neuronal-specific STAT3 KO and WT identified changes in levels for nine proteins. Two proteins of the nine were of higher levels in KO animals: Uqcrfs1 (141%), (Mdh1) (508%). Seven proteins were of lower levels in KO mice: 14-3-3 protein alpha/beta (Ywhab) (39%), 14-3-3 protein zeta/delta (Ywhzd) (15 and 26%), myotrophin (Mtpn) (30%), transcriptional activator protein pur-alpha (Tappa) (62%), (Qdpr) (65%), (Hprt1) (48%) and CB1 cannabinoid receptor-interacting protein (Cnrip1) (34%).

Validation experiments

For the validation of proteomics data, the expression levels of Pin-1 protein, identified by mass spectrometry analysis, were analyzed by western Blotting. The results shown in Fig. 3 demonstrate a decrease of Pin-1 in ipsilateral KO

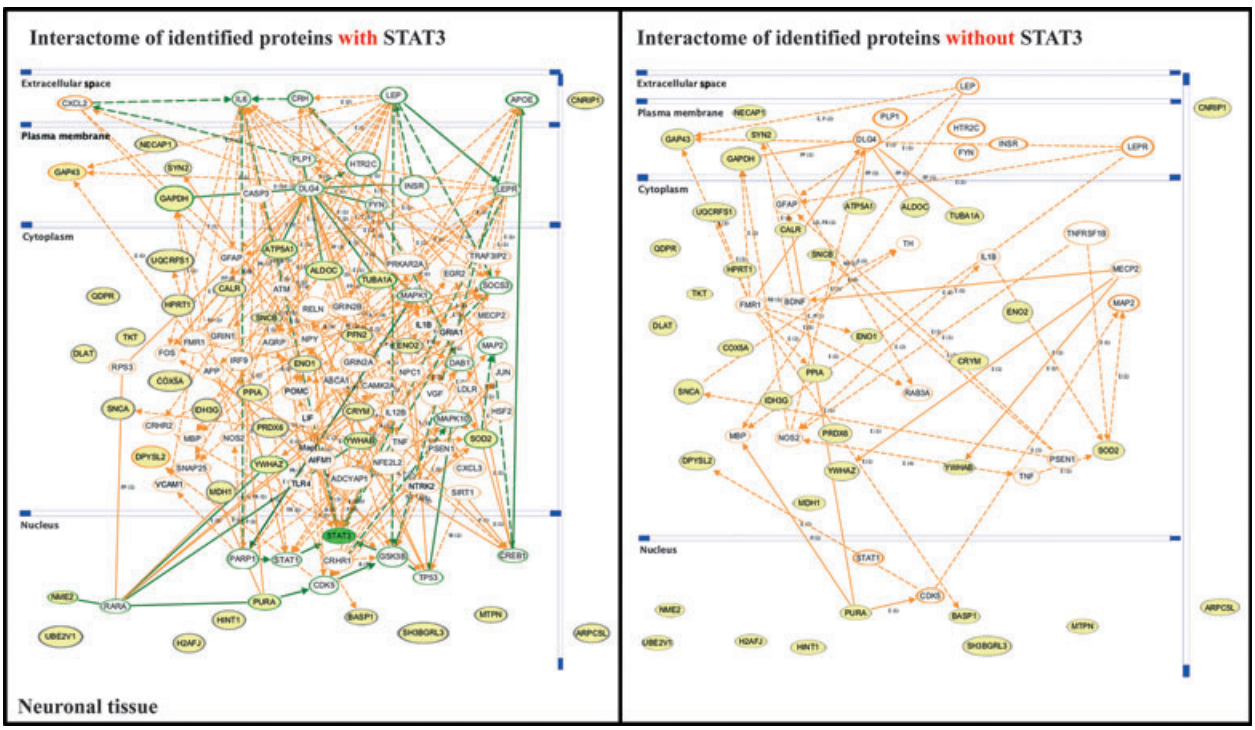

Fig. 4 Interactome maps showing direct and indirect relationship between the proteins identified in this study with altered expression (yellow) in presence or absence of STAT3 in neuronal tissue; LEFT: direct and indirect interaction of all the proteins between them, with and trough STAT3, the green lines represent the direct interaction of identified protein and STAT3, the orange lines represents indirect interaction with STAT3 and direct and indirect interaction of the proteins between them; RIGHT: direct and indirect interactions of all the proteins between them without STAT3 (orange lines). A description of the interactome software is given in Materials and methods.

female samples compared to ipsilateral WT female samples, which confirm the levels obtained by proteomics analysis.

Discussion

Several proteomics studies have analyzed the involvement of STAT3 and the influence of gender in the events following ischemia/reperfusion (Haqqani *et al.* 2007; Di Domenico *et al.* 2010; Koh 2010; Sung *et al.* 2010). However, none of these studies analyzed these two variables in the outcome from stroke together.

In our previous study we revealed, by proteomic analysis of brain from WT and neuron-specific STAT3 KO mice, the molecular pathways directly or indirectly controlled by STAT3 underscoring its role in brain development and maintenance (Di Domenico *et al.* 2010). Moreover, by using both female and male mice we were able to investigate the different processes that could contribute to the sexual dimorphic behavior observed in the incidence of neurological and mental diseases. This current study represents a further step in the discovery of STAT3 activity in the brain. Here, WT and neuron-specific STAT3 KO mice were subjected to MCAO to analyze the involvement of STAT3 activation in processes following ischemia/reperfusion. Several studies demonstrated the activation and the beneficial effects of STAT3 after ischemia/reperfusion both in brain and heart. In the heart, STAT3 is activated during ischemia preconditioning, ischemia, and reperfusion with beneficial effect (Merkel *et al.* 2010; Boengler 2011). In addition, STAT3 phosphorylation is induced by different classes of cardioprotective pharmacological agents (Boengler 2011). In primary cortical neurons IL-10, known to reduce ischemia-triggered pro-inflammatory responses, increased neuronal survival after exposure to oxygen-glucose deprivation (OGD) or glutamate toxicity via activation of STAT3 and PI3K/AKT signal transduction pathways (Sharma *et al.* 2011). *In vivo*, STAT3 phosphorylation levels and DNA binding increase in neurons, microglia, and astroglia mainly during reperfusion (Dinapoli *et al.* 2010; Lei *et al.* 2011). In ischemic aged rats, which showed enhanced neurodegeneration and increased production of pro-inflammatory cytokines when compared to ischemic young rats, the duration of STAT3 activation was reduced (Dinapoli *et al.* 2010), supporting the protective effect of STAT3 signaling in the maintenance of brain physiology.

Similar to neurological diseases, stroke displays a sexual dimorphic behavior with females being protected until menopausal age for the advantageous properties of sexual hormones such as estrogen. Estrogen's neuroprotective effects are complex and embrace chemical, biochemical and genomic mechanisms and are comprised of three broad categories: antioxidant, defense, and viability (Nilsen and Brinton 2004). In particular, estrogen was demonstrated to have vascular effects leading to better preservation of cerebral blood flow during ischemia (Alkayed *et al.* 1998; McCullough *et al.* 2001), cytoprotective effects resulting in a greater tolerance of the brain to ischemia (Alkayed *et al.* 2001; Simpkins *et al.* 2005), and remarkable anti-inflammatory properties (Santizo *et al.* 2000; Vegeto *et al.* 2000, 2003; Alkayed *et al.* 2001).

While ovariectomy increased infarct size, physiologically relevant amounts of 17β- estradiol (E2) attenuated infarct size (Rusa *et al.* 1999; Alkayed *et al.* 2000).

The E2 exhibits acute and chronic effects. The chronic effects of E2 are mediated via alterations in gene expression while acute, rapid effects are mediated via non-genomic mechanisms, such as changes in protein phosphorylation (O'Lone *et al.* 2004; Bryant *et al.* 2005). Recent studies suggest that estrogen rapidly activates signal transduction pathways that ultimately lead to increased gene transcription (Bjornstrom and Sjoberg 2005). The protective effect of E2 to reduce infarct volume appeared to be mediated via phospho-STAT3 because STAT3 phosphorylation is increased by E2 in an aortic endothelial cell line (Bjornstrom and Sjoberg 2002) and pharmacological inhibition of pospho-STAT3 abolished the protective effect of E2 on infarct size (Dziennis *et al.* 2007). It was recently reported that E2 replacement increases the phosphorylation of STAT3 after transient focal ischemia relative to ovariectomized female rats (Dziennis *et al.* 2007).

The aim of this present study was to evaluate alteration at the brain proteome level caused by ischemia/reperfusion, the involvement of STAT3 in modulation of the altered outcomes following stroke and the influence of gender in such mechanisms of alteration and protection. An overall evident effect was the induction of changes in protein levels in the contralateral non-ischemic hemisphere even in WT mice. A similar result was previously reported by Focking *et al.*, from which we speculate that this phenomenon may be associated with brain plasticity (Focking *et al.* 2006). Moreover building an interactome map (Fig. 4) we were able to demonstrate how the lack of neuronal STAT3 impairs the interaction pathways of the proteins found with altered expression levels in this study. Indeed, we show that most of the proteins are strictly connected with STAT3 signaling and among them when STAT3 is steadily expressed in murine brain. However, in brain of neuronal-specific STAT3 KO mice, the interactions among all the proteins drop consistently, suggesting that the loss of neuronal STAT3 impairs the molecular pathways dependent on STAT3 expression through the deprivation of protein–protein interactions and alterations in expression levels.

Mitochondrial, Energy-related proteins (Table 2)

In the evaluation of brain tissue in WT and STAT3 KO following MCAO it appears that even after 96 h of reperfusion the expression pattern of proteins involved in energy metabolism is still altered in the contralateral and

Table 2 Expression levels changes of proteins identified in all the groups compared

	Ipsilateral versus contralateral		Ipsilateral versus contralateral		Knock out versus wild type		Knock out versus wild type	
	Wild type		Knock Out		Contralateral		Ipsilateral	
Up ↑ Down ↓	Female	Male	Female	Male	Female	Male	Female	Male
Mitochondrial, energy-related proteins								
Gamma enolase (ENO2)				0.65 ↓		4.09 ↑		3.08 ↑
				0.35 ↓				
		0.62 ↓						2.28 ↑
Alpha-enolase (ENO1)				0.55 ↓		6.87 ↑		
Cytochrome c oxidase subunit 5A (COX5A)						2.00 ↑		
Dihydrolipoyllysine-residue acetyltransferase (DLAT)		2.14 ↑	5.89 ↑	2.18 ↑		2.92 ↑		2.83 ↑
Fructose-bisphosphate aldolase C (ALDOC)						0.64 ↓		
Cytochrome b-c1 complex (UQCRFS1)							0.57 ↓	
					1.41 ↑			
Manganese superoxide dismutase (MnSOD)		0.72 ↓		1.87 ↑			0.63 ↓	
Aconitate hydratase (ACO2)		0.71 ↓					1.44 ↑	
Transketolase (TKT)	2.02 ↑							
Malate dehydrogenase, cytoplasmic (MDH1)	0.59 ↓	0.55 ↓	0.15 ↓		5.08 ↑	2.50 ↑		2.55 ↑
Isocitrate dehydrogenase (IDH)			0.07 ↓			3.48 ↑		
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)								2.11 ↑
Cell signaling, synaptic transmission proteins								
14-3-3 protein zeta/delta					0.15 ↓	0.35 ↓		0.41 ↓
					0.26 ↓			
14-3-3 protein beta/alpha			2.41 ↑		0.39 ↓	0.62 ↓		
Histidine triad nucleotide-binding protein 1 (HINT1)			0.38 ↓				0.33 ↓	
			0.42 ↓					
CB1 cannabinoid receptor-interacting protein (CNRIP1)					0.34 ↓			
Alpha synuclein (SNCA)						2.06 ↑		
Beta synuclein (SNCB)								2.50 ↑
Synapsin II (SYN2)		0.57 ↓						
Structural, endocytic proteins								
Neuromodulin (Gap43)				1.72 ↑				
Histone H2A.J (H2A.J)				0.30 ↓		2.40 ↑		
Profilin-2 (PFN2)		0.51 ↓				0.46 ↓		
Tubulin alpha-1A chain (TUBA1A)			0.15 ↓	0.16 ↓		2.08 ↑	3.45 ↑	
Adaptin ear-binding coat-associated protein (NECAP1)			5.88 ↑			2.02 ↑	2.39 ↑	
Actin-related protein 2/3 complex subunit 5 (ARPC5L)							2.99 ↑	
Dihydropirimidase related protein 2 (CRMP2)						2.21 ↑		
Myotrophin (Mtpn)					0.30 ↓			
Antioxidant, folding proteins								
Peroxiredoxin 6 (PRDX6)		0.75 ↓	1.79 ↑					
SH3 domain-binding glutamic acid-rich-like protein (SH3BGRL3)	0.56 ↓					0.52 ↓		
PIN 1							0.39 ↓	

Table 2 (*Continued*)

Up ↑ / Down ↓	Ipsilateral versus contralateral		Ipsilateral versus contralateral		Knock out versus wild type		Knock out versus wild type	
	Wild type		Knock Out		Contralateral		Ipsilateral	
	Female	Male	Female	Male	Female	Male	Female	Male
Transciptional proteins								
Brain acid soluble protein 1 (BASP1)						2.99 ↑		
Transcriptional activator protein (PURA)	0.32 ↓				0.62 ↓			
Calreticulin (CALR)								2.44 ↑
Others proteins								
Mu-crystallin homolog (CRYM)				0.66 ↓				
Dihydropteridine reductase (QDPR)	0.71 ↓				0.65 ↓			
Hypoxanthine-guanine phosphoribosyltransferase (HPRT1)	0.52 ↓				0.48 ↓			
Nucleoside diphosphate kinase B (NME2)							0.40 ↓	
Ubiquitin-conjugating enzyme (UBE2V1)	2.60 ↑						0.47 ↓	
Up	2	1	4	3	3	12	4	7
Down	5	7	5	6	7	5	6	1
Total	7	8	9	9	10	17	10	8

ipsilateral sides of both WT and STAT3 KO mice. Many of the identified proteins are cytoplasmic enzymes of glycolysis and the tricarboxylic acid cycle (TCA) cycle, as a consequence of the remodeling of anaerobic and aerobic metabolism in response to the variation in oxygen and nutrients availability. Among the listed proteins enolase, aldolase and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are prone to oxidative modifications in neurodegenerative disorders, such as mild cognitive impairment, Alzheimer's disease, and Parkinson's disease (Butterfield *et al.* 2011). Other than their susceptibility to oxidation, both enolase and GAPDH have been previously described as proteins involved in many regulatory pathways other than glucose metabolism in brain (Butterfield and Lange 2009; Butterfield *et al.* 2010).

Enolase activity is modulated by oxygen availability and by plasminogen, it is a debated candidate for ischemia outcome prediction and it is known for its role in supporting anaerobic metabolism in tumor cells (Brea *et al.* 2009; Kaca-Orynska *et al.* 2010; Capello *et al.* 2011). Decreased enolase expression was observed only in the ischemic brain of male mice, while no change in enolase levels were observed in any of the female groups, suggesting a possible modulation of enolase expression by hormone-related signaling as already observed in rat ovary (Yoshioka *et al.* 2011). In the direct comparison between STAT3 KO and WT mice, enolase was increased in both sides of male STAT3 KO ischemic brain, reminiscent of the behavior observed in the progression of Alzheimer's disease and mild cognitive impairment in which alpha enolase was found to be consistently up-regulated and oxidatively modified (Butterfield and Lange 2009).

In male STAT3 KO and WT we observed modulation of glycolytic and TCA cytosolic enzymes only, while in the ipsilateral side of STAT3 KO female mice the high potential 2Fe-2S Rieske protein UQCRFS1 and MnSOD were reduced. Both enzymes are localized in mitochondria and their reduction could be a consequence of prolonged post-ischemic oxidative damage to the respiratory chain system in the ipsilateral side of STAT3 KO female brain.

Malate dehydrogenase (MDH) is part of the malate-aspartate shuttle system regulating the exchange of reducing equivalents between cytosol and mitochondria. MDH can be regulated by nutrients and hormones among which are E2, androsterone and nandrolone (Danis and Farkas 2009). Malate dehydrogenase activity was found increased in brain of aged rats, in skeletal muscle of rats trained under hypoxic condition and in human hypertrophic heart (Patnaik 1990; Takahashi *et al.* 1996; Lo *et al.* 2005). In STAT3 KO mice, MDH levels were increased in the contralateral side of both males and females and in the ipsilateral side of males only, while aconitase was increased in the ipsilateral side of female STAT3 KO. The consistent increase of different TCA cycle enzymes in STAT3 KO mice compared with their WT counterparts might suggest a delay in the adaptation to the post-ischemia/reperfusion conditions. In line with this view is the transketolase increase, which is specifically observed in the ipsilateral side of WT female only. Similar to the other listed proteins transketolase takes part in energy transduction but is also part of the pathway that generates ribose for the synthesis of nucleic acids (Alexander-Kaufman and Harper 2009), indicating a possible recovery phase in the ischemic side of WT but not STAT3 KO female mice.

Signaling proteins (Table 2)

Major alterations were found in the levels of protein involved in synaptic transmission and signaling. Changes in expression are more pronounced between WT and KO mice brain either in the contralateral or the ipsilateral hemispheres than between the two hemispheres in transgenic and WT animals, suggesting that signaling and neurotransmission pathways are more affected by persistent transgenic condition than by transient ischemia/reperfusion. We previously reported that neuron specific STAT3 KO mice compared with WT animals present expression differences in synaptic and signaling proteins in both females and males (Di Domenico *et al.* 2010). Here we found that STAT3 KO female animals demonstrate mainly decreased levels for brain proteins identified in both hemispheres, while males does not show a peculiar trend. We suggest that this effect is related to the loss of neuronal STAT3 that influences estrogen activity on female brain but not in male.

Several signaling pathways are induced by estrogen and it is widely recognized that estrogen signaling leads to activation of MAPK to exploit its anti-inflammatory effects, and to the activation of the cAMP/PKA/CREB and PI3K/AKT protective pathways (Nilsen and Brinton 2004; Liu *et al.* 2009).

Among the signaling proteins identified with the altered levels was the protein 14-3-3 either in the z/d or a/b isoform in mice KO for STAT3 from both sexes. In males, the results support our previous belief about the influence of STAT3 on 14-3-3 expression and downstream pathways. In females the alteration of 14-3-3 protein does not appear strictly related to the transgenic condition but more on ischemic/reperfusion, since increased 14-3-3 a/b levels were observed between the ischemic and the non-ischemic hemisphere in females KO mice while decreased 14-3-3, both isoforms, were found in the ipsilateral hemisphere only. This result suggests a sex-related mechanism of alteration of 14-3-3 protein that is estrogen-independent. Another signaling protein altered is Hint1, which is decreased in the ipsilateral hemisphere of female neuron–specific STAT3 KO mice in both comparisons, suggesting that the decrease is due to the simultaneous action of neuron-specific STAT3 KO and MCAO effects.

Cannabinoid receptor 1 (CB1) plays a prominent role in synaptic neurotransmission and has been correlated with cerebral ischemia/reperfusion injury (Zhang *et al.* 2008a; Rojo *et al.* 2011). Our analysis identified decreased levels of CB1 interacting protein in the contralateral hemisphere of female neuronal-specific STAT3 KO compared to WT mice suggesting a correlation between the lack of STAT3, the activity of CB1 and the putative neuronal plasticity event ongoing in the non-ischemic hemisphere.

Structural proteins (Table 2)

Alteration of structural proteins represents another common outcome of the transgenic condition in both males and females, as previously seen (Di Domenico *et al.* 2010). However, in this study sex related alterations in structural proteins are present in the comparison between neuronal-specific STAT3 KO and WT in male contralateral region and in female ipsilateral region. The alterations in levels of proteins in brain from female transgenic mice subjected to MCAO is, again, related to the loss of STAT3-mediated protective effect, induced by estrogen. Several studies show that estrogen affects neurite elongation and differentiation by directly regulating the transcription of genes required for growth such as the microtubule-associated protein tau or different synaptic proteins (Dziennis and Alkayed 2008; Liu *et al.* 2009), and, as we demonstrated in our previous study (Di Domenico *et al.* 2010), structural protein alterations follow the sexual dimorphic behavior driven by estrogen. The same trend is followed also by the endocytotic protein necap1, supporting once again our findings. Male, in contrast, being outside estrogen action need accessory mechanisms that might account for such differences in proteins alterations.

An interesting result concerns the overexpression of GAP43 in the ipsilateral hemisphere of male neuronal-specific STAT3 KO mice. Previous studies in adult rats show that the elevated expression of GAP43 is due to transneuronal regression of SNr neurons in response to deafferentation of the GABAergic inputs (Gregersen *et al.* 2001). In line with these studies our results suggest that male KO mice subjected to MCAO have increased damage at the neuronal structure level than that of WT. Neuronal damage present in males mice is mostly related to the lack of neuronal STAT3, as demonstrated by the high number of proteins altered in the contralateral hemisphere of STAT3 KO animals compared. WT. CRMP2, an axonal guidance protein, was increased in the contralateral hemeshpere of transgenic male animals. Several studies from our and other labs correlated CRMP2 alterations with stroke and Alzheimer disease outcome (Castegna *et al.* 2002; Chen *et al.* 2007; Owen *et al.* 2009; Cuadrado *et al.* 2010; Koh 2011) suggesting CRMP2 impairment as a common feature of the neurodegenerative process.

Antioxidant, folding and transcriptional proteins (Table 2)

Among the proteins identified, peroxiredoxin 6 (prdx6), belonging to cell antioxidant response, was found altered in the comparison between ischemic and non-ischemic brain hemispheres. Interestingly, in this work we report for WT animals lower levels in males and no change in females, while in KO animals no changes in male and increased levels in females, supporting an involvement of prdx6 in neuroprotection events that are induced after ischemia/reperfusion by the lack of STAT3. We previously reported differences of Prdx6 levels between males and females, with the latter being increasingly protected from oxidant species (Di Domenico *et al.* 2010). A previous proteomics study on male rats

subjected to MCAO, support our finding showing that ischemic injury induces neuronal cell death by modulating several antioxidant proteins such as peroxiredoxin (Koh 2010).

Our previous study suggested that Pin-1 levels reflect the estrogen STAT3 mediated induction of pro-survival pathway(s) (Di Domenico *et al.* 2010), this conclusion is supported here where decreased levels of Pin-1 are present in ischemic brain of KO female, while no changes were found in brain from male mice.

Transcriptional proteins (Table 2)

It is well established that transcription factors such as NF-kB are activated during MCAO-induced ischemia/reperfusion injury. Further STAT3 activation leads to the induction of transcription at different levels (Dziennis and Alkayed 2008). In the current study, altered levels of proteins involved in transcription events are mainly related with the transgenic condition than with stroke; therefore, increased levels of CALR and Brain acid soluble protein were found in male neuronal-specific STAT3 KO compared to WT and decreased levels of transcriptional activator protein were observed in female neuronal-specific STAT3 KO compared to WT. Again the trend of alterations appears to be sex specific.

Conclusion

The expression pattern observed in our model supports the role of STAT3 as a mediator of neuronal function after injury by controlling metabolic, synaptic, structural and transcriptional pathways. Analyzing the different class of proteomics-identified proteins it appears that in neuronal-specific STAT3 KO mice the adaptive response to ischemia/reperfusion injury is delayed as a consequence of inadequate activation of pro-survival/repair, antioxidant, and energy-related activities. The differences observed between males and females confirm the role of sex hormones in response to brain injury and in the modulation of STAT3 signaling, emphasizing the importance of an appropriate choice of sex in the design of ischemia/reperfusion experiments and in the study of ischemic neuroprotection to ensure a complete and adequate interpretation of the results.

Acknowledgements

All authors state that they have no conflicts of interest. This work was supported in part by NIH grants NS070837 to NJA and AG05119 to DAB.

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