

HIPPOCAMPAL HEAT SHOCK PROTEIN 25 EXPRESSION IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

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Abstract

Hippocampal abnormalities are believed to increase the risk of cognitive decline in diabetic patients. The underlying mechanism is unknown, but both hyperglycemia and oxidative stress have been implicated. Cellular stresses induce expression of heat shock protein 25 (HSP25) and this results in cytoprotection. Our aim was to assess hippocampal expression of HSP25 in experimental diabetes. Mice were rendered diabetic by streptozotocin injection. Ten weeks after diabetes onset hippocampal HSP25 expression was studied by immunoblotting and immunohistochemistry (IHC). Expression of glial fibrillary acidic protein (GFAP), nitrotyrosine, iNOS, HSP72, HSP90, and Cu/Zn superoxide dismutase (SOD) was assessed by either IHC or immunoblotting, Cu/Zn-SOD activity by enzymatic assay, and malondialdehyde (MDA) content by colorimetric assay. Hippocampal HSP25 was significantly increased in diabetic as compared to non-diabetic animals and localised predominantly within the pyramidal neurons layer of the CA1 area. This was paralleled by overexpression of nitrotyrosine, iNOS, SOD expression/activity, and enhanced MDA content. In experimental diabetes, HSP25 is overexpressed in the CA1 pyramidal neurons in parallel with markers of oxidative stress.

Keywords: HSP25, oxidative stress, experimental diabetes, hippocampus

Introduction

Multiple organ systems are adversely affected by diabetes, including the brain, which undergoes changes that may increase the risk of cognitive decline (Ryan et al., 1993; Ott et al., 1999; Rstow, 2004). Studies in experimental diabetes have demonstrated progressive astrogliosis, pyramidal neuron apoptosis, altered synaptic plasticity, and reduced dendritic complexity within the hippocampus (Magariños and McEwen, 2000; Saravia et al., 2002; Valastro et al., 2002). The underlying mechanisms of these abnormalities are unknown; however, both hyperglycemia and oxidative stress have been implicated (Mastrocola et al., 2005; Nishikawa et al., 2000).

Heat Shock Proteins (HSPs) are ubiquitous, highly evolutionary conserved intracellular proteins (Ellis and van der Vies, 1991). Thermal, oxidative, hemodynamic, osmotic, and hypoxic stresses induce HSPs expression, and this stress response results in cytoprotection (Ellis and van der Vies, 1991; Feder and Hofmann, 1999). Specifically, HSPs prevent nonspecific protein assembly, assist in denatured protein refolding, and interfere with proapoptotic pathways (Yenari et al., 2005).

Studies on the expression of HSPs in the diabetic hippocampus have shown that HSP60, a mitochondrial-specific molecular chaperon, is overexpressed in the hippocampal CA1 region and that HSP60 expression strongly correlates with that of superoxide dismutase (SOD) (Yuan et al., 2006), a marker of mitochondrial oxidative stress. By contrast, no abnormalities in HSP70 expression were observed (Güven et al., 2009).

HSP25 is a chaperone, binds to F-actin, and protects cells from both oxidative stress and apoptosis (Mehlen et al., 1997; Sanz et al., 2001). In addition, HSP25 interacts with HSP90 and synergizes with HSP72 in preventing neural cell death (Patel et al., 2005). HSP25 is weakly expressed in the normal hippocampus, but strongly induced in hippocampal neurons exposed to thermal stress (Kirbach and Golenhofen, 2011). Recent studies have shown that HSP25 expression is enhanced in the retina and the kidney (Barutta et al., 2008; Pinach et al., 2011) from mice made diabetic with streptozotocin (STZ), an antibiotic that causes pancreatic β -cell destruction, resulting in a state of

insulin-dependent type 1 diabetes mellitus. Whether HSP25 is also altered in the brain, another important target organ of diabetes-induced injury, remains unknown.

The aim of the present study was to assess HSP25 expression in the hippocampus of streptozotocin-induced diabetic mice in parallel with markers of oxidative stress.

Experimental procedures

Materials

All materials were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Animals and induction of diabetes

Eight-week-old male C57BL6 mice from Jackson Laboratories (Bar Harbor, ME) were used in the present study. All animal procedures were in accordance with the Italian law (D.L.116/1992) and animals were maintained on a normal diet under standard animal house conditions. Diabetes was induced by intraperitoneal injections of STZ in citrate buffer, pH 4.5 (55 mg/kg body wt/day), delivered in five consecutive daily doses. Mice sham injected with sodium citrate buffer were used as controls. Diabetes onset was confirmed by blood glucose levels >250 mg/dl 4 weeks after the first dose of STZ. After 10 weeks of experimental diabetes, mice were euthanized by decapitation. Brain hemispheres were fixed in 4% neutral buffered formaldehyde and then paraffin embedded for light microscopy. The hippocampus was isolated, frozen in N₂, and stored at -80°C for protein analysis.

Metabolic and physiological parameters

Before euthanasia blood samples were taken via saphenous vein puncture on alert 4 hours fasted animals, and glucose levels measured using a glucometer (Accu-check; Roche Applied Science). Glycated hemoglobin was measured in whole blood samples obtained at the time of killing by quantitative immunoturbidimetric latex determination (Sentinel Diagnostic, Milan, Italy).

Immunohistochemistry

Immunohistochemical staining was performed on 4- μ m paraffin sections of fixed tissue. Briefly, sections were dewaxed, rehydrated, and immersed in 0.01M citrate buffer pH 6.0 at 100°C for

antigen retrieval. Endogenous peroxidase activity was quenched by incubation with 3% H₂O₂. Endogenous avidin-binding activity was inhibited by sequential treatment with avidin-biotin, and non-specific binding sites blocked with 3% BSA. For immunodetection, sections were incubated overnight with mouse anti-glial fibrillary acidic protein (GFAP, Calbiochem, Darstadt, Germany), rabbit anti-HSP25 (Stressgen, Ann Arbor, MI, USA), or rabbit anti-nitrotyrosine (anti-N-Tyr Abcam, Cambridge, UK) antibodies, then the specific staining was detected using the high sensitive Labeled StreptAvidin Biotin (LSAB) + system-HRP (Dako, Glostrup, Denmark) that uses a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. Sections were counterstained with Mayer's hemallume and visualized with an epifluorescence microscope (Olympus-Bx4I) connected by a photographic attachment (Carl Zeiss, Oberkochen, Germany). For each antibody, a negative control was included in which the primary antibody was replaced with a non-immune isotypic control antibody. In semiquantitative immunohistochemical analysis, the cellular area of nucleated positive cells with a staining intensity over an established threshold was calculated by a computer-aided image-analysis system (Axiovision 4.7; Carl Zeiss) on digital images of hippocampal sections, as previously described (Saravia et al., 2002). Results were then expressed as percentage increase over non-diabetic controls. In addition, GFAP staining was also calculated as the number of GFAP positive cells per area (65x10³ μm²). Evaluations were performed by two independent investigators in a blinded fashion.

Cu/Zn-SOD activity

Cu/Zn-SOD activity was evaluated in cytosolic hippocampal extracts by a kinetic enzymatic commercial assay kit (Cayman Chemical, Ann Arbor, MI, USA), following manufacturer's instructions.

Malondialdehyde measurement

Malondialdehyde (MDA), a marker of lipid peroxidation, was measured by a commercial colorimetric assay kit (BioVision, Milpitas, USA) following manufacturer's instructions. MDA

levels in hippocampal sample homogenates were expressed as nanomoles of MDA per milligram of protein.

Immunofluorescence

Expression of Cu/Zn SOD was assessed by indirect immunofluorescence using a sheep anti-mouse primary antibody (Calbiochem-Merck, Milan, Italy) and a FITC-conjugated donkey anti-sheep secondary antibody (Dako). Sections were examined using an Olympus epifluorescence microscope (Olympus Bx4I) and digitised with a high resolution camera (Carl Zeiss).

Double immunofluorescence

Double immunofluorescence was performed for HSP25 and either Cu/Zn-SOD or GFAP on hippocampal sections. After blocking, sections were incubated with either an anti-Cu/Zn-SOD antibody or an anti-GFAP antibody for 1 hour, followed by a labelled secondary antibody (Cu/Zn-SOD: FITC-conjugated donkey-anti sheep antibody; GFAP: TRITC-conjugated goat anti-mouse antibody). After washing, sections were incubated with an anti-HSP25 antibody for 18 h at 4°C, followed by 1 h incubation with a biotinylated swine anti-rabbit IgG (Dako) and then with FITC/Alexa 555-conjugated streptavidin (Invitrogen, Milan, Italy). Sections were examined using an Olympus epifluorescence microscope (Olympus Bx4 I) with photographic attachment (Carl Zeiss). The images were colour-combined and assembled into photomontages by using Adobe Photoshop (Universal Imaging, West Chester, PA).

Protein Extraction

For total protein extraction isolated hippocampal tissue was homogenised in a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 µg/mL leupeptin, 1 µg/ml aprotinin, 1 mM PMSF). Lysates were then centrifuged at 10,000 g for 40 min at 4° and protein concentration measured by DC protein (Bio-Rad Laboratories, Hercules, CA, USA). Cytosolic and nuclear extracts were prepared as previously described (Collino et al, 2006 Collino et al, 2009). Briefly, tissues were homogenized at 10% (wt/vol) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ) using a homogenization buffer

containing 20 mM HEPES (pH 7.9), 1 mM MgCl₂, 0.5 mM EDTA, 1% Nonidet P-40, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, and 2.5 µg/ml leupeptin. Homogenates were centrifuged at 1000 × g for 5 min at 4 C. Supernatants were removed and centrifuged at 105,000 × g at 4 C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, and 2.5 µg/ml leupeptin and incubated on ice for 30 min for high-salt extraction, followed by centrifugation at 15,000 × g for 20 min at 4 C. The resulting supernatants containing nuclear proteins were carefully removed and stored at -80 C.

Immunoblotting

Equal amounts of total protein were separated on a 10% SDS-polyacrylamide gel, then transferred to nitrocellulose membrane (Amersham Biosciences, Braunschweig, Germany). Following blocking of non-specific sites with 5% non-fat milk, membranes were probed with anti-HSP25, anti-HSP72 (Stressgen, Ann Arbor, MI, USA), anti-HSP90 (Stressgen, Ann Arbor, MI, USA), anti-GFAP, anti-nitrotyrosine, anti-iNOS (Cell Signalling, MI, Italy), anti-USF2 (Santa Cruz, CA, USA) antibodies overnight at 4°C. After washing, a secondary HRP-linked (Amersham) antibody was added. Immunoreactive bands were detected by SuperSignal West Femto chemiluminescence (Pierce, Rockford, IL, USA), visualised on a Gel-Doc system (Bio-Rad), and quantified by image J software. Tubulin (Santa Cruz Biotechnology) was used as internal control. In addition, USF-2 and β-tubulin serve as markers of nuclear and cytosolic fractions, respectively.

Statistical Analysis

The results are expressed as means ± SD. Comparisons were performed by unpaired two-tailed Student's t-test. P<0.05 was considered statistically significant.

Results

Metabolic and physiological parameters

As shown in Table 1, after 10 weeks of diabetes both blood glucose and glycated haemoglobin levels were significantly higher in diabetic (DM) than in non-diabetic (ND) mice. Furthermore, compared to sham-injected control animals, diabetic mice showed a significant decrease in body weight.

Hippocampal astrogliosis

GFAP protein expression was studied in the hippocampus from both control and diabetic mice by western blotting. Immunoblots showed a band migrating at ~50 kDa, corresponding to the reported molecular weight of GFAP and densitometric analysis demonstrated that GFAP protein expression was significantly increased in the diabetic mice (Figure 1I), indicating the presence of hippocampal astrogliosis in experimental diabetes. To evaluate the distribution of GFAP staining within the hippocampus, GFAP expression was also assessed by immunohistochemistry. Specificity of the antibody binding was confirmed by staining disappearance when the antibody was replaced with a non-immune isotype control antibody (Figure 1A). Consistent with our immunoblotting results, hippocampal positive staining for GFAP appeared greater in diabetic mice as compared to control animals in all hippocampal subfields, though the difference was particularly marked in the stratum lacunosum-moleculare (Figure 1 B,C). As shown in higher magnification images, GFAP positive staining in the CA1 area was greater in diabetic mice than in controls (Figure 1 D,E), but in diabetic animals there were no differences in GFAP staining between CA1 and CA3 areas (Figure 1G,H). Semiquantitative analysis performed in the CA1 area showed that diabetic mice had a significant 2.5 fold increase of the GFAP-positive cellular area (Figure 1F) and a significant 3-fold increase in the number of GFAP positive cells (ND: $23 \pm 1,05$ vs DM 61 ± 0.79 ; $p < 0.001$).

Hippocampal HSP25 expression in experimental diabetes

To establish whether HSP25 expression is modulated by diabetes, we studied HSP25 protein expression in total hippocampus from both diabetic and control mice by western blotting. Total HSP25 protein expression was greater in diabetic mice and in densitometric analysis we found a significant 2.8-fold increase of HSP25 expression in diabetic animals as compared to controls

(Figure 2A). On the contrary, hippocampal HSP72 and HSP90 expression was similar in diabetic and control animals (Figure 2B,C).

Distribution of HSP25 staining in the diabetic hippocampus

To assess HSP25 distribution within the hippocampus, we studied HSP25 expression in hippocampal sections by immunohistochemistry. Staining for HSP25 was enhanced in diabetic mice and localised predominantly to the CA1 pyramidal neurons layer (Figure 2 D,E and GH). Antibody specificity was confirmed by disappearance of the signal when the antibody was replaced with a non-immune isotype control antibody (Figure 2 F,I). Double immunofluorescence for both HSP25 (Figure 2J) and GFAP (Figure 2K) confirmed the presence of a predominant HSP25 staining in the pyramidal neurons layer (Figure 2L), but revealed that some GFAP-positive activated astrocytes (Figure 2K) also stained positively for HSP25 (Figure 2L). At high magnification, HSP25 and GFAP exhibited differences in their spatial distribution within astrocytes. GFAP localised mainly in the cellular processes, while HSP25 was observed predominantly in the somata (Figure 2L insert).

Markers of oxidative stress are enhanced in the diabetic hippocampus

As oxidative stress is a known inducer of HSP25, an array of oxidative stress markers was also studied in the hippocampus from control and diabetic mice. As shown in Figure 3, in the diabetic hippocampus, there was a significant increase in nitrotyrosine staining, as assessed by both immunohistochemistry (Figure 3A,B) and immunoblotting (Figure 3C), that was paralleled by iNOS overexpression (Figure 3D). Hippocampal content of MDA, a marker of lipid peroxidation, was significantly higher in diabetic animals than in controls (Figure 3 E). Finally, in diabetic mice we also observed an enhanced immunofluorescent staining for Cu/Zn SOD (Figure 3F,G) and a significant increased in hippocampal SOD enzymatic activity (Figure 3H). Double-labelling immunofluorescence showed that within the pyramidal neurons layer the positive staining for Cu/Zn SOD (Figure 3J) and HSP25 (Figure 3I) appears to localize to the same cell. However,

overlapping was very modest as Hsp25 showed a more nuclear localization, whereas Cu/Zn SOD was predominantly localised in the perinuclear area (Figure 3K).

Intracellular distribution of HSP25 in the diabetic hippocampus

To investigate the intracellular distribution of HSP25, western blotting analysis was performed separately for nuclear and cytosolic hippocampal protein extracts. USF2, a nuclear marker, and α -tubulin, a cytosolic marker, were used to confirm nuclear and cytosolic fraction purity (Figure 4A). As shown in Figure 4B, in diabetic mice HSP25 expression increased significantly in both the cytosolic and the nuclear fractions. However, HSP25 upregulation was greater in protein extracts from the nuclei, suggesting that HSP25 overexpression was paralleled by nuclear translocation (Figure 4B).

Discussion

In this study we have provided evidence that in experimental diabetes HSP25 is overexpressed in the hippocampus, predominantly in CA1 pyramidal neuron layer, in parallel with enhanced oxidative stress and astrogliosis. In hippocampal protein extracts HSP25 expression was greater in diabetic than in control mice as assessed by immunoblotting. On the contrary, no difference were observed in HSP72 and HSP90, suggesting that diabetes has a specific effect on HSP25. Previous studies have shown that hippocampal HSP25 is overexpressed in response to hyperthermia, preconditioning, ischemia, epileptic seizure (Benn et al., 2002; Franklin et al., 2005; Kirbach and Golenhofen, 2011), but this is the first evidence of HSP25 overexpression in the hippocampus in experimental diabetes. In diabetic mice, HSP25 was predominantly expressed by the pyramidal neurons of the CA1 region, as assessed by immunohistochemistry. This is not surprising as the CA1 region is highly sensitive to oxidative damage due to high anion superoxide production by CA1 pyramidal neurons (Wang et al., 2005). A HSP25 immunoreactivity of CA1 pyramidal neurons has been previously reported in vivo (Kato et al., 1999). Furthermore, a recent study has shown that cultured hippocampal neurons overexpress HSP25 in response to heat stress, suggesting a direct cytoprotective role of HSP25 in this cell type (Kirbach and Golenhofen, 2011).

Consistent with a previous report (Saravia et al., 2002), we found that expression of GFAP, a reliable marker of reactive astrocytes, was enhanced in the hippocampus from diabetic mice, confirming the presence of astrogliosis in experimental diabetes. The difference in GFAP staining between diabetic and control mice was particularly marked in the stratum lacunosum1 moleculare. HSP25, however, was only sporadically expressed by activated astrocytes. At variance, in experimental models of ischemia, epilepsy, and hyperthermia, HSP25 expression is restricted to reactive astrocytes (Benn et al., 2002; Franklin et al., 2005; Kirbach and Golenhofen, 2011). The underlying mechanism of this discrepancy is unclear; however, it is possible that the predominant HSP overexpression in CA1 pyramidal neurons is a specific feature of diabetes-induced damage. In line with this hypothesis, hippocampal HSP60 is induced in CA1 pyramidal neurons in STZ-induced diabetic animals (Yuan et al., 2006), while is overexpressed by astrocytes after transient ischemia (Hwang et al., 2007).

In diabetic mice hippocampal HSP25 overexpression occurred not only in the cytosol, but also in the nuclei. This finding is of functional relevance as in cultured hippocampal cells HSP25 nuclear translocation is important for HSP25 protective effect against DNA fragmentation and in conferring resistance to cellular stresses (Geum et al., 2002). Furthermore, in cultured neurons, nuclear HSP25 potentiates SP1-dependent neurotrophic gene transcription, suggesting that HSP25 may exert a cytoprotective effect also through modulation of transcriptional activity (Friedman et al., 2009).

The underlying mechanism of HSP25 overexpression is unknown. However, oxidative stress is a likely candidate as it is a potent inducer of HSP expression (Calabrese et al., 2003; Mehlen et al., 1993) and is enhanced in the diabetic hippocampus (Aragno et al., 2005; Mastrocola et al., 2005). Alternatively, proteasomal stress can also be implicated as a diabetes-induced insufficiency of the proteasome system may triggers a stress response through heat shock factor 1 activation, resulting in HSP25 induction (Dantuma et al, 2010). However, lack of HSP72/HSP90 overexpression in our model makes less likely this possibility.

To further explore the role of oxidative stress in diabetes-induced HSP25 hippocampal expression, we have also studied an array of oxidative stress markers. Cu/Zn SOD, which catalyzes the conversion of superoxide radicals to hydrogen peroxide, represents an important defence against superoxide production and an indirect index of oxidative stress. Consistent with previous results in STZ-induced diabetic rats (Huang et al., 1999), we found an increase in Cu/Zn SOD both expression and activity in the diabetic hippocampus. In addition, we found that SOD was predominantly expressed in the CA1 pyramidal neuron layer and that SOD and HSP25 localize to the same cell, though with a diverse intracellular distribution. A recent report has demonstrated that, in the context of global cerebral ischemia, hyperglycemia can exacerbate superoxide production in hippocampal CA1 neurons, suggesting hyperglycemia as a possible mechanism of SOD induction (Muranyi and Li, 2005).

The presence of nitrotyrosine on proteins is a marker of *in vivo* peroxynitrite formation from nitric oxide and superoxide. An increased nitrotyrosine expression in CA1 pyramidal neurons has been previously demonstrated in foetuses exposed to hyperglycemia because of maternal diabetes (Hockett et al., 2004), but our work provides the first evidence of enhanced hippocampal nitrotyrosine in an adult model of experimental diabetes and identifies the CA1 pyramidal neuron layer as a specific site of enhanced peroxynitrite formation. Of interest a recent study has shown a strong nitrotyrosine immunoreactivity of CA1 pyramidal neurons in mice expressing a mutated form of SOD (Cha et al., 2000), providing evidence of a causal link between oxidative stress and nitrotyrosine expression in this region. On the other hand, in our study we also found that iNOs protein expression was greater in diabetic than in control mice. Therefore, increased levels of nitrotyrosine containing proteins may, at least in part, reflect an increased NO production likely secondary to diabetes-induced endothelial dysfunction (Nagareddy et al., 2005).

Reactive oxygen species degrade polyunsaturated lipids, forming MDA. This reactive aldehyde, which can cause cellular toxic stress, is thus used as a marker of oxidative stress and lipid peroxidation. In our study, hippocampal content of MDA was found significantly enhanced in

diabetic mice as compared to controls. This finding is in agreement with previous studies (Grillo et al., 2003; Cosar et al., 2008). Furthermore, it provides a more convincing and direct evidence of the presence of enhanced oxidative stress in our experimental model. HSP25 decreases reactive oxygen species and enhances levels of glutathione, the most important intracellular antioxidant (Mehlen et al., 1997; Preville et al., 1999). Furthermore, therapeutic strategies, leading to HSP25 upregulation, diminish hippocampal neuron apoptosis in rodents (Akbar et al., 2003; An et al., 2009; Jin et al., 2006). Therefore, the upregulation of HSP25, herein reported, may represent a protective mechanism against diabetes-induced oxidative stress and neuronal death. However, no causal relationship can be drawn from morphological studies and further investigations are required to establish whether HSP25 is solely an indicator of oxidative stress or plays a role in neuroprotection of the diabetic hippocampus.

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Figure Legends

Figure 1. Expression of GFAP in the hippocampus from control and diabetic mice.

GFAP expression was assessed by immunohistochemistry in hippocampal sections from nondiabetic (ND n=4) and diabetic (DM n=4) mice. Representative images (x40) of the whole hippocampus: (A) negative control, (B) non-diabetic mice, (C) diabetic mice. Images (x200) of the hippocampal CA1 region in (D) non-diabetic and (E) diabetic mice. Results of semiquantitative analysis in the CA1 region are shown in the graph (F) (* $p < 0.001$ DM vs ND). Images (x200) comparing hippocampal CA1 (G) and CA3 (H) regions in diabetic mice. GFAP protein expression was assessed by immunoblotting in total hippocampal protein extracts. A representative immunoblot and results of densitometry analysis are shown (I). Tubulin was used as internal control (n=4; * $p < 0.01$ DM vs ND).

Figure 2. HSP25 expression and localisation in the diabetic hippocampus.

HSP25 protein expression were studied by immunoblotting on hippocampal protein extracts from non-diabetic (ND) and diabetic mice (DM). Tubulin was used as internal control. A representative immunoblot and results of densitometry analysis are shown (A) (n=4; * $p < 0.01$ DM vs. ND). Protein expression of both HSP72 and HSP90 was also assessed in total protein hippocampal extracts (B,C). HSP25 expression was analyzed by immunohistochemistry on hippocampal sections from non-diabetic mice (ND n=4) (D,G) and diabetic (DM n=4) (E,H) mice. Specificity of antibody binding was confirmed with a non-immune isotype control antibody (F,I) (D-F: 40X and G-I: 400X magnification). Double immunofluorescence for HSP25 (J) and GFAP (K) showed no colocalisation (L), except for sporadic astrocytes (L insert).

Figure 3. Markers of oxidative in the diabetic hippocampus.

A number of oxidative stress markers were assessed in the hippocampus from non-diabetic (ND n=4) and diabetic (DM n=4) mice. Representative immunohistochemical images of nitrotyrosine (N-tyr) in hippocampal sections from ND (A) and DM mice (B) (magnification 400X). Immunoblots and results of densitometry analyses of N-tyr (C) and iNOS (D) protein expression in

hippocampal protein extracts from ND and DM mice (n=4, *p \leq 0.01 DM vs ND, tubulin: internal control). Malondialdehyde (MDA) levels (E) in hippocampal homogenates from ND and DM mice (n=4, *p<0.001 DM vs ND). Cu/Zn superoxide dismutase (SOD) both staining (F,G) and activity (H) in the hippocampus from ND (F) and DM mice (G), assessed by immunofluorescence (magnification 400X) and enzymatic assay (n=4, *p<0.05 DM vs ND), respectively. Double immunofluorescence for HSP25 (I) and SOD (J) and merged image (K) in hippocampal sections from DM mice.

Figure 4. Intracellular HSP25 hippocampal distribution

HSP25 protein expression was studied in hippocampal both cytosolic and nuclear protein extracts from non-diabetic (ND n=3) and diabetic (DM n=3) mice by immunoblotting. Tubulin was used as internal control. USF2, a nuclear marker, and beta-tubulin, a cytosolic marker, were used to confirm purity of nuclear and cytosolic extracts (A). Representative immunoblots (B) and results of densitometry analysis (C) are shown (*P<0.05; #P<0.01 DM vs. ND).

Table.1 Metabolic and physiologic parameters of control and diabetic mice

	Body weight (grams)	Blood Glucose (mmol/L)	Glycated Hb (%)
Control (6)	29.08 ± 0.78	4.28 ± 0.53	5.94 ± 1.16
Diabetes (6)	24.64 ± 0.93*	20.78 ± 2.95*	11.19 ± 2.37*

References

- Akbar MT, Lundberg AM, Liu K, Vidyadaran S, Wells KE, Dolatshad H, Wynn S, Wells DJ, Latchman DS, de Belleruche J (2003), The neuroprotective effects of heat shock protein 27 overexpression in transgenic animals against kainate-induced seizures and hippocampal cell death. *J Biol Chem* 278:19956-199565.
- An JJ, Lee YP, Kim DW, Sohn EJ, Jeong HJ, Kang HW, Shin MJ, Kim MJ, Ahn EH, Jang SH, Kang JH, Kang TC, Won MH, Kwon OS, Cho SW, Lee KS, Park J, Eum WS, Choi SY (2009), Transduced HSP27 protein protects neuronal cell death by enhancing FALSassociated SOD1 mutant activity. *BMB Reports* 42:136-141.
- Aragno M, Mastrocola R, Medana C, Restivo F, Catalano MG, Pons N, Danni O, Boccuzzi G (2005), Up-regulation of advanced glycated products receptors in the brain of diabetic rats is prevented by antioxidant treatment. *Endocrinology* 146:5561-5567.
- Barutta F, Pinach S, Giunti S, Vittone F, Forbes JM, Chiarle R, Arnstein M, Perin PC, Camussi G, Cooper ME, Gruden G (2008), Heat shock protein expression in diabetic nephropathy. *Am J Physiol Renal Physiol* 295:F1817-F1824.
- Benn SC, Perrelet D, Kato AC, Scholz J, Decosterd I, Mannion RJ, Bakowska JC, Woolf CJ (2002), Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival. *Neuron* 36:45-56.
- Calabrese V, Scapagnini G, Colombrita C, Ravagna A, Pennisi G, Giuffrida Stella AM, Galli F, Butterfield DA (2003), Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach. *Amino Acids* 25:437-444.
- Cha CI, Chung YH, Shin CM, Shin DH, Kim YS, Gurney ME, Lee KW (2000), Immunocytochemical study on the distribution of nitrotyrosine in the brain of the transgenic mice expressing a human Cu/Zn SOD mutation. *Brain Res* 853:156-161.

- Collino M, Castiglia S, Manoni M, Salsini L, Chini J, Masini E, Fantozzi R (2009), Effects of a semi-synthetic N-,O-sulfated glycosaminoglycan K5 polysaccharide derivative in a rat model of cerebral ischaemia/reperfusion injury. *Thromb Haemost* 102:837-845.
- Collino M, Aragno M, Mastrocola R, Gallicchio M, Rosa AC, Dianzani C, Danni O, Thiemermann C, Fantozzi R (2006), Modulation of the oxidative stress and inflammatory response by PPAR-gamma agonists in the hippocampus of rats exposed to cerebral ischemia/reperfusion. *Eur J Pharmacol* 530:70-80.
- Cosar M, Songur A, Sahin O, Uz E, Yilmaz R, Yagmurca M, Ozen OA (2008), The neuroprotective effect of fish n-3 fatty acids in the hippocampus of diabetic rats. *Nutr Neurosci* 11:161-166.
- Ellis RJ, van der Vies SM (1991), Molecular chaperones. *Annu Rev Biochem* 60:321-347
- Dantuma NP, Lindsten K, Stressing the ubiquitin-proteasome system (2010), *Cardiovascular Research* 85:263-271.
- Feder ME, Hofmann GE (1999), Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 61:243-282.
- Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW (2005), The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. *Int J Hyperthermia* 21:379-392.
- Friedman MJ, Li S, Li XJ (2009), Activation of gene transcription by heat shock protein 27 may contribute to its neuronal protection. *J Biol Chem* 284:27944-27951.
- Geum D, Son GH, Kim K (2002), Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. *J Biol Chem* 277:19913-19921.
- Grillo CA, Piroli GG, Rosell DR, Hoskin EK, Mcewen BS, Reagan LP (2003), Region specific increases in oxidative stress and superoxide dismutase in the hippocampus of diabetic rats subjected to stress. *Neuroscience* 121:133-140.

- Guven A, Yavuz O, Cam M, Comunoglu C, Sevi'nc O (2009), Central nervous system complications of diabetes in streptozotocin-induced diabetic rats: a histopathological and immunohistochemical examination. *Int J Neurosci* 119:1155-1169.
- Hockett PK, Emery SC, Hansen L, Masliah E (2004), Evidence of oxidative stress in the brains of fetuses with CNS anomalies and islet cell hyperplasia. *Pediatr Dev Pathol* 7:370-379.
- Huang WC, Juang SW, Liu IM, Chi TC, Cheng JT (1999), Changes of superoxide dismutase gene expression and activity in the brain of streptozotocin-induced diabetic rats. *Neurosci Lett* 275:25-28.
- Hwang IK, Ahn HC, Yoo KY, Lee JY, Suh HW, Kwon YG, Cho JH, Won MH (2007), Changes in immunoreactivity of HSP60 and its neuroprotective effects in the gerbil hippocampal CA1 region induced by transient ischemia. *Exp Neurol* 208:247-256.
- Jin Y, Fan Y, Yan EZ, Liu Z, Zong ZH, Qi ZM (2006), Effects of sodium ferulate on amyloid-beta-induced MKK3/MKK6-p38 MAPK-Hsp27 signal pathway and apoptosis in rat hippocampus. *Acta Pharmacol Sin* 27:1309-1316.
- Kato K, Katoh-Semba R, Takeuchi IK, Ito H, and Kamei K (1999), Responses of Heat Shock Proteins hsp27, α B-Crystallin, and hsp70 in Rat Brain After Kainic Acid-Induced Seizure Activity. *J Neurochem* 73:229–236.
- Kirbach BB, Golenhofen N (2011), Differential expression and induction of small heat shock proteins in rat brain and cultured hippocampal neurons. *J Neurosci Res* 89:162-175.
- Magariños AM, McEwen BS (2000), Experimental diabetes in rats causes hippocampal dendritic and synaptic reorganization and increased glucocorticoid reactivity to stress. *Proc. Natl. Acad. Sci. U.S.A.* 97:11056-11061.
- Mastrocola R, Restivo F, Vercellinatto I, Danni O, Brignardello E, Aragno M, Boccuzzi G (2005), Oxidative and nitrosative stress in brain mitochondria of diabetic rats. *J Endocrinol* 187:37-44.

- Mehlen P, Briolay J, Smith L, Diaz-latoud C, Fabre N, Pauli D, Arrigo AP (1993), Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the Drosophila 27-kDa heat-shock protein. *Eur J Biochem* 215:277-284.
- Mehlen P, Hickey E, Weber LA, Arrigo AP (1997), Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochem Biophys Res Comm* 241:187-192.
- Muranyi M, Li PA (2005), Hyperglycemia increases superoxide production in the CA1 pyramidal neurons after global cerebral ischemia. *Neurosci Lett* 393:119-121.
- Nagareddy PR, Xia Z, McNeill JH, MacLeod KM (2005), Increased expression of iNOS is associated with endothelial dysfunction and impaired pressor responsiveness in streptozotocin-induced diabetes. *Am J Physiol Heart Circ Physiol* 289:H2144-H2152.
- Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardini I, Brownlee M (2000), Normalizing mitochondrial superoxide production blocks three pathways of hyperglycemic damage. *Nature* 404:787-790.
- Ott A, Stolk R, van Harskamp F, Pols H, Hofman A, Breteler M (1999), Diabetes mellitus and the risk of dementia: the Rotterdam study. *Neurology* 58:1937-1941.
- Patel YJ, Payne Smith MD, de Belleruche J, Latchman DS (2005), Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells. *Brain Res Mol Brain Res* 134:256-274.
- Pinach S, Burt D, Berrone E, Barutta F, Bruno G, Porta M, Perin PC, Gruden G (2011), Retinal heat shock protein 25 in early experimental diabetes. *Acta Diabetol* 2011 doi: 10.1007/s00592-011-0346-1.
- Preville X, Salvemini F, Giraud S, Chaufour S, Paul C, Stepien G, Ursini MV, Arrigo AP (1999), Mammalian small stress proteins protect against oxidative stress through their ability to

increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. *Exp Cell Res* 247:61-78.

Ristow M (2004), Neurodegenerative disorders associated with diabetes mellitus. *J Mol Med* 82:510-529.

Ryan CM, Williams TM, Finegold DN, Orchard TJ (1993), Cognitive dysfunction in adults with type 1 (insulin-dependent) diabetes mellitus of long duration: effects of recurrent hypoglycaemia and other chronic complications. *Diabetologia* 36:329-334.

Sanz O, Acarin L, González B, Castellano B (2001), Expression of 27 kDa heat shock protein (Hsp27) in immature rat brain after a cortical aspiration lesion. *Glia* 36:259-270.

Saravia FE, Revsin Y, Gonzalez Deniselle MC, Gonzalez S, Roig P, Lima A, Homo-Delarche F, De Nicola AF (2002), Increased astrocyte reactivity in the hippocampus of murine models of type I diabetes: the nonobese diabetic (NOD) and streptozotocin treated mice. *Brain Res* 957:345-353.

Valastro B, Cossette N, Lavoie N, Gagnon S, Trudeau F, Massicotte G (2002), Up-regulation of glutamate receptors is associated with LTP defects in the early stages of diabetes mellitus. *Diabetologia* 45:642-650.

Wang X, Pal R, Chen XW, Limpeanchob N, Kumar KN, Michaelis EK (2005), High intrinsic oxidative stress may underlie selective vulnerability of the hippocampal CA1 region. *Brain Res Mol Brain Res* 140:120-126.

Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG (2005), Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. *Ann N Y Acad Sci* 1053:74- 83.

Yuan J, Young BJ, Martinus RD (2006), Expression of chaperonin 60 in the hippocampus of the streptozotocin diabetic rat. *Neuroreport* 17:239-242.

Figure 1

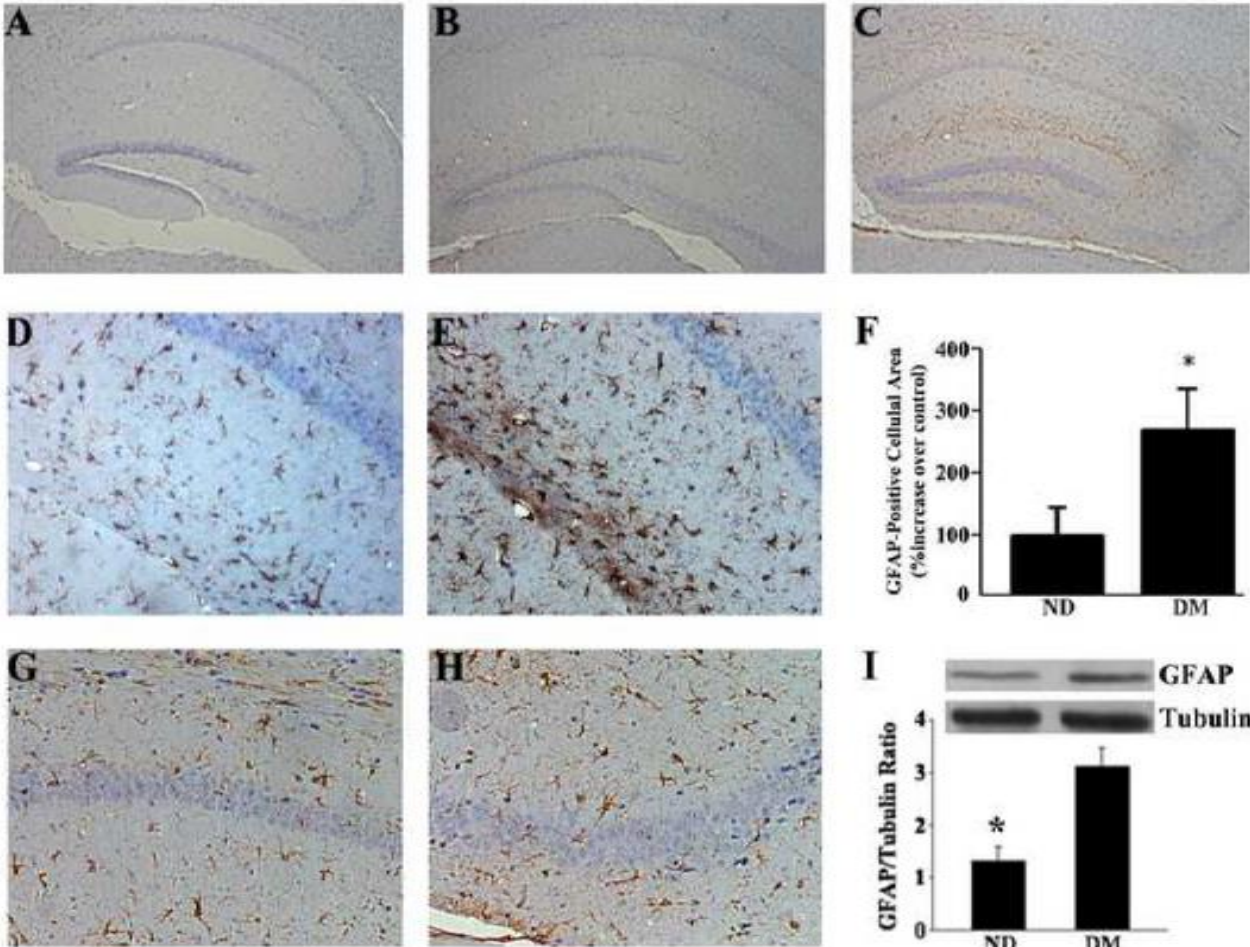


Figure 2

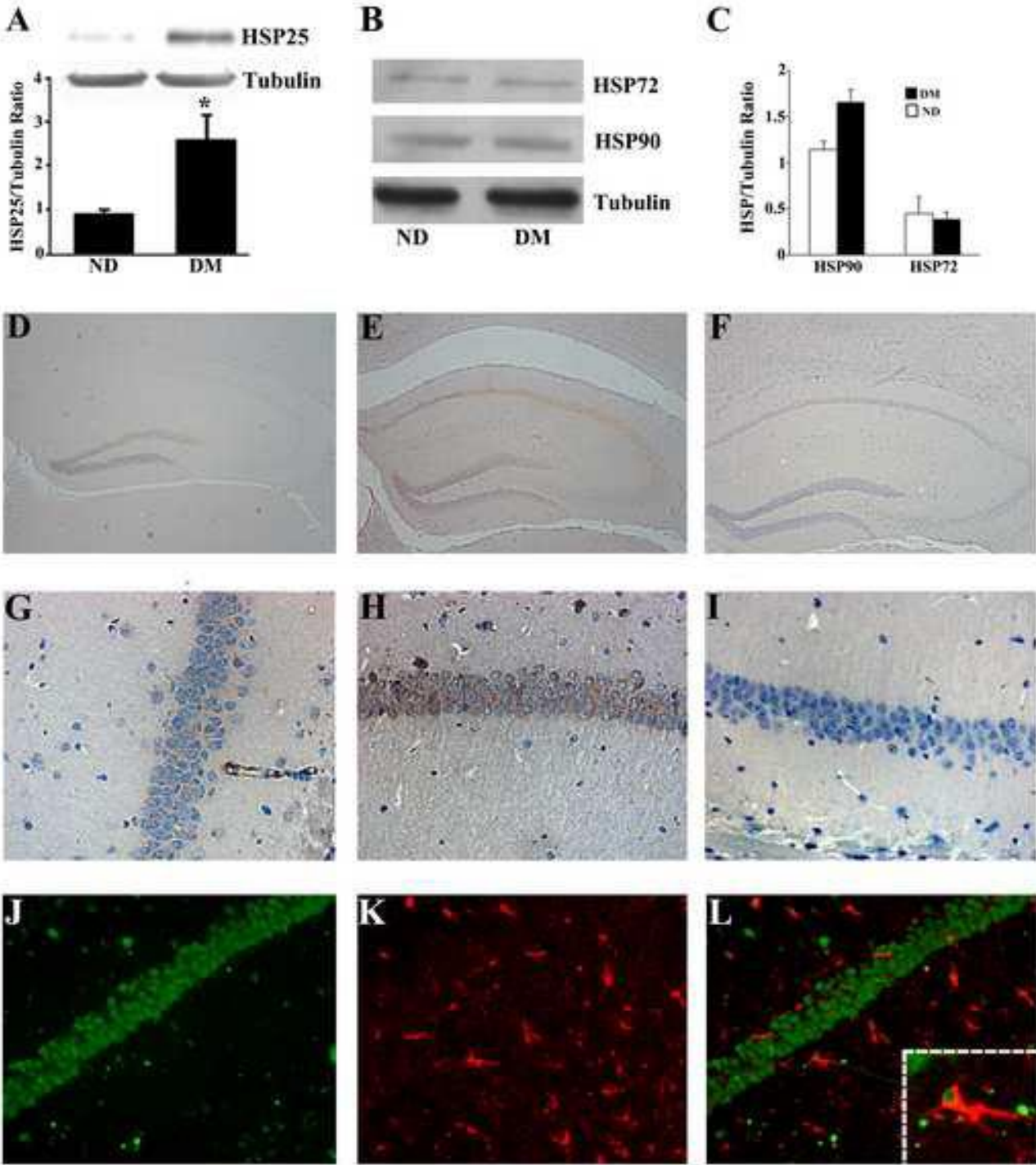


Figure 3

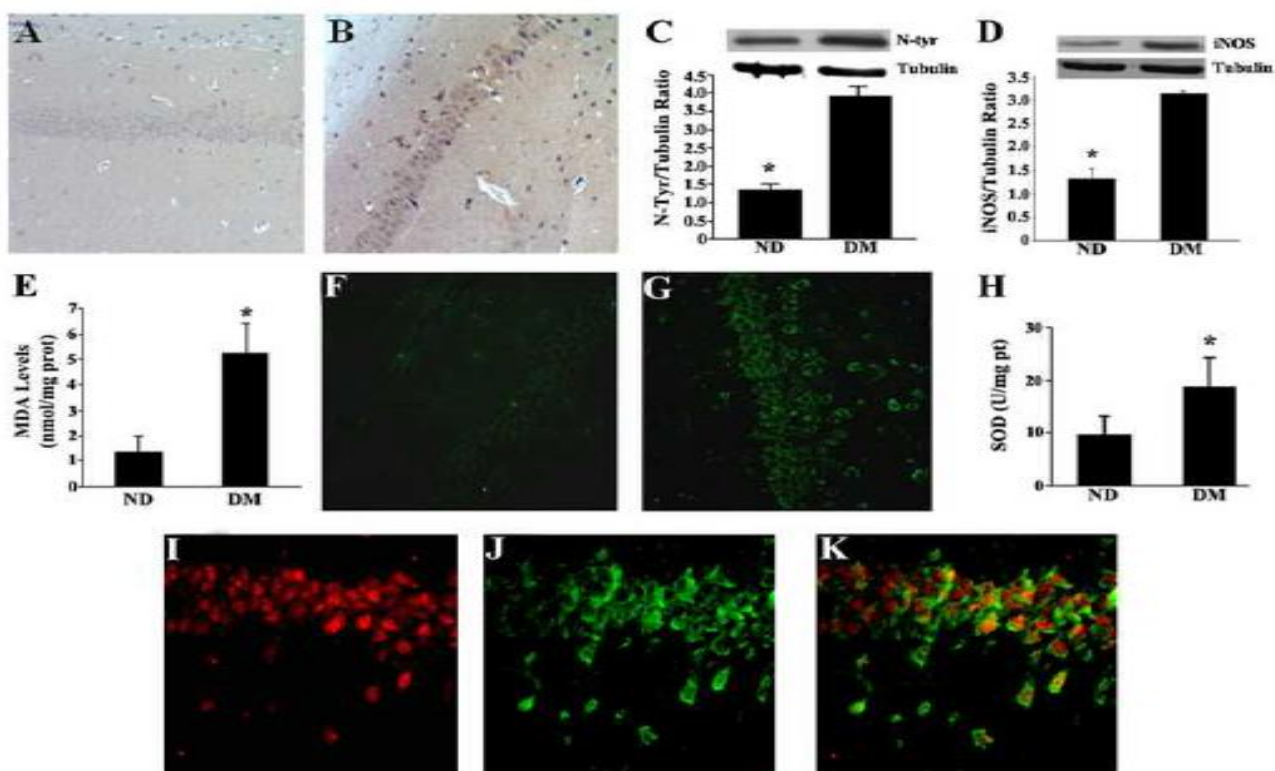


Figure 4

