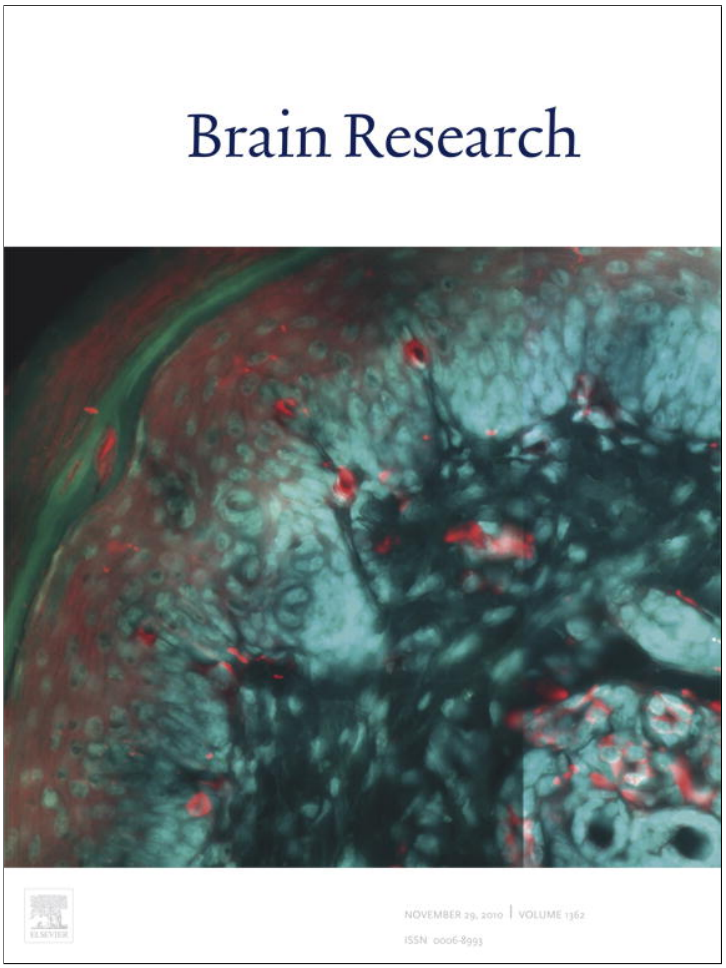

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BRAIN RESEARCH

Research Report

Involvement of stat3 in mouse brain development and sexual dimorphism: A proteomics approach

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ABSTRACT

Although the role of STAT3 in cell physiology and tissue development has been largely investigated, its involvement in the development and maintenance of nervous tissue and in the mechanisms of neuroprotection is not yet known. The potentially wide range of STAT3 activities raises the question of tissue- and gender-specificity as putative mechanisms of regulation. To explore the function of STAT3 in the brain and the hypothesis of a gender-linked modulation of STAT3, we analyzed a neuron-specific STAT3 knockout mouse model investigating the influence of STAT3 activity in brain protein expression pattern in both males and females in the absence of neurological insult. We performed a proteomic study aimed to reveal the molecular pathways directly or indirectly controlled by STAT3 underscoring its role in brain development and maintenance. We identified several proteins, belonging to different neuronal pathways such as energy metabolism or synaptic transmission, controlled by STAT3 that confirm its crucial role in brain development and maintenance. Moreover, we investigated the different processes that could contribute to the sexual dimorphic behavior observed in the incidence of neurological and mental disease. Interestingly both STAT3 KO and gender factors influence the expression of several mitochondrial proteins conferring to mitochondrial activity high importance in the regulation of brain physiology and conceivable relevance as therapeutic target.

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1. Introduction

The signal transducer and activator of transcription 3 (STAT3) is a molecule capable of rapidly responding to stimulation by cytokines, growth factors, and hormones and was initially recognized as a mediator of the acute response (Levy and Lee, 2002; Raz et al., 1994). In latent conditions STAT3 dynamically shuttles between the cytoplasm and the nucleus until it is recruited to the plasma membrane by an activated receptor. The subsequent phosphorylation of Tyr 705 allows the dimerization of STAT3 that becomes competent to bind the DNA and to activate the expression of target genes (Zhong et al., 1994). It is now evident that STAT3 function is not limited to the mediation of the immune response. Indeed, it appears to have pleiotropic activity relevant also to cell survival and differentiation (Levy and Darnell, 2002; Levy and Lee, 2002; Lutticken et al., 1994).

STAT3 is characterized by a multi-domain structure consisting of a coiled-coil domain, a DNA binding domain, a connector domain, an Src homology 2 domain (SH2), and the transcription activation domain at the carboxy terminus (Becker et al., 1998) containing the Tyr705 whose phosphorylation is the prerequisite for the stimulation of genic expression. The activity of STAT3 as a transcription factor can be positively or negatively modulated by a second phosphorylation event on Ser727 (Decker and Kovarik, 2000).

Most of STAT3 biological activities, including its neuroprotective and oncogenic functions depend on the phosphorylation status of a tyrosine residue located at position 705 (Tyr^{705}). The classical view of STAT3 actions states that tyrosine phosphorylation at this position promotes STAT3 dimerization and ability to bind target genes in the nucleus. However, recent reports indicate that unphosphorylated STAT3 also exhibits biological activities. For example, unphosphorylated STAT3 contributes to cholinergic anti-inflammation, prevents systemic inflammation, and improves survival in sepsis (Pena et al., 2010). Furthermore, unphosphorylated STAT3 can also affect gene expression in the nucleus; one mechanism is through binding to nuclear factor B (NF-B) and mediating its nuclear import (Yang and Stark, 2008). Finally, Gough et al. (2009) have discovered another role of STAT3 that is independent of tyrosine phosphorylation: the promotion of cellular transformation by the H-Ras oncogene. This function, which is dependent on serine phosphorylation of STAT3, takes place in mitochondria, where STAT3 is also believed to play a role in cellular respiration (Wegrzyn et al., 2009). Taken together, these observations suggest that unstimulated STAT3 plays an important role in diverse biological functions, and that STAT3 deletion will produce a phenotype under unstimulated (unstressed) conditions.

The basic role of STAT3 in cell physiology and tissue development is demonstrated by the fact that total STAT3 knockout animals die prior to the end of gestation (Takeda et al., 1997), and its importance in development and maintenance of nervous tissue has been largely investigated (Dziennis et al., 2007), although the mechanisms of neuroprotection are not yet known. Taken together, STAT3 shows the features of a highly regulated "multi-task" molecule involved in the regulation of different cellular pathways.

The potentially wide range of STAT3 activities raises the question of tissue- and gender-specificity as putative mechanism of regulation. To investigate the hypothesis of a gender-linked modulation of STAT3 we analyzed a neuron-specific STAT3 knockout mouse model in order to gain insight into whether STAT3 activity determines differences in the pattern of expressed brain protein in males and females also in absence of injury (Dziennis and Alkayed, 2008; Gao et al., 2004). STAT3 activation plays an important role during the development of estrogen-related sexual dimorphism in the brain. Interestingly, in a mouse model of brain ischemia it has been shown that the STAT3 protective effect is mediated by estradiol (Dziennis et al., 2007). The regulation of STAT3 function by sexual hormones has been proposed as well in a rat model of heart ischemia (Wang et al., 2009). The link between estradiol production and STAT3 is represented by the capacity of estradiol to phosphorylate STAT3, modulating its functionality; however, whether estradiol enhances STAT3 phosphorylation in brain regions during degenerative process or whether STAT3 activation contributes to estradiol's neuroprotective action has not been established yet (Dziennis et al., 2007; Dziennis and Alkayed, 2008).

In recent years, several studies show that estrogens play an important role in the central nervous system (CNS). Steroid hormones synthesized by the gonads and adrenal glands cross the blood–brain barrier and accumulate within the nervous tissues. Moreover, the nervous system is not only a target for sex hormones, but also a source of sex steroids (Genazzani et al., 2007; Plassart-Schiess and Baulieu, 2001; Veiga et al., 2004). Estrogen controls the differentiation and plasticity of distinct neuronal populations, enhances neurogenesis, modulates synaptogenesis and influences axonal sprouting (Candore et al., 2006; Czlonkowska et al., 2005; Kupina et al., 2003; Rodriguez-Navarro et al., 2008). Further, estrogen reportedly exerts a neuroprotective role in the brain through the inhibition of lipid peroxidation, the improvement in cerebral blood flow, induction of anti-apoptotic bcl-2, ER activation, activation of the MAP kinase pathway and the attenuation of NMDA receptor activation (Brann et al., 2007; Gandy, 2003; Green and Simpkins, 2000; Lloret et al., 2008; Nilsen and Brinton, 2004).

The implication of sexual dimorphism in the incidence of numerous neurological and mental disease like Parkinson's disease (PD), Alzheimer's disease (AD) or amyotrophic lateral sclerosis (ALS) has been largely investigated in the past years. Women showed lower incidence of PD, ALS or stroke compared to men, while for AD the incidence increased for women compared to men (Alkayed et al., 1998; Candore et al., 2006; Czlonkowska et al., 2005; Hall et al., 2005; Kupina et al., 2003; Vina et al., 2007; Zhang et al., 2008). A common factor for the incidence of these diseases was their reliance on individuals' age and the loss of ovarian steroids at menopause, particularly estrogens, correlates with the age-dependence of sexual dimorphism in many neurological diseases like AD (Behl and Manthey, 2000; Liu et al., 2009; Ragonese et al., 2006; Rodriguez-Navarro et al., 2008).

The purpose of the current study was to identify by a proteomic study, using male and female NF-L-Cre neg STAT3 flox/+ (CTR) and neuron-specific STAT3 KO mice brain samples, proteins controlled directly or indirectly by STAT3. We expected to gain new insight into the involvement of

STAT3 in brain development and maintenance. Moreover, performing a proteomic inter-gender study, we report differences in protein expression between male and female CTR and STAT3 KO mice that might lead to sexual dimorphism in the occurrence of neurological and mental disease.

2. Results

Two-dimensional electrophoresis offers an excellent tool for the screening of abundant protein changes in various disease states (Butterfield, 2004). The current proteomic study has been carried out to analyze the impact of the neuron-specific knock out of the STAT3 gene and the influence of the gender on mice brain protein expression pattern. We investigated in total for this study four groups of 6 brain samples each consisting of CTR Female, CTR Male, STAT3 KO Female and STAT3 KO Male, from 8 months old mice presenting differences for gender and transgenic condition. Each sample was processed by 2D-gel electrophoresis to obtain the corresponding gel protein map. All the gels were compared by software matching at the same time and then divided in four groups for comparison according to transgenic status or gender. The four groups of samples compared were CTR female vs. KO Female, CTR Male vs. KO Male, CTR Female vs. CTR Male and KO Female vs. KO Male. Each group was investigated for protein expression to have a more complete view of the phenomenon and to assess the proteome differences depending on the STAT3 KO condition or gender and to understand how these two factors may act in concert.

2.1. *CTR vs. STAT3 KO females*

The comparison between CTR females and neuron-specific STAT3 KO female mice brain samples allowed the identification of the proteins with altered expression induced by the Knock out condition in female animals. Each value is expressed in percent normalized to the CTR, set as 100% (Table 1A, Fig. 1a). Interestingly, all the seven proteins identified presented a decreased expression in the knock out animal relative to CTR animals. The proteins identified and percent decrease levels were: dynamin-1, 41%: aspartate aminotransferase (AAT), 70%; aconitate hydratase mitochondrial (Aco2), 53%; ATP synthase, (ATP Syn) 52%; peroxiredoxin 3 (Prdx3), 36%: glutamine syntethase (GS), 28%; and synapsin II (Syn II), 24%.

2.2. *CTR vs STAT3 KO males*

In male animals the comparison between neuron-specific STAT3 KO and CTR strain gave the identification of eight proteins with altered expression. CTR male values were set as 100%. Five brain proteins presented a decreased expression in transgenic mice and were: 14-3-3 zeta/delta, 30%; peroxiredoxin 6 (Prdx6), 56%: pyruvate dehydrogenase (PDH), 3%; hydroxybutyrate dehydrogenase (HDH), 11%; and synapsin II, 16%. Increased expression in STAT3 KO animals compared to CTR was identified for peptidyl prolyl cis/trans isomerase (Pin-1), 189%; voltage dependent anion channel 3 (VDAC3), 137%; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 218% (Table 1B, Fig. 1b).

2.3. *CTR females vs. CTR males*

The study of gender influence in CTR animal protein expression allowed the identification of 11 proteins with altered levels. CTR males were set as 100%. Beta synuclein with 206%; Peroxyredoxin 6 (Prdx6) with 205%; dynamin-1 (Dyn-1) with 236%; aconitate hydratase mitochondrial (Aco2) with 209%; manganese superoxide dismutase (MnSOD) with 151%; GAPDH with 272%; and voltage dependent anion channel 2 (VDAC2) with 312% showed increased levels in female animals compared with males. Conversely, Pin-1 with 53%; and isocitrate dehydrogenase (IDH) with 29% showed decreased expression in female animals (Table 2A, Fig. 1c).

Table 1 – (A) Summary of the proteins with different expression identified by mass spectrometry in CTR vs. STAT3 KO female mouse brain; CTR female is set as 100%. (B) Summary of the proteins with different expression identified by mass spectrometry in CTR vs. STAT3 KO male mouse brain; CTR male is set as 100%.

Protein	SwissProt Accession	*p*	Fold-change	*p*-value	Function
(A) CTR female vs. STAT3 KO female					
Dyn-1	P39053	6.00E-06	41% ↓	0.018	Synaptic vescicle formation
AAT	P05201	1.00E-06	70% ↓	0.046	Energy metabolism
Aco2	Q99KI0	7.00E-07	53% ↓	0.020	Energy metabolism
ATP Syn	Q03265	6.00E-08	52% ↓	0.023	Energy metabolism
Prdx 3	P20108	0.0005	36% ↓	0.028	Mitochondrial antioxidant
GS	P15105	2.00E-12	28% ↓	0.009	Glutamate metabolism
Syn II	Q64332	7.00E-14	24% ↓	0.001	Neurotrasmitters release
(B) CTR male vs STAT3 KO male					
14-3-3 z/d	P63101	6.00E-11	30% ↓	0.025	Structure/signalling
Prdx 6	O08709	3.00E-06	56% ↓	0.037	Cytosolic antioxidant
PDH	P35486	6.00E-04	3% ↓	0.048	Energy metabolism
Pin-1	P17742	9.00E-05	189% ↑	0.005	Signalling/proliferation
VDAC 3	Q60931	2.00E-05	137% ↑	0.025	Mitochondrial exchange
GAPDH	P16858	2.00E-07	218% ↑	0.049	Energy metabolism
HDH	Q99L13	3.00E-10	11% ↓	1.4E-06	Ketone bodies metabolism
Syn II	Q64332	7.00E-14	16% ↓	0.007	Neurotrasmitters release

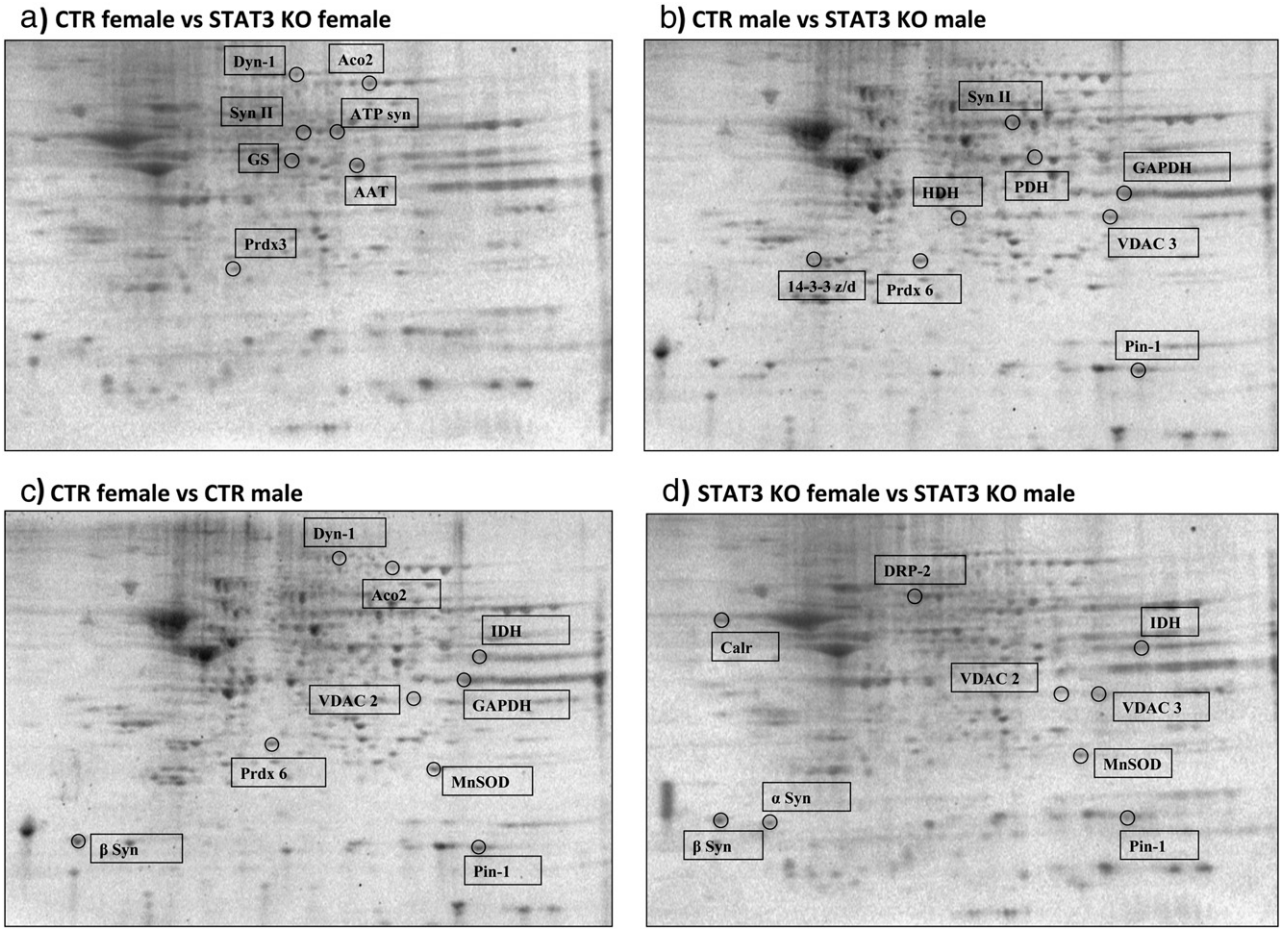

Fig. 1 – Proteomic profile with protein differentially expressed in the fourth groups of matching. (a) female CTR vs. STAT3 KO; (b) male CTR vs. STAT3 KO; (c) female CTR vs. male CTR; (d) female STAT3 KO vs. male STAT3 KO.

2.4. *STAT3 KO females vs. STAT3 KO males*

As the last analysis we compared neuron-specific STAT3 KO female with neuron-specific STAT3 KO male animals to investigate the impact of the gender influence on transgenic animals and identify the proteins whose expression might be influenced by STAT3 expression. STAT3 KO male values were set as 100%. Nine proteins presented a different expression pattern in brain in female vs. male study. Alpha and beta synuclein (α Syn, β Syn) with 188% and 154%; VDAC2 with 224%; calreticulin (Calr) with 163%; dihydropirimidase related protein 2 (DRP2) with 535%; and MnSOD with 147% showed increased expression in female animals. Peptidyl prolyl cis/trans isomerase (Pin-1) with 62%; voltage dependent anion channel 3 (VDAC3) with 68%; and isocitrate dehydrogenase (IDH) with 49% showed decreased expression in STAT3 KO female animals compared with STAT3 KO male (Table 2B, Fig. 1d).

2.5. *Proteomics data validation*

To verify the proteomics data, validation studies on the expression levels of three proteins identified by mass spectrometry analysis have been performed. The modulation of protein expression level was validated by WB analysis of GAPDH, MnSOD and Pin-1. The results are shown in Fig. 2 demonstrating an increase of GAPDH in CTR males vs. STAT3 KO male, an increase in MnSOD in CTR female vs. CTR male, and an increase of Pin-1 in STAT3 KO male vs. female, confirming the identification and levels obtained by proteomics.

2.6. *Protein carbonyls measurements*

Several neurodegenerative diseases like Alzheimer or Parkinson disease are characterized by extensive oxidative stress in the CNS. The most widely studied marker of oxidative stress-induced protein oxidation is the formation of carbonyl groups (Sultana et al., 2006b). Protein carbonylation by free radical leads to oxidation of side-chains (mostly hystidine, arginine and lysine residues), backbone fragmentation, formation of new reactive species, release of further radicals, and occurrence of chain reactions. Protein carbonylation is generally associated with loss of function and may lead to either the unfolding or degradation of the damaged proteins. Our results in Fig. 3 show low levels of protein carbonyls in CTR animals with a slightly non-significant difference between male and female. The total content of protein carbonyls increased significantly in STAT3 KO animals, respectively, about 131% for female and 142% for male compared to CTR female

Table 2 – (A) Summary of the proteins with different expression identified by mass spectrometry in CTR female vs. CTR male mouse brain; CTR female is set as 100%. (B) Summary of the proteins with different expression identified by mass spectrometry in STAT3 KO female vs. STAT3 KO male mouse brain; STAT3 KO female is set as 100%.

Protein	Accession number	*p*	Fold-change	*p*-value	Function
(A) CTR female vs CTR male					
β Syn	Q91ZZ3	5.00E-09	206% ↑	0.048	Synaptic
Prdx 6	O08709	1.00E-06	205% ↑	0.026	Antioxidant
Pin-1	P17742	7.00E-05	53% ↓	0.032	Signaling/proliferation
Dyn-1	P39053	6.00E-06	236% ↑	0.039	Vescicle formation
Aco2	Q99KI0	7.00E-07	219% ↑	0.047	Energy metabolism
MnSOD	P09671	3.00E-06	151% ↑	0.046	Antioxidant
GAPDH	P16858	2.00E-07	272% ↑	0.0002	Energy metabolism
VDAC2	Q60930	9.00E-06	328% ↑	0.007	Mitochondrial exchange
IDH	O81796	2.00E-04	29% ↓	0.007	Energy metabolism
(B) STAT3 KO female vs STAT3 KO male					
β Syn	Q91ZZ3	5.00E-09	188% ↑	0.012	Synaptic
α Syn	O55042-1	1.00E-11	154% ↑	0.036	Synaptic
VDAC 2	Q60930	2.00E-06	224% ↑	0.045	Mitochondrial exchange
Calreticulin	P14211	7.00E-06	164% ↑	0.002	Protein folding
DRP-2	O08553	3.00E-08	535% ↑	0.025	Neuron structure
Pin-1	P17742	9.00E-05	62% ↓	0.016	Signaling/proliferation
MnSOD	P09671	3.00E-06	172% ↑	0.042	Antioxidant
VDAC 3	Q60931	2.00E-05	68% ↑	0.002	Mitochondrial exchange
IDH	O81796	2.00E-04	49% ↑	0.016	Energy metabolism

animals. The direct comparison between female and male STAT3 KO animals shows a small increase of protein cabonyls content (8.4%) in male brain (Fig. 3).

3. Discussion

In order to have a more detailed understanding of the impact of STAT3 on brain molecular physiology we chose an approach consisting in both intra- and inter-gender comparison of CTR and STAT3 KO mice. Further, we analyzed by measurement of protein carbonyls the amount of oxidative stress in CTR and KO animals to correlate the influence of the stress condition to proteins levels. The phenotype shown by our model and described in the Experimental procedures section potentially explain the increased protein carbonyl content in knock out brain compared with CTR animals.

The proteomics data obtained from the comparison of the females CTR vs. females STAT3 KO (Fig. 4) indicated a tight connection between STAT3 and energy production in brain. Previous reports showed the importance of STAT3 in the regulation of the mitochondrial oxidative phosphorylation system (Gough et al., 2009; Wegrzyn et al., 2009). Our data further support this hypothesis showing in an ex-vivo model that, in female mouse brain, the absence of STAT3 is not only linked to the reduction of the mitochondrial aconitate hydratase and ATP synthase, but also to the reduction of the cytosolic aspartate aminotransferase. The reduction of Aco2 and AAT can be linked to a limited anaplerotic metabolism and to an impaired metabolism of glutamate whose potentially toxic effect for the neuronal cell is further increased in the case of an inadequate ATP availability (Meredith et al., 2009).

The hypothesis of a dysfunction in glutamate metabolism is supported by the reduction of GS, an enzyme that catalyzes the ATP-dependent conversion of glutamate to glutamine, a non-essential amino acid whose homeostasis appears to be of high relevance for mitochondrial activity (Mates et al., 2009).

The importance of STAT3 signaling for mitochondrial activity was strengthened by the reduction, in KO mice, of Prdx3, a mitochondrial matrix specific enzyme involved in the scavenging of hydrogen peroxide (Cox et al.). Our data and the reported contribution of STAT3 in the regulation of MnSOD in ischemic models (Jung et al., 2009a,b) would suggest that STAT3 is a regulator of both mitochondrial metabolism and redox homeostasis in mouse female brain. Moreover, elevated GAPDH and VDAC levels in male STAT3 KO neurons (Table 1B) propose an increase in glycolysis. GAPDH controls a critical step of glycolysis and is one of the major target of oxidative modification in Alzheimer's disease (Butterfield et al., 2010; Sultana et al., 2007). VDAC3, responsible of the transport of various metabolites across the outer mitochondrial membrane, has been localized in the active presynaptic zone (Morciano et al., 2009) and is part of the mitochondrial permeability transition pore, implying that differential VDAC expression could influence mitochondrial activity and apoptosis. STAT3 may play a role in mitochondrial energy production by either inducing mitochondrial mass increase and/or enhancing oxidative phosphorylation enzyme complexes. In contrast, female STAT3 KO neurons present reduced mitochondrial oxidative phosphorylation enzymes, or more likely, the mitochondrial mass is reduced, as evidenced by reduction of Aco2 and ATP synthase (Table 1A).

Two other proteins, concerned the synaptic transmission machinery, are downregulated in the brain of STAT3 KO female. Dyn-1 is a GTPase involved in vesicular trafficking processes and in synaptic vesicle recycling, with a basic role as component of the mitochondrial fusion-fission machinery (Ingerman et al., 2005). Recently, it has been shown that Dyn-1 is downregulated

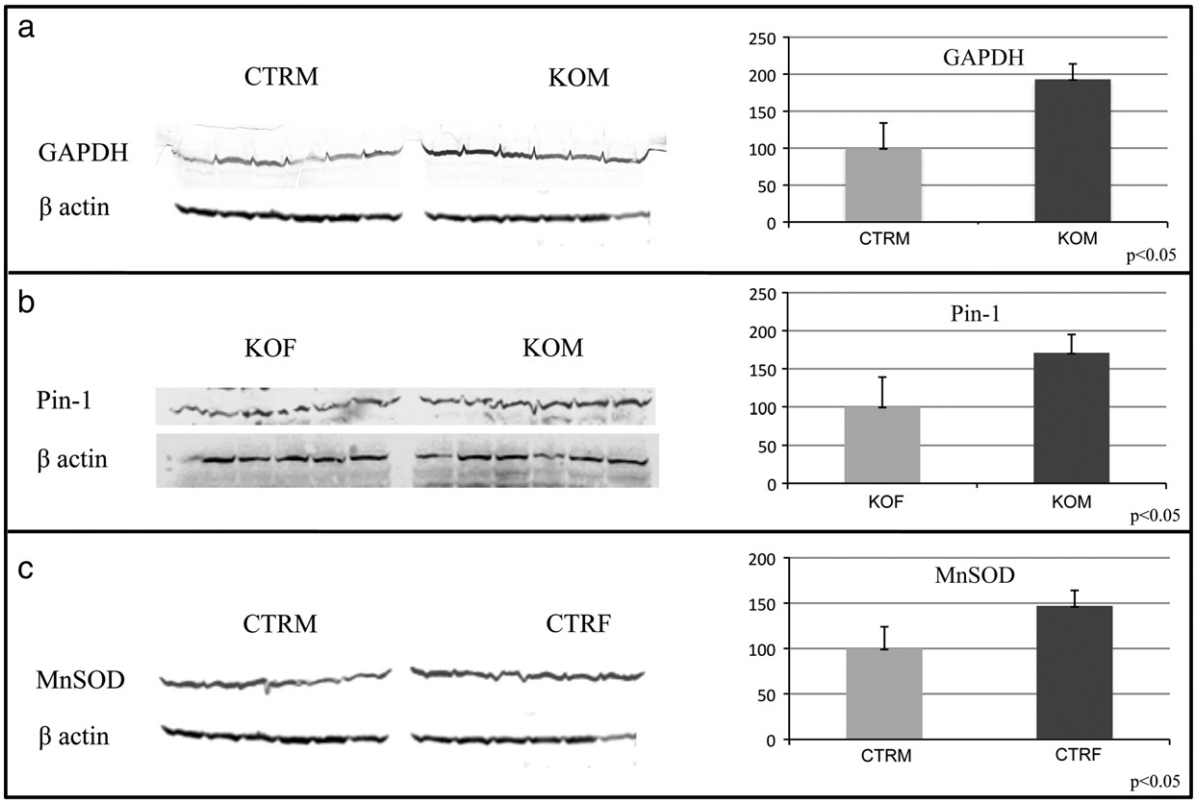

Fig. 2 – Western blot analysis of GAPDH, Pin-1 and MnSOD protein levels. Total proteins (50 μg) extracted from CTR male and female and STAT3 KO male and female brains were loaded onto a 8–16% SDS-PAGE gel), blotted onto nitrocellulose membrane and challenged with specific GAPDH, Pin-1 and MnSOD antibodies. Western blot analysis confirmed proteomic results of increased levels of GAPDH in KO male compared to CTR male (a), increased levels of Pin-1 in STAT3 KO males compared to STAT3 KO females (b) and increased levels of MnSOD in CTR females compared to CTR males (c). Immunoblots were analyzed by densitometry and all values were normalized to β-actin. Densitometric values are given as percentage of the relative control values ($n=6$) and represent the mean ± S.E.M. of three independent experiments.

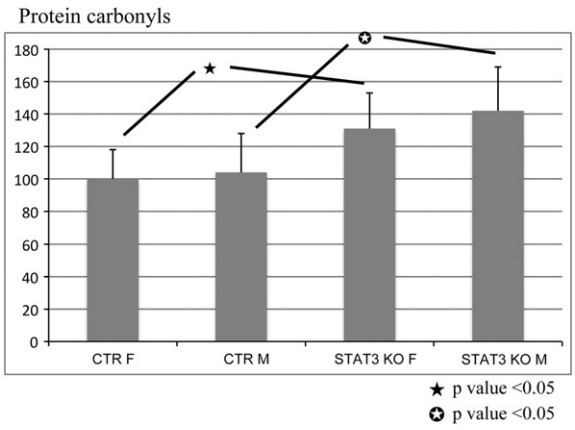

Fig. 3 – Slot blot analysis of protein carbonyls levels in CTR and STAT3 KO female and male samples. Data represent the increase of the protein carbonyl levels in STAT3 KO female and male compared to CTR female and male mouse brain using traditional immunochemical detection. Error bars indicate S.E.M for 6 samples in each group. Measured values are normalized with the mean of the control sample (CTRF).

by Aβ oligomers treatment in rats, and its downregulation correlates with memory impairment (Watanabe et al.) conferring relevance to this protein in the molecular processes of brain physiology.

The reduced expression of Syn II supports the hypothesis of a weaker neurotransmission system in the in STAT3 KO females. Syn II is involved in the modulation of neurotransmitter release and synaptogenesis (Bennett and Scheller, 1994), with an established role in mice learning mechanisms (Silva et al., 1996). Syn II variants have been proposed as risk factor in the susceptibility to schizophrenia (Chen et al., 2004) and small alterations in its activity could have long-term effects in the central nervous system.

Pin-1 is an essential nuclear peptidylprolyl cis-trans isomerase with a fundamental role for the neuronal physiology, as demonstrated by its role in neurodegeneration (Butterfield et al., 2006; Sultana et al., 2006a). In Alzheimer's disease Pin-1 expression is inversely correlated with predicted neuronal vulnerability and actual neurofibrillary degeneration (Liou et al., 2003). In our system Pin-1 overexpression in mice could confirm a stimulation of protective mechanism to counteract the effect of absence of STAT3 signaling in male neurons.

The hypothesis of an unstable equilibrium redox in males STAT3 KO is supported by the decreased expression of Prdx6.

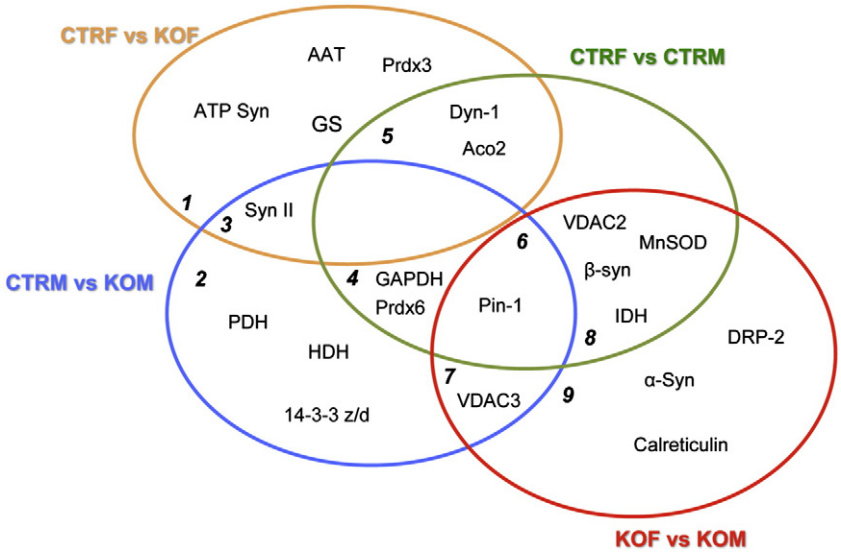

Fig. 4 – Venn diagram on overlapping proteins between the four different group of analysis. Every group and their overlapping parts have been categorized in 9 different subgroups numbered from 1 to 9. (1) AAT, ATP synthase, GS and Prdx3 present only in female intra-gender group; (2) PDH, HDH and 14-3-3 z/d present in male intra-gender group; (3) Syn II, overlapped between both intra-gender group; (4) GAPDH and Prdx6, overlapped between both intra-gender group and CTR inter-gender group; (5) Dyn-1 and Aco2, overlapped between female intra-gender group and CTR inter-gender group; (6) Pin-1, overlapped between male intra-gender group and CTR and KO inter-gender groups; (7) VDAC3, overlapped between male intra-gender group and KO inter-gender group; (8) VDAC2, MnSOD, IDH and β-Syn, overlapped between CTR and KO inter-gender group; (9) DRP-2, calreticulin and α-Syn, present only in KO inter-gender group.

HDH is a mitochondrial membrane enzyme involved in the NAD-dependent metabolism of ketone bodies. Our data showed that in STAT3 KO male brain the expression of both PDH and HDH is severely down regulated, thus leading to a condition in which mitochondria could be deprived of substrates for TCA cycle. Therefore, in males the knock out of STAT3 gene likely results in a deficit in the mitochondrial capacity to adapt to changes in substrates availability, which could make them more vulnerable upon stress inducing events.

14-3-3 z/d is a protein able to induce a shift in the cellular machinery toward a pro-survival execution program (Dong et al., 2007; Masters et al., 2002; Mhawech, 2005). Our results on 14-3-3 support the control of STAT3 on 14-3-3 z/d expression (Chong and Maiese, 2007; Quayle and Sadar, 2007). The only protein that shows the same behavior in both genders is Syn II suggesting that the common endpoint of lack of STAT3 in neuronal cells could be the alteration in synaptic transmission and consequently an elevated susceptibility to neurodegeneration and brain injuries.

According to several reports of modulation of STAT3 activity by hormones (Bjornstrom and Sjoberg, 2002; Dziennis et al., 2007; Wang et al., 2009), our data demonstrate that males and females respond to the STAT3 KO by modulating proteins expression in different ways. Interestingly, while in females the absences of STAT3 signaling modifies the expression of metabolic proteins, in transgenic males we observed the regulation of both metabolic and signaling proteins probably associated with a more complex series of events. Nevertheless, the major effects of the STAT3 KO in male as in female brain appear to reside in mitochondrial and oxidative metabolism. The overexpression in GAPDH, VDAC3 and Pin-1 reflect that males are characterized by an increased stress and oxidative damage in STAT3 absence, probably derived from the lack of the neuroprotective action of female hormones.

The precise mechanisms responsible for naturally occurring sexual dimorphism in the prevalence and clinical course of numerous neurological disorders remains unclear (Veiga et al., 2004); however, gender-related differences in neuroanatomy, and neurochemistry have been described in several mammals especially in human and rodents (Cerghet et al., 2009; Guevara et al., 2009; Ramirez and Jimenez, 2002; Zhang et al., 2002). The value of the gender aspect showed relevance also in several mouse models of AD or ischemia particularly (Alkayed et al., 1998; Liu et al., 2009; Schuessel et al., 2004; Wang et al., 2003).

As previously described by Alkayed et al., STAT3 expression is strongly correlated with sexual dimorphism in neurodegenerative damage and recovery, due to the interaction of STAT3 with estradiol (Dziennis et al., 2007; Dziennis and Alkayed, 2008). STAT3 could be considered one of the main signaling players in several pathways affected by estrogen-mediated sexual dimorphism (Bjornstrom and Sjoberg, 2002).

In our study we used a mouse model that was sacrificed at 8 months old, when female mice were under the influence of sexual hormones. We analyzed, by proteomics, the protein expression differences of male versus female CTR and neuron-specific STAT3 KO mice brain, inferring the influence of gender in normal and neuron-specific STAT3 KO condition. The inter-gender analysis comparison evoke several intra-gender results (i.e., Dyn-1), supporting our previous

hypothesis about the involvement of STAT3 in brain development and maintenance and attributing to STAT3 a considerable role in sexual dimorphism, probably as a mediator of estrogen signaling. In contrast, the upregulation of proteins repeated in females in both inter-gender analysis (i.e., MnSOD) could be associated with estrogen action in a STAT3-independent manner. Proteins altered either in male or female STAT3 KO and not in CTR could be influenced by the increased stress condition supported by the protein carbonyl analysis.

The inter-gender study shows the effect of STAT3 expression on Aco2 levels, described in the intra-gender analysis, suggesting indeed that in the brain this difference might be fueled by estrogen production. GAPDH, instead, increases in STAT3 KO male probably as a consequence of the increased stress condition caused by the STAT3 deficit in neurons. Also the increased expression of IDH in both CTR and STAT3 KO males possibly reflects a compensation mechanism adopted by the neuron to maintain high levels of α-ketoglutarate for energy production and neurotransmission through glutamate. Given that several proteins related to energy metabolism are impaired in neurodegenerative disease like AD (Reed et al., 2009; Sultana et al., 2006b; Sultana et al., 2006c), gender differences in energy metabolism are consistent with the hypothesis that sexual dimorphism directly influences the incidence of neurodegenerative disease.

Interestingly, earlier studies showed that estrogen mediated overexpression of MnSOD decreases oxidant production in young females and protect mitochondria against the increase in peroxide production caused by Aβ peptide (Guevara et al., 2009; Schuessel et al., 2004; Vina et al., 2007). Our data support this hypothesis showing that MnSOD over expression is strictly related with female gender. Moreover, the female protection from oxidant species might be associated with the overexpression of Prdx6 in a STAT3-dependent manner.

Both VDAC3 and VDAC2 are differently expressed in males and females and they might be influenced by stress condition and sexual dimorphism, respectively.

The synaptic transmission machinery presents as well a sexual dimorphic behavior with the overexpression of Dyn-1 and β Syn in females, suggesting the involvement of estrogens in synaptic transmission in mammals CNS. Moreover alteration on DRP-2, calreticulin and α Syn levels might depend on the sex-related activation of pro-survival pathways to counterbalance the STAT3 deficiency and the increased oxidative stress. DRP-2 overexpression might also suggest the involvement of cellular plasticity in neuronal adaptation to stress condition.

Pin-1 overexpression occurs in males in both inter-gender studies. suggesting it as a protective response to a lower hormone-related induction of pro-survival pathway. The involvement of Pin-1 in neuronal disease such as AD (Butterfield et al., 2006; Sultana et al., 2006a) correlates with our hypothesis.

The inter-gender analysis in CTR and STAT3 KO mice support part of the data from the intra-gender analysis evaluating sexual-related STAT3-dependent differences and verifying a prominent role of STAT3 in sexual dimorphism. These data correlate with gender-related incidence of several neurodegenerative disease and provide new insight to understand the trophic and protective actions of estrogen in the brain.

The overall results of our study enhance the already well-known relevance of mitochondrial activity in the regulation of brain physiology showing how neuronal cells adapt to altered signaling (i.e., STAT3 KO) by modulating mitochondrial and mitochondrial-associated proteins. In hippocampal neurons the role of leptin mediated-STAT3 signaling on mitochondrial physiology is shown by the induction of ROS-scavenger manganese superoxide dismutase and anti-apoptotic Bcl-xL enhancing mitochondrial resistance to apoptosis and excitotoxicity (Guo et al., 2008). A recent report confirms the connection between STAT3 and mitochondria showing that the mitochondrial toxin MPP(+) decreases STAT3 prosurvival pathway in cerebellar granule cells, therefore supporting the hypothesis that the neuroprotective role of STAT3 can be related to mitochondrial function (Junyent et al., 2010). Furthermore, Wegrzyn et al. (2009) demonstrated the location of STAT3 within mitochondria of cultured cells and primary tissues, and its capability to modulate respiration by regulating the activity of Complexes I and II in pro-B. Several data show that the mechanisms of estrogen-inducible neuroprotection against degenerative insults depend from estrogen regulation of mitochondrial bioenergetics and activation of pro-survival pathways. We suggest, as other authors proposed previously (Nilsen and Brinton, 2004), mitochondria as effective therapeutic targets of estrogen actions in the prevention of neurodegenerative diseases. However additional studies are needed in order to verify our finding on STAT3 and sexual dimorphism in human brain.

4. Experimental procedures

4.1. *Animals*

4.1.1. *Neuronal STAT3 knockout (STAT3 KO) Mice*

NF-L-Cre and STAT3$^{flox/flox}$ mice were originally provided by Dr. Michael Sendtner, Institute for Clinical Neurobiology, University of Würzburg, Würzburg, Germany to Dr. Nabil Alkayed, Oregon Health & Science University, Portland, OR who performed crosses between these two lines to generate the neuronal STAT3 KO and control mice used in these studies. NF-L-Cre transgenic mice express Cre recombinase under the control of the human neurofilament light chain (NF-L) promoter in brain and spinal cord and are neuron-specific (Schweizer et al., 2002). The NF-L-Cre mouse line is maintained on a CD-1 background by breeding hemizygotes to CD-1 mice (Hsd:ICR (CD-1®); Harlan Laboratories, Inc., Indianapolis, IN). Mice hemizygous for the NF-L-Cre transgene are viable, fertile, and do not display any gross physical or behavioral abnormalities. Primer sequences for genotyping by PCR are as follows (Schweizer et al., 2002): NF-L-Cre 5′-TCG CAG GCT GCG TCA GGA G-3′ and Pmc-cRE 5′-GGT ATG CTC AGA AAA CGC C-3′. These sequences detect the unique combination of the human NF-L promoter and Cre cDNA and produce a 250 bp product.

Mice with the loxP-flanked (floxed) *Stat3* gene (STAT3$^{flox/flox}$) possess two loxP sites flanking a neomycin resistance (*neo*) gene within intron 21 and one loxP site within intron 22 of the *Stat3* gen (Akira, 2000; Takeda et al., 1998). STAT3$^{flox/flox}$ mice are viable, fertile, and do not display any gross physical or behavioral abnormalities. PCR is performed with the following primers for the STAT3-loxP: mStat3e22fwd 5′-CCT GAA GAC CAA GTT CAT CTG TGT GAC-3′ and mStat3e23rev 5′-CAC ACA

AGC CAT CAA ACT CTG GTC TCC-3′. Wild-type and floxed STAT3 alleles yield 250 and 350 bp products, respectively.

Neuronal STAT3 KO mice are generated by crossing NF-L-Cre mice with $STAT3^{flox/flox}$ mice as previously described (Schweizer et al., 2002). To detect Cre-mediated recombination of the floxed *Stat3* gene, the following primers are used for PCR: mStat3loxP 5′-GAT TTG AGT CAG GGA TCC ATA ACT TCG-3′ and mStat3e23rev. The recombined STAT3 allele yields a product of 150 bp. Controls for all experiments were mice negative for NF-L-Cre and heterozygous for the floxed STAT3 allele.

The NFl-Cre/Floxed STAT3 mice used in our study show similar body weight as their wild type counterparts, in agreement with another report using the same mice (Schweizer et al., 2002).

4.2. *Sample preparation*

All the mice have been sacrificed at the age of 8 months. Mice were briefly anesthetized with isoflurane (2–3%) and decapitated. The brain is rapidly removed from skull, and placed on pre-chilled metal plate on ice for dissection. The olfactory bulbs and cerebellum are removed, the remaining brain tissue is bisected into two hemispheres, and each hemisphere is dipped into 2-methylbutane on dry ice (–50 °C). The whole procedure between decapitation and freezing takes less than 1 min. The brains were then manually homogenized in ice-cold buffer (10 mM Tris pH 8, 0.32 M Sucrose, 0.1 mM $MgCl_2$, 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 BCA method (Pierce, Rockford, IL, USA).

Proteins (150 μg) were precipitated in 15% final concentration of trichloroacetic acid for 10 min in ice. Samples were then spun down at 14000 rpm for 5 min and precipitates were washed in ice-cold ethanol-ethyl acetate 1:1 solution four times.

The final pellets were dissolved in 200 μl of 8M urea, 2% CHAPS, 2 M thiourea, 20 mM dithiothreitol, 0.2% of ampholytes (Bio-Rad, Hercules, CA, USA) and bromophenol blue, incubated at room temperature for 90 minutes and sonicated for 5 s.

4.3. *Two-dimensional electrophoresis*

Samples (200 μl) were loaded on 110-mm pH 3–10 immobilized pH gradients (IPG) strips in a Bio-Rad IEF Cell system (Bio-Rad, Hercules, CA). Following 18 h of active rehydration (50 V) isoelectric focusing was performed as previously reported (Reed et al., 2009). The focused IEF 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 (SDS), 20% glycerol containing first 2% dithiothreitol and then 2.5% of iodoacetamide.

SDS PAGE was performed in Criterion Tris-HCl Gels 8-16% (Bio-Rad, Hercules, CA, USA) at 200 V for 1 h.

4.4. *SYPRO ruby staining*

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, Hercules, CA, USA).

After destaining in deionized water, gels were scanned with a STORM UV transilluminator (λex=470 nm, λem=618 nm, Molecular Dynamics, Sunnyvale, CA, USA).

4.5. *Image analysis*

SYPRO ruby-stained gel images were obtained using a STORM phosphoimager as indicated above and saved in TIFF 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 (Control and AD or Control and MCI) 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. Based on these parameters the software defines spot centers for the gel. If the software "misses" spots that are obvious to the naked eye, the user can manually assign a spot center. This process generates a large pool of data, approximately 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 manger 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. A Boolean analysis set was created that identified overlapping spots from the aforementioned quantitative and statistical sets. These spots were selected for subsequent mass spectrometric analysis.

4.6. *In-gel trypsin digestion*

Protein spots statistically different than controls were digested in-gel by trypsin using protocols previously described and modified by Thongboonkerd et al. (2002). Spots were taken from individual gels and not 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) at room temperature for 15 min. Acetonitrile was then added to the gel pieces and incubated at room temperature 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 DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT 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 room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 ml of 50 mM NH_4HCO_3 and incubated at

room temperature 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) 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.

4.7. *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 8 most intense parent ions measured in the FT at 60,000 resolution 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, and P-value (protein and peptide)<0.01. IPI accession numbers were cross-correlated with SwissProt accession numbers for final protein identification.

4.8. *Western blot*

Protein (50 μg) were added to sample buffer, denaturated for 5 min at 100 °C, loaded on 8–16% precast Criterion gels (Biorad) 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 (BSA) in phosphate-buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST). The membranes were incubated with primary antibody anti-GAPDH (Chemicon International Inc, Billerica, MA), anti-MnSOD (AbCAM Inc, Cambridge, MA) and anti-Pin1 (Santa Cruz Biotech. Inc., Santa Cruz, CA) for expression analysis in PBST for 2 h with gentle rocking at room temperature. The membranes were then washed three times for 5 min with PBST followed by incubation with anti-mouse horseradish peroxidase conjugate secondary antibody (Sigma, MO) in PBST for 2 h at room temperature. Membranes were then washed three times in PBST for 5 min and developed using ECL plus WB detection reagents (Amersham Biosciences, Pittsburgh, PA) for horseradish peroxidase conjugate secondary antibody. Blots were dried, scanned in TIF format using STORM UV transilluminator (λex=470 nm, λem=618 nm, Molecular Dynamics, Sunnyvale, CA, USA) for chemiluminescence. The images were quantified with ImageQuant TL 1D version 7.0 software (GE Healthcare).

4.9. *Protein carbonyls detection*

Protein carbonyls are an index of protein oxidation and were determined as described previously (Stadtman and Berlett, 1997). Samples (5 μg of protein) were derivatized with 10 mM 2,4-dinitrophenylhydrazine in the presence of 5 μl of 12% SDS for 20 min at room temperature. The samples were neutralized with 7.5 μl of the neutralization solution (2 M Tris in 30% glycerol). The resulting solution was loaded into each well on nitrocellulose membrane under vacuum using a slot-blot apparatus (250 ng/lane). Then the membrane was washed with wash buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20], blocked by incubation in the presence of 3% BSA, followed by incubation with rabbit polyclonal anti-DNPH antibody as primary antibody for 1 h. The membrane was washed with wash buffer and further incubated with alkaline phosphatase (ALP)-conjugated goat anti-rabbit antibody as secondary antibody for 1 h. Blot was developed using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) color developing reagent, scanned in TIF format using Adobe Photoshop on a Canoscan 8800F (Canon) and quantified with ImageQuant TL 1D version 7.0 software (GE Healthcare).

4.10. *Statistical analysis*

Statistical analysis of protein levels matched with spots on 2D-gels from AD and MCI 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.

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