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Proteomic Analysis of Hippocampus in Mice Following Long-term Exposure to Low Levels of Copper

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Abstract

Recent studies suggest that copper exposure, even at very low levels, can produce significant toxic effects on the brains of mice. This study is aimed to explore the effects of low levels of copper on the hippocampal proteome of mice. Two-dimensional fluorescence difference gel electrophoresis was performed on hippocampal homogenate obtained from mice, which were given either drinking water only (control) or water supplemented with 0.13 ppm copper (copper-treated) for a period of 8 months beginning at an age of 3 months. A total of 9 differentially expressed proteins between copper-treated mice and control mice were identified. Protein functional analysis revealed that the altered proteins mainly involved energy metabolism-related proteins, synaptic proteins, molecular chaperones and cellular structural components. Among these differentially expressed proteins, serine racemase (SRR) and glial fibrillary acidic protein (GFAP) were significantly down-regulated and up-regulated, respectively, in the hippocampus of copper-treated mice compared with control mice. SRR was shown to be involved in memory formation. The increased expression of GFAP, an astrocyte marker, indicated that long-term low levels of copper exposure caused activation of the inflammatory response, a process linked to spatial memory impairment. In agreement with the data from proteomic analysis, memory impairment was observed in copper-treated mice as measured by the Morris water maze test. In summary, this study has identified a number of abnormally expressed proteins in the hippocampus of copper-treated mice, and the identified protein, such as SRR, together with inflammatory responses, as evidenced by the increased expression of GFAP, could

contribute to memory impairment resulting from copper exposure. Our findings provide insights for a better understanding of copper neurotoxicity at the protein level in response to low levels of copper exposure.

Keywords: Copper; Learning and memory; Proteomics

Introduction

Copper (Cu) is an essential trace element and a key constituent of the respiratory enzyme complex cytochrome C oxidase that plays an important role in the detoxification of oxygen radicals in the cytosol. In humans, the main organs in which Cu is found are the liver, brain, muscle and bone. In human blood, 85-95% of Cu is safely bound to ceruloplasmin, and the other 5-15% is loosely bound to albumin, transcuprein and small molecules [1, 2]. Although Cu is indispensable, an excess of Cu in its labile or free form (i.e., not bound to ceruloplasmin in serum) is toxic. Cu toxicities due to excess Cu accumulation in organs of the body have been reported in diseases such as Wilson's disease (WD), in which at least 5 mutations of ATP7B (the WD gene) have been identified. In addition, excess Cu accumulation is linked to Alzheimer's disease (AD), with evidence that Cu promotes tau polymerization [3, 4] and that Cu chelation is protective [5]. Furthermore, defective ceruloplasmin activity is also found in connection with Parkinson's disease (PD) and AD [6].

A body of evidence indicates that environmental Cu exposure may be a risk factor for

cognitive impairment[7, 8]. Recently, we showed that exposure to a high concentration of Cu (250 ppm) caused synaptic loss and spatial memory impairment in mice[9]. A study by Kitazawa et al. [10] showed that chronic exposure to a 250 ppm concentration of Cu aggravated both amyloid-β (Aβ) and tau pathology in a mouse model of AD. However, in real-life situations, humans are rarely exposed to such a high concentration of Cu. Thus, exposure to low levels of Cu is gaining more and more attention in studies of Cu neurotoxicity. A recent study showed that a Cu concentration of as little as 0.12 ppm in drinking water (less than 1/10 the level set by the United States EPA as the standard for drinking water [1.3 ppm]) disrupted brain Aβ homeostasis by altering its production and clearance [11]. However, there is a lack of overall understanding regarding the effects of long-term exposure to low levels of Cu on the brain at the protein level.

In this study, we used an unbiased and cutting-edge proteomics technique, namely, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled with MALDI-TOF-MS/MS. We determined the effects of low levels of Cu on protein expression in the hippocampus (a brain region closely related to spatial learning and memory) and identified the potential key molecules that may be involved in memory deficits caused by Cu exposure.

Material and Methods

Ethics statement

The authors declare no actual or potential conflict of interest. Shenzhen Center for Disease Control and Prevention approved the animal experiment. All experimental procedures using animals were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised in 1985) and the Regulations for Animal Care and Use from the Committee of the Experimental Animal Center at Shenzhen Center for Disease Control and Prevention in Shenzhen, Guangdong Province, China.

Materials

High purity cupric chloride ($CuCl_2$) was purchased from Sigma. The source information for all other assay reagents and materials is described below in each of their respective assay methods.

Animals and treatment

Mice (strain: B6129SF2/J) with the same genetic background were purchased from Jackson Laboratory as described previously [12]. All mice used in this study were 3 months old. The mice were provided with either drinking water (control mice) or drinking water that contained 0.13 ppm [11] $CuCl_2$ (Cu-treated mice) for a period of 8 months. This level of Cu was equivalent to 1/10 of the level that the United States EPA allows in drinking water (1.3 ppm). The drinking water was freshly prepared every week. The mice were housed in groups of 10 mice per cage (dimensions of 470 mm × 350 mm × 200 mm) with free access to food and water and maintained on a

12-hour light-dark cycle with the light on from 7:00 am to 7:00 pm at a stable temperature (20±2 ºC) and humidity (55±5%). Every effort was made to minimize animal suffering and reduce the number of mice used.

Morris water maze test

The Morris water maze test was performed as previously described by D'Hooge et al. [13] and Sharma et al. [14]. The testing apparatus mainly consisted of a circular pool (170-cm diameter) filled with water to a 30 cm-depth that was made opaque by adding powdered milk and maintained at 22±1 ºC. The maze was divided into four quadrants (I–IV). Around and away from the tank, a white curtain with different black geometric patterns (i.e., square, rectangle and triangle) was used to present spatial visual cues.

In the training stage, a black escape platform (10 cm in diameter) approximately 1 cm below the surface of the water was randomly fixed in the center of quadrant II (target quadrant). Each mouse was trained to locate the escape platform hidden under the water from four different starting points for 5 consecutive days, and every test lasted for 60 s. The mice were put on the escape platform for 15 s to ensure that the mice were aware of this platform in the water, regardless of whether the mice were able to find the escape platform. The next trial did not begin until all mice had completed the previous trial. The data were automatically recorded by a camera (SONY SSC-DC488P) hung above the center of the pool.

In the memory testing stage, one week after the training stage, the platform was removed. The mice were individually placed into the water at the quadrant opposite from the target quadrant. The mice were allowed to swim freely for 120 s. Various parameters, including the latency to find the platform, the time spent in each quadrant, the number of platform crossings, and the swimming velocity, were analyzed to evaluate changes in learning and memory.

Protein sample preparation

The mice were killed after the 8-month experimental period. Their hippocampi were isolated on an ice-cold plate and immediately frozen in liquid nitrogen and stored at -80 ºC until use. The hippocampal tissues were suspended in DIGE-specific lysis solution (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS, pH 8.5), then ultrasonicated with a Fisher 550 Sonic Dimenbrator (Pittsburgh, PA, USA) to fully break them apart. Subsequently, the hippocampal tissue samples were centrifuged at 20,000 g at 4 ºC for 60 min, and then, the supernatants were ultrafiltered at 14,000 g for 30 min to remove salt and other impurities. The protein solutions were collected and the protein concentrations were determined using a 2-D Quant Kit (GE Healthcare, USA) in accordance with the manufacturer`s protocol.

Protein labeling with CyDye

Each vial of CyDye (GE Healthcare, USA) was reconstituted in 99.8% anhydrous N, N-Dimethylformamide (DMF, Sigma 227056) to achieve a final dye concentration of

1 nmol/L as a stock solution. The CyDye working solutions of 200 pmol/L that were used to label protein were generated by diluting stock solutions with DMF. All samples were diluted to 5 μg/L. The protein samples were labeled with CyDyes. In brief, 25 μg of each sample was labeled with Cy3 or Cy5 minimal dye, and 25 μg of protein taken from the mixture of all proteins was used as an internal standard on each gel and was labeled with Cy2. In parallel, each group sample was labeled with either Cy3 or Cy5, and the dyes were scrambled within each group to avoid possible dye bias. The proteins were incubated on ice in the dark for 30 min and subsequently quenched by 10 mM lysine (Sigma, L5626) for 10 min. Then, the Cy2-, Cy3- and Cy5-labeled samples were mixed together and an equal volume of 2×lysis buffer (8 M urea, 2% CHAPS, 0.2% DTT, 2% (v/v) IPG buffer, pH 3-11 non-linear, 0.002% bromophenol blue) was added to each mixture and incubated on ice for 10 min. Rehydration buffer was then added to adjust the total sample volumes to 450 μL.

Isoelectric focusing

The first-dimension isoelectric focusing (IEF) was performed using the Ettan IPGphor Isoelectric Focusing System (GE Healthcare, USA). Equal amounts of labeled samples (75 μg) were taken up into 24-cm, pH 3-11, non-linear, IPG strips (GE Healthcare, USA). Prior to the second-dimension separation, the focused strips were equilibrated at 37 ºC for 30 min. Then, 2 mL of mineral oil was added to cover each strip to reduce solvent evaporation. Proteins were taken up into strips by active rehydration at 50 V for 18 h. The IEF conditions were as follows: step 300 V for 12 h,

step 500 V for 2 h, step 1000 V for 2 h, gradient 8000 V for 8 h, and step 8000 V for 8 h.

SDS-PAGE

Prior to second-dimension gel electrophoresis, the focused strips were equilibrated with a reducing equilibration buffer containing 6 M urea, 75 mM Tris-HCl buffer (pH 8.8), 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT for 15 min at room temperature. Subsequently, the strips were re-equilibrated in the same buffer containing 4.5% IAA rather than DTT. The equilibrated strips were loaded on the top of 12.5% SDS-PAGE gels with a 0.5% (w/v) ultra-low melting point agarose sealing solution (25 mM Tris, 192 mM glycine, 0.1% SDS, 0.5% (w/v) agarose, 0.02% bromophenol blue). Electrophoresis was performed using an Ettan DALTsix Electrophoresis System (GE Healthcare, USA) with the following conditions: 1 W/gel for 1 h and subsequently 10 W/gel for 6 h in the dark at 15 ºC. The gels were immediately scanned using a Typhoon TRIO Variable Mode Imager (GE Healthcare, USA).

Image analysis

According to the manufacturer's instructions, the data were analyzed using the DeCyder software package (Version 6.5, GE Healthcare, USA). Approximately 1500 spots were matched across all three analytical gels. The spots were matched using an Ettan Spot Picker (GE Healthcare, USA) combined with manual screening, and then,

the spots with a significant difference (P<0.05) were further analyzed.

In-gel tryptic digestion

A total of 1000 μg of hippocampal protein was used to run 2-DE using the identical conditions as above. The gel was stained with a Coomassie blue solution (0.12% Coomassie Brilliant Blue G-250, 20% ethanol, 10% phosphoric acid, 10% ammonia sulfate). The spots of interest that were detected by Decyder software analysis were manually excised from the Coomassie blue stained gel. Gel pieces were destained and digested overnight at 37 ºC with trypsin (Promega Corp., WI, USA) as described by Robinson[15]. The tryptic peptides were used for MALDI-TOF-MS/MS analysis.

Mass spectrometry identification

Tryptic peptides were analyzed using MALDI-TOF-MS/MS (AB SCIEX MALDI-TOF/TOF 5800 mass spectrometer). Briefly, 1μL of peptide extraction was used for the MALDI-TOF-MS analysis and crystallized with 1 μL of 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile (ACN) directly in the target and dried at ambient temperature. The spectra were externally calibrated. MASCOT was used for database searching against the SwissProt databases (Matrix Science, UK). The search was carried out in Mus musculus and conducted with a tolerance on mass measurement of 100 ppm in MS mode and 0.5 Da in MS/MS mode. Up to two missed cleavages per peptide was allowed. A fixed carbamidomethyl modification was taken into account. Protein

molecular weight (MW) and isoelectric point (pI) information were also considered to evaluate the protein identification based on the location of the excised protein spot from the 2-D gel.

Western-blot analysis

Hippocampal proteins from each group of mice (i.e., Cu-treated mice or control mice) were extracted by using lysis buffer (Beyotime, China) with a phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA). Protein samples were mixed with loading buffer and heated for 5 min at 100 ℃, then separated on 10% SDS-PAGE and transferred onto PVDF membranes. Then, membranes were blocked by 5% nonfat-dried milk at room temperature for 2 h. Western-blot analysis was performed using monoclonal anti-serine racemase (diluted 1:2000, Abcam, USA), monoclonal anti-glial fibrillary acidic protein (diluted 1:5000, Abcam, USA), monoclonal anti-β-actin (diluted 1:1000, Santa Cruz, USA), or monoclonal anti-GAPDH (diluted 1:1000, Santa Cruz, USA) as the primary antibodies. Anti-rabbit or anti-mouse IgG HRPs (diluted 1:2000, Thermo Fisher Scientific, USA) were used as secondary antibodies. After washing in TBST, the membranes were detected using chemiluminescence with an ECL kit (Pierce Biotechnology, USA) on a phosphor imager, and the optical density was analyzed based on Image Quant 1D software (GE Healthcare, USA).

Statistical analysis

Data were expressed as the mean ± SEM and analyzed using SPSS 19.0 statistical software (SPSS Inc., Chicago, Illinois, USA). The significance of the differences between the two groups of mice was determined by an unpaired t-test. The level of statistical significance was set at $P<0.05$.

Results

2D-DIGE and image analysis of hippocampal proteins from Cu-treated mice and control mice

To determine the effects of low levels of Cu on the hippocampal protein expression, comparative proteomic analysis by 2D-DIGE, image analysis, in-gel digestion, and MS identification were performed on the hippocampal proteins obtained from the mice with or without Cu treatment. Typical 2D-DIGE gel images of hippocampal proteins isolated from mice with or without Cu treatment are shown in Fig. 1A-D. Spots with a fold-change of |1.1| or greater and a p-value ≤ 0.05 were considered to be differentially expressed.

A total of 10 differentially expressed protein spots were found, and 9 of them were identified with MS and annotated (Fig. 1E) in the form of protein ID. The Mascot scores; theoretical MW of these proteins, which were obtained from the SwissProt database; and the fold-change ratio and *p*-values, which were obtained from DeCyder, are shown in Table 1.

Of these 9 differentially expressed and identified proteins, 5 proteins were

significantly up-regulated and 4 proteins were significantly down-regulated in the hippocampus of Cu-treated mice compared with control mice. The significantly up-regulated hippocampal proteins were tubulin alpha-1A chain (TUBA1A), heterogeneous nuclear ribonucleoprotein D-like (HNRDL), heat shock-related 70-kDa protein 2 (HSP72), GFAP, and guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2 (GBG2). The significantly down-regulated proteins were SRR, complexin-2 (CPLX-2), ATP synthase subunit delta (ATPD), and stathmin (STMN1). The main functions of these proteins analyzed by Gene Ontology (GO) are also shown in Table 1. The proteins were identified with at least two different peptide sequences and multiple peptide hits corresponding to every MS/MS event. The MS/MS spectra for two exemplary proteins, SRR and GFAP, are presented in Supplementary Fig. 1 and Supplementary Fig. 2, respectively. To determine the functions of the individual proteins, the 9 differentially expressed proteins were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The main pathways and disease-related proteins obtained from the KEGG website (http://www.genome.jp/kegg/) are shown in Table 2.

Validation of differentially expressed hippocampal proteins by western blot analysis

To confirm the data obtained by 2D-DIGE, western-blot analysis was performed. SRR and GFAP were selected for the validation. In agreement with the data from 2D-DIGE, a significantly decreased expression of SRR (Fig. 2A, 2B) and a significantly

increased expression of GFAP (Fig. 2C, 2D) were observed in the hippocampus of Cu-treated mice compared with control mice.

Effect of long-term exposure to low levels of Cu on spatial memory in mice

To determine the effects of long-term low levels of Cu on spatial learning and memory of mice, the Morris water maze test was performed. In the learning acquisition test, no significant differences in the escape latency were observed between Cu-treated mice and control mice during the 5-day training period (Fig. 3A), indicating that low levels of Cu exposure did not affect the spatial learning ability. One week after the training session, the long-term memory of the mice was evaluated by analyzing the changes of the probe time (i.e., time to find the platform), platform crossing number and preference for the target quadrant. The probe time and the platform crossing number were not significantly different between Cu-treated mice and control mice (Fig. 3B, C). The percentage of time spent in the correct quadrant and the percentage of the distance traveled in the target quadrant were significantly decreased in Cu-treated mice compared with control mice (Fig. 3D, E, F). No significant differences in the total traveled distance or the average swimming speeds were observed between the two groups (Fig. 3G, H). These data suggest that long-term low levels of Cu exposure caused spatial memory impairment in mice.

Discussion

In this study, we evaluated the effects of long-term low levels of Cu exposure on the

hippocampal proteome and spatial learning and memory in mice. We successfully identified 9 proteins in the hippocampus that were differentially expressed in Cu-treated mice compared with control mice using 2D-DIGE combined with MALDI-TOF-MS/MS. Protein functional analysis revealed that these altered proteins mainly involved energy metabolism-related proteins, synapse-associated proteins, molecular chaperones and cellular structural components. KEGG analysis indicated that GFAP is associated with diseases, such as Alexander disease, and ATPD is associated with AD, PD and Huntington's disease (HD) in which Cu toxicity is implicated, whereas GBG2 is associated with cancer such as malignant melanoma.

Synapse-associated proteins

The memory consolidation phase in the mammalian brain is accompanied by the widespread reorganization of synapses 16. The proteins identified in this study included synapse-associated proteins, such as STMN1, CPLX2 and SRR. The changes of these proteins imply reorganization of synapses that could be induced by long-term low levels of Cu exposure and thus represented an aspect of the toxic effects of Cu. STMN1, as a cytosolic protein that binds tubulin, is involved in the maintenance of axonal integrity [17] and is highly enriched in growth cones [18]. As a marker for neuritic sprouting, STMN1 regulates the cell cycle and acts to destabilize microtubules [19]. The down-regulated expression of STMN1 is required for the phenotypic changes and classical activation of macrophages upon exposure to pro-inflammatory cytokines and pathogenic stimuli [20]. CPLX2 is one of presynaptic

proteins that binds to the synaptic SNARE (soluble N-ethylmaleimide-sensitive fusion-attachment protein receptor) complex to initiate the fusion of synaptic vesicles into the plasma membrane. Mice lacking CPLX2 show impaired long-term potentiation (LTP), a processes correlated with spatial learning and memory, in the hippocampal CA1 and CA3 regions [21], indicating that decreased expression of CPLX2 could be involved in the toxic effects of Cu on the behavior of mice, which we observed. SRR is an enzyme that generates D-serine from L-serine. D-serine is an endogenous co-agonist of the N-methyl-D-aspartate (NMDA)-type glutamate receptor [22, 23], whose activation is required for LTP in the hippocampus [24] . The mechanism has been implicated in memory formation. The significant down-regulation of SRR in the hippocampus of Cu-treated mice that we observed suggested that SRR could also be involved in the memory impairment of mice caused by long-term low levels of Cu exposure. Taken together, we propose that the change in the expression levels of STMN1, CPLX2 and SRR that were triggered by our experimental conditions may have caused some degree of reorganization of some synapses in the hippocampus. This in turn may be the cause of the impaired memory that we detected.

Cytoskeleton-associated proteins

The cytoskeleton plays a role in controlling cell proliferation, cell cycle and apoptosis [25]. In this study, we identified two differentially expressed cytoskeleton-associated proteins, TUBA1A and GFAP, in the hippocampus of Cu-treated mice. TUBA1A is encoded by the *Tuba1a* gene and forms a dimer with β-tubulin to make up the

microtubules, a process that is essential for cell division. Thus, it is implied that the structure of microtubules could be affected by long-term low levels of Cu. GFAP is an intermediate filament protein and serves as an astrocyte-specific activation marker [26]. The increased expression that we observed indicated that a neuro-inflammatory response was triggered by long-term low levels of Cu exposure, which is consistent with the common toxic mechanisms of Cu by which Cu initiates or propagates an inflammatory response [27]. Neuro-inflammation contributes to memory impairment [28]. Most direct evidence comes from a study showing that nonsteroidal anti-inflammatory drugs could prevent neuro-inflammation-induced memory deficits [29]. Thus, neuro-inflammation, as evidenced by the increased expression of GFAP, could also contribute to spatial memory impairment caused by long-term low levels of Cu exposure.

Energy metabolism-related proteins

In this study, we also observed changes of the energy metabolism-related proteins ATPD, HNRDL, and GBG2 by long-term low levels of Cu exposure. ATPD is a subunit of oligomycin sensitivity conferral protein (OSCP) in mitochondrial ATPase, which is an important enzyme that generates energy for cells through the synthesis of adenosine triphosphate. HNRDL can regulate the expression of genes involved in the cell cycle, proliferation, survival, senescence, and stress responses [30, 31]. Nuclear HNRDL was shown to link the maintenance of telomere length and normal aging to the attenuation of inflammatory cytokine expression [32]. Increased expression of

HNRDL results in deregulation of mRNAs, including c-myc, c-jun, c-fos, and tumor necrosis factor-α, thus promoting tumorigenesis [30, 33]. GBG2 is a subunit of G protein complexes that acts as molecular switch inside cells. G proteins regulate metabolic enzymes, ion channels, transporters, and other parts of the cell machinery, which in turn regulate diverse systemic functions, such as embryonic development, learning and memory, and homeostasis [34]; GBG2 is associated with cancer, such as malignant melanoma. The changes of these energy metabolism-related proteins could also be involved in the toxic effects of Cu exposure.

Molecular chaperones

Molecular chaperones are responsible for protein folding. The altered expression of molecular chaperones is indicative of endogenous stress conditions, such as oxidative stress [35]. HSP72 as a molecular chaperone works to guide the folding of proteins and to aid the translocation of proteins across membranes [36]. Further study is required to investigate the specific role of HSP72 in toxic effects due to long-term low levels of Cu.

In summary, this study identified a number of abnormally expressed proteins in the hippocampus of mice caused by long-term low levels of Cu exposure. The identified protein, such as SRR, together with inflammatory responses, as evidenced by the increased expression of GFAP, could contribute to the observed memory impairment caused by Cu exposure. Our findings provide insights for a better understanding of Cu neurotoxicity at the protein level.

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Declaration of Conflict of Interest

None of the authors have any conflicts of interest to disclose.

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Figure Legends

Fig. 1. A representative 2D-DIGE gel image of hippocampal proteins from

Cu-treated mice and control mice.

The hippocampal protein samples from Cu-treated mice and control mice (n=6 for each group) were labeled with Cy-Dye, multiplexed, and underwent isoelectric focusing on 24-cm, pH 3-11, nonlinear IPG strips. The proteins were subsequently separated on large-format 12.5% gels. Spots of interest were manually excised, digested and subjected to identification by MALDI-TOF-MS/MS. (A) Cy3-labeled hippocampal proteins from the control mice. (B) Cy5-labeled hippocampal proteins from the Cu-treated mice. (C) Cy2-labeled proteins as internal standards. (D) The merged image showing Cy2-, Cy3- and Cy5-labeled proteins. (E) Grayscale 2D-DIGE gel image showing 9 differentially expressed protein spots identified by MALDI-TOF-MS/MS (black numbers with white square) in the hippocampus of Cu-treated mice compared with control mice.

Fig. 2. Confirmation of differential expression of SRR and GFAP in the hippocampus by western blot analysis.

(A, B) The relative levels of SRR in hippocampus by western blot analysis; (C, D) The relative levels of GFAP in hippocampus by western blot analysis. The graphed results depict the mean ± SEM. **P*<0.05 for Cu-treated mice vs control mice (n=6 for each group).

Fig. 3. The effect of long-term exposure to low levels of Cu on learning and memory in mice.

For the navigation test, the escape latency was measured to determine the effects of Cu exposure on the spatial learning ability (A). For the probe test, the probe time (B), the platform crossing number (C), the representative swimming path (D), the percentage of time spent in the target quadrant (E), the percentage of distance traveled in the target quadrant (F), the total distance traveled (G), and the average swimming speed (H) are shown. The graphed results depict the mean ± SEM. *P < 0.05, **P<0.01 for Cu-treated mice vs control mice (n = 8-10 for each group).

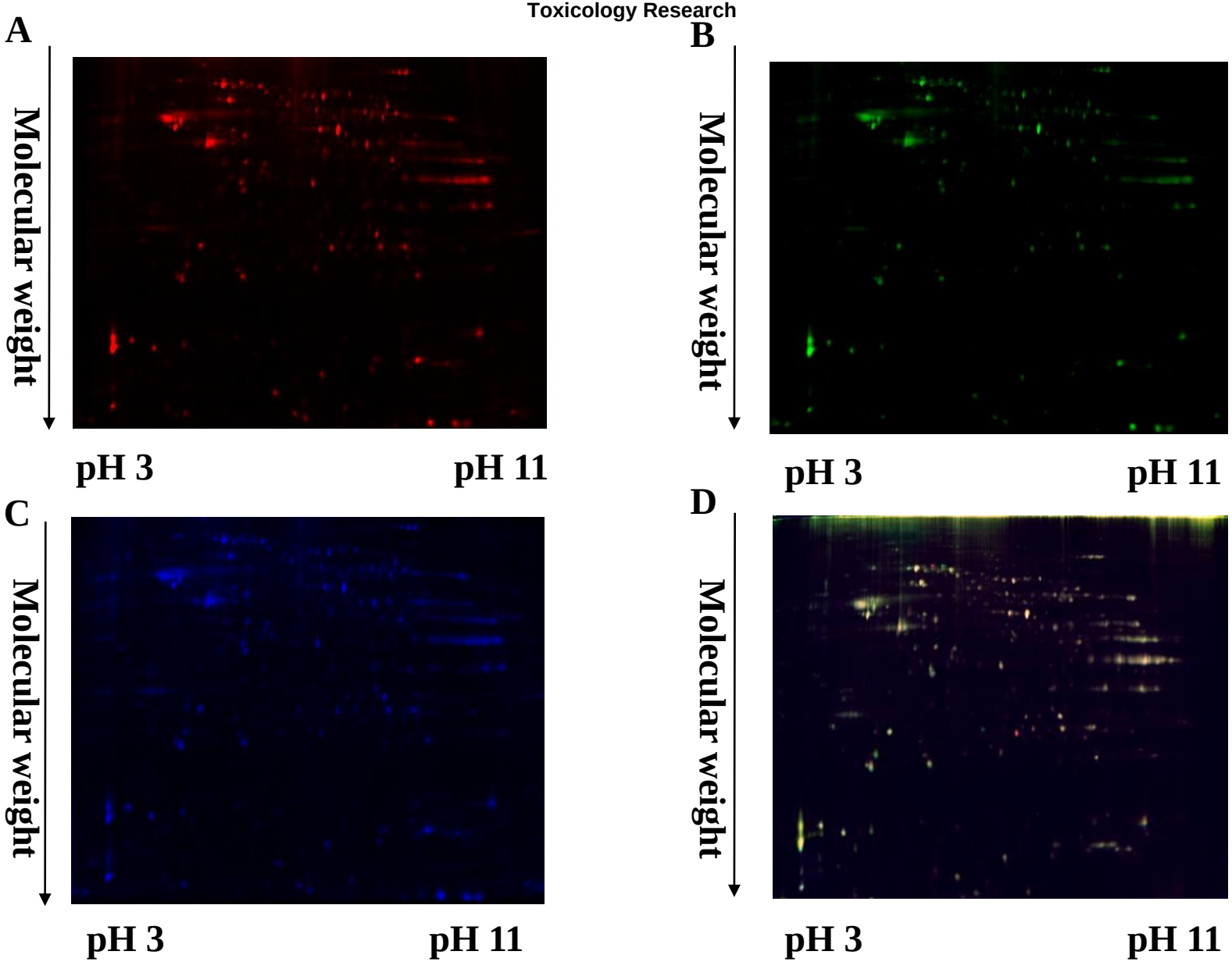

Figure 1

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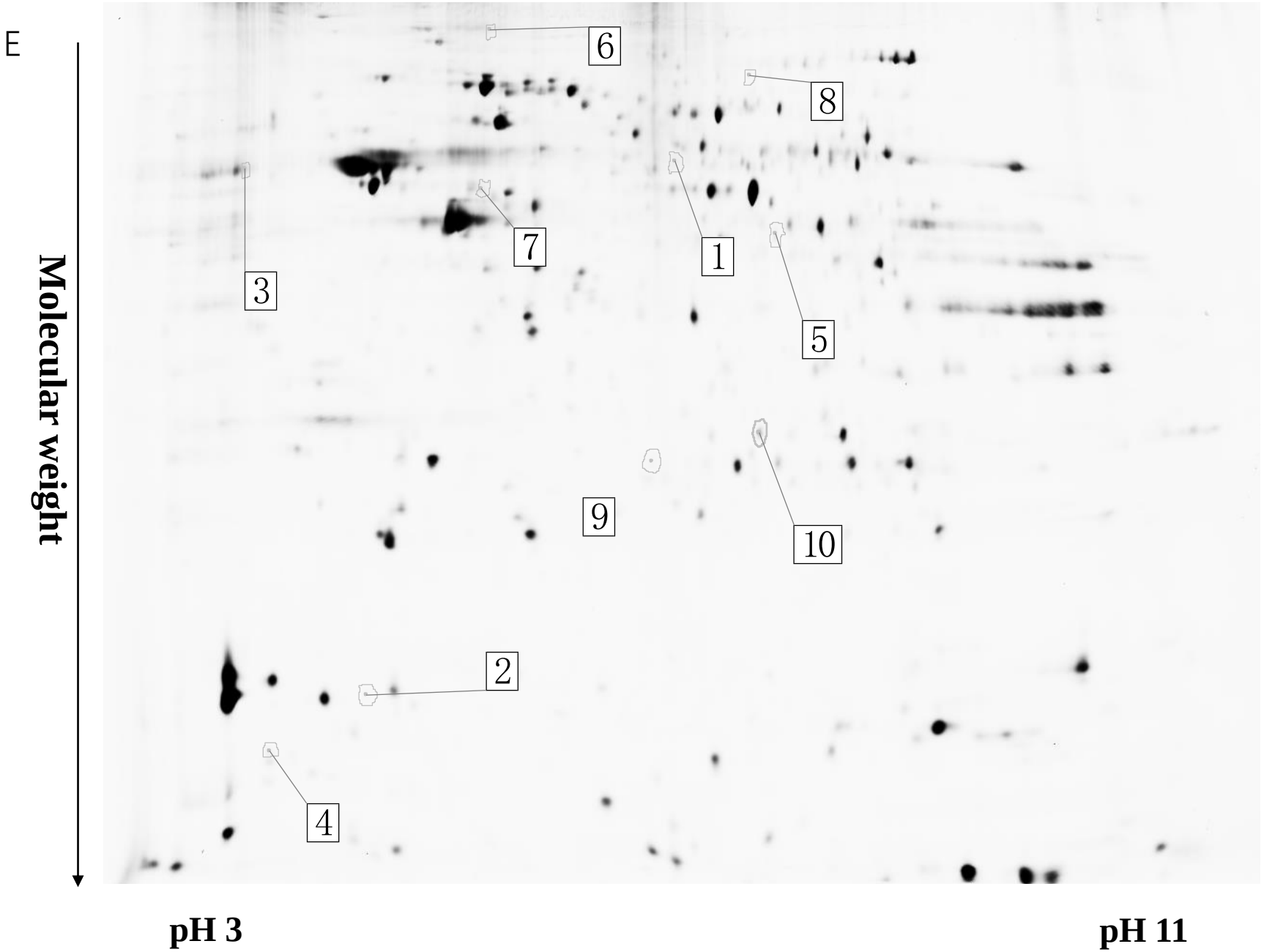

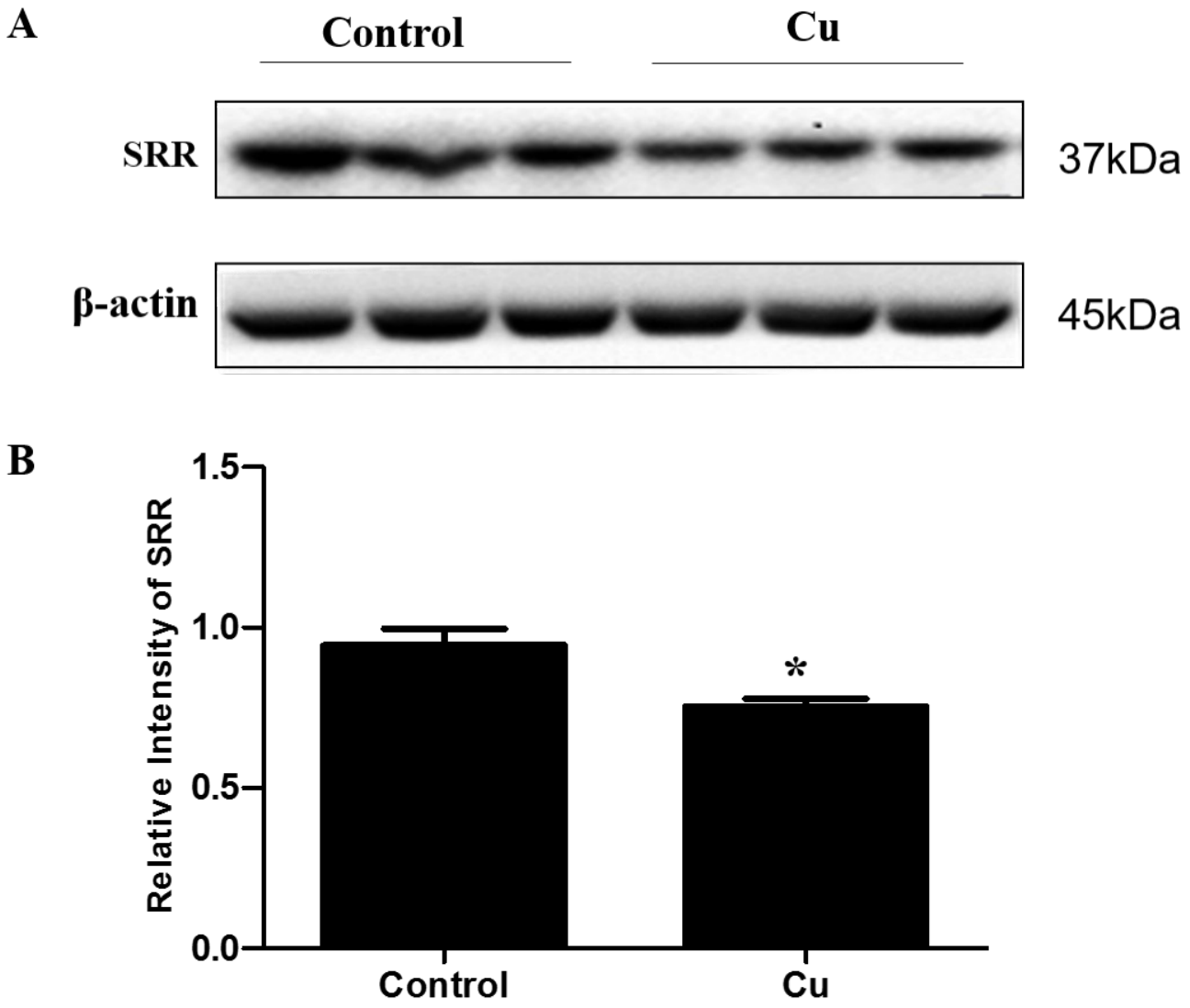

Figure 2

Sun et al., 2015

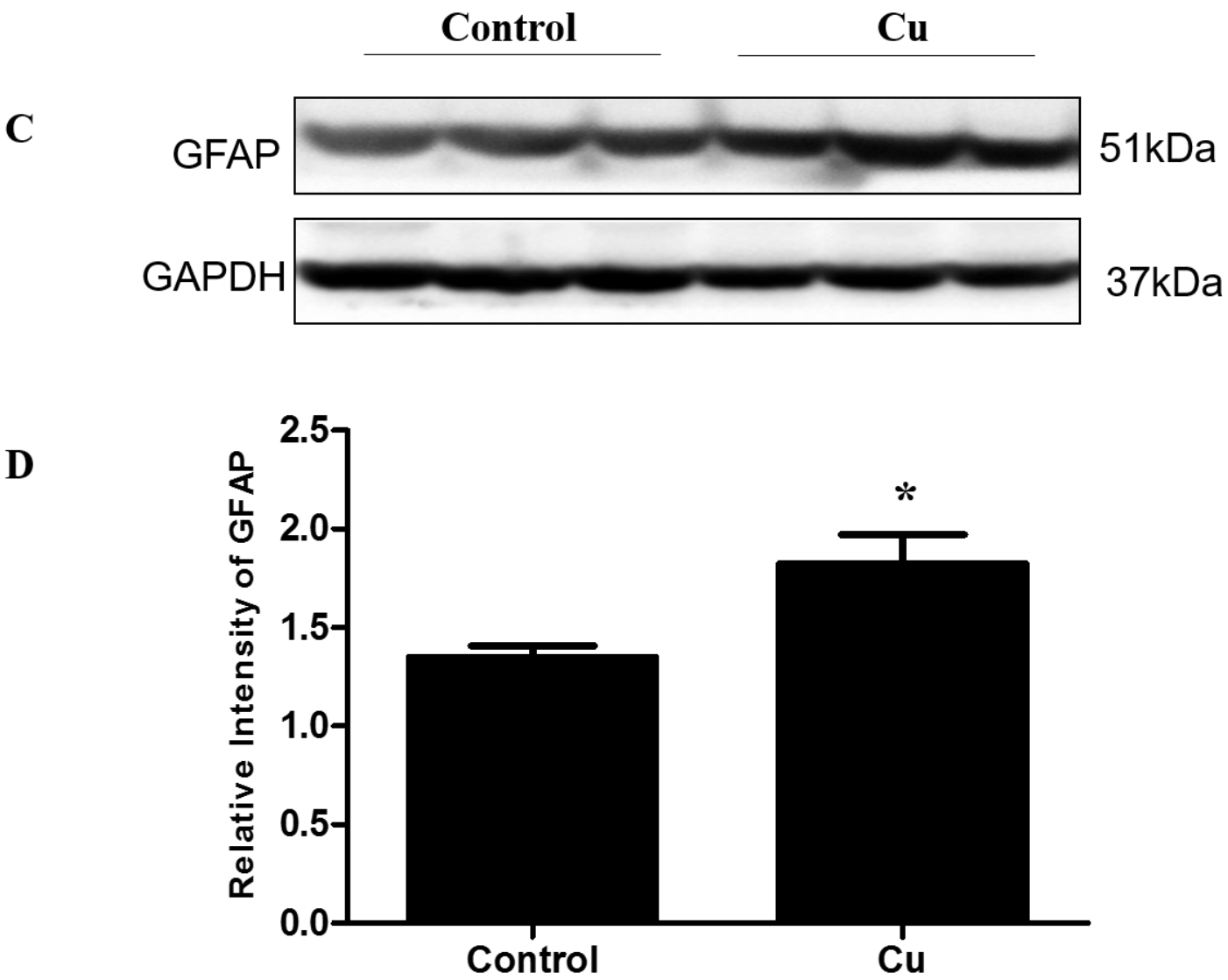

Figure 2

Sun et al., 2015

A

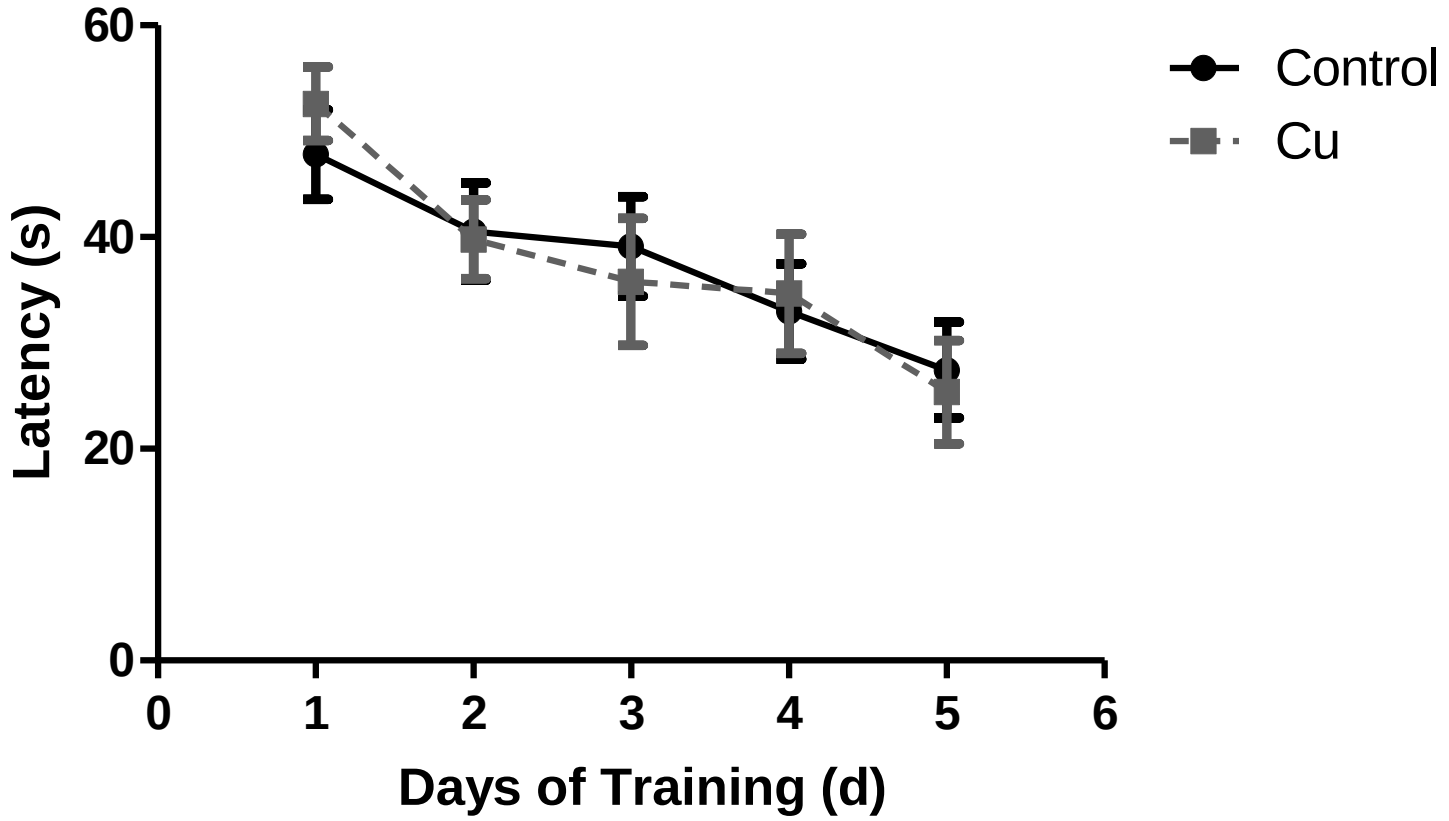

Figure 3

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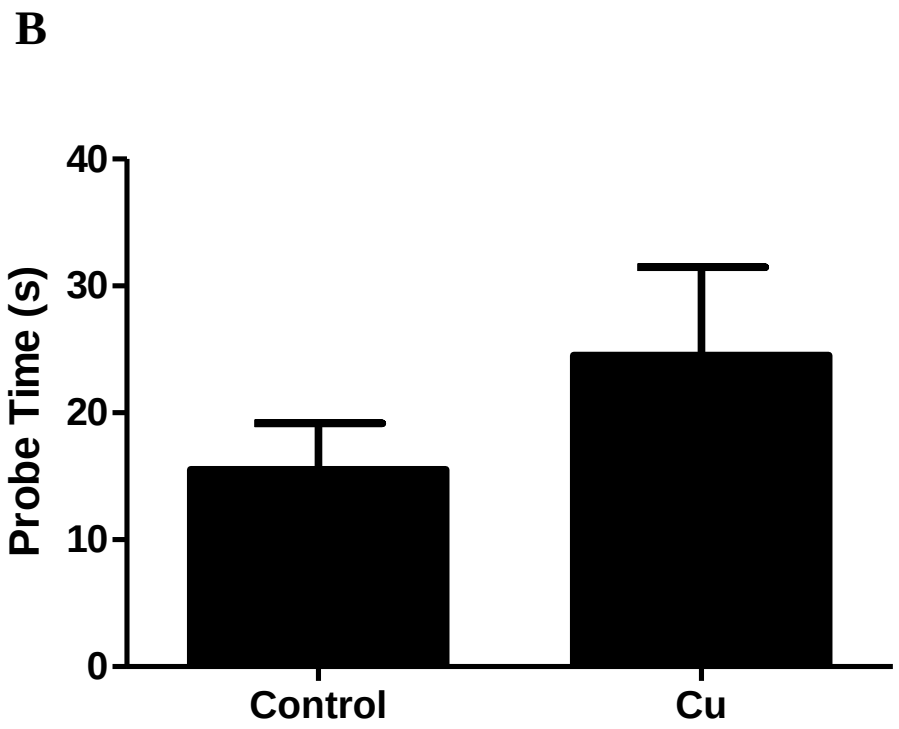

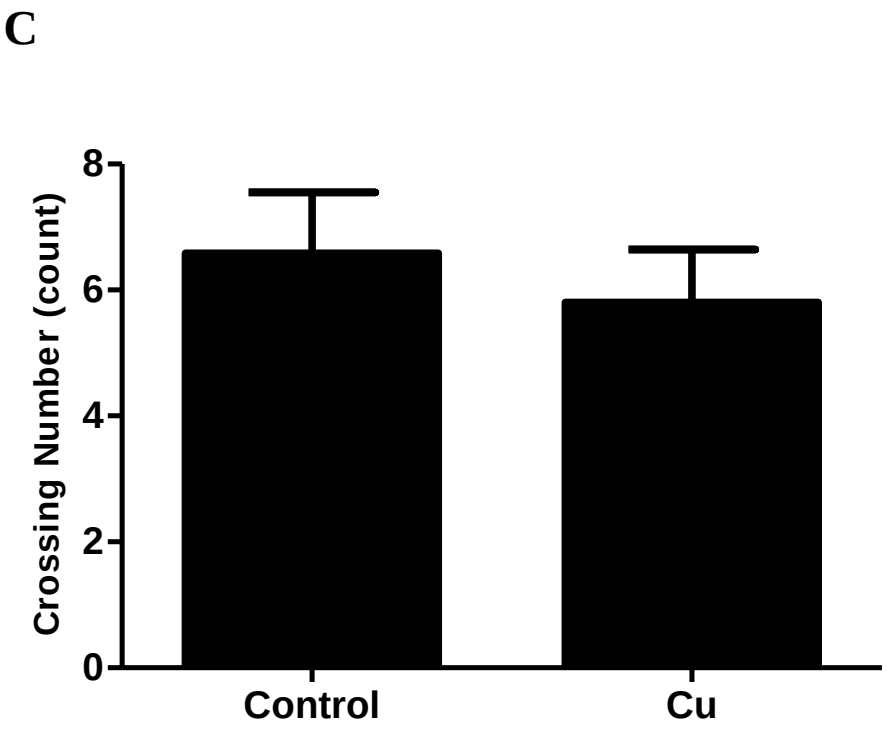

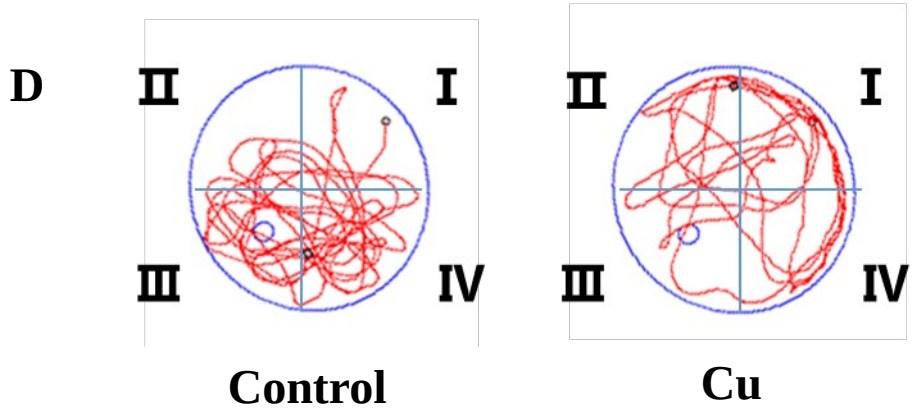

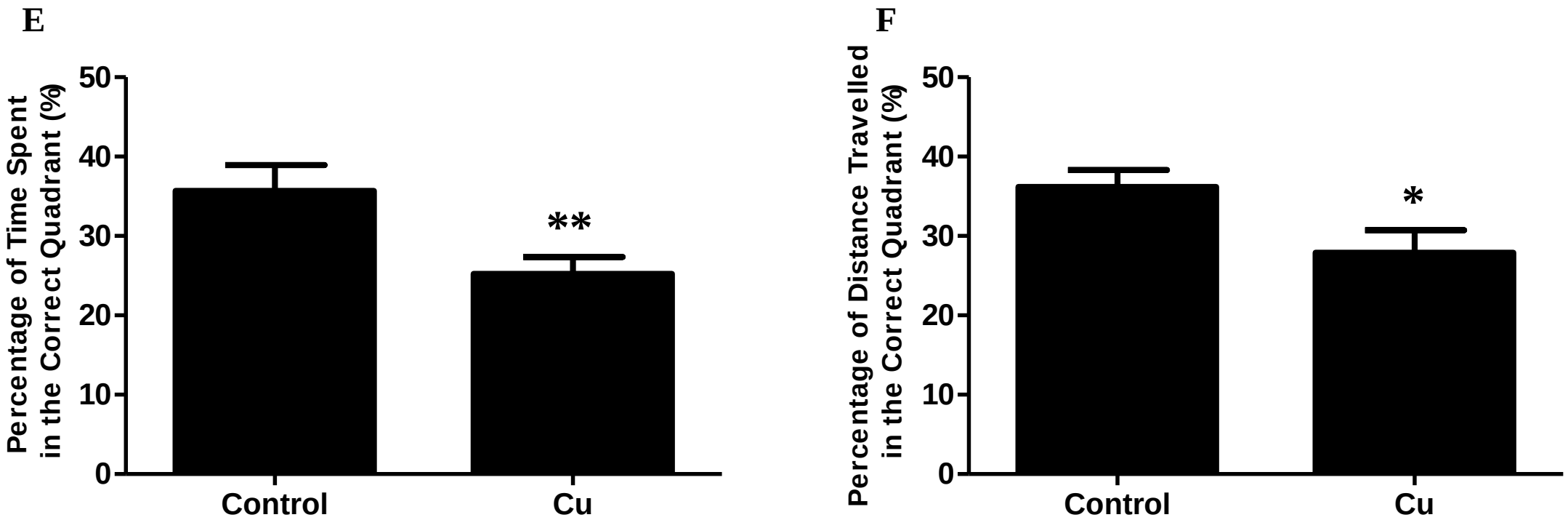

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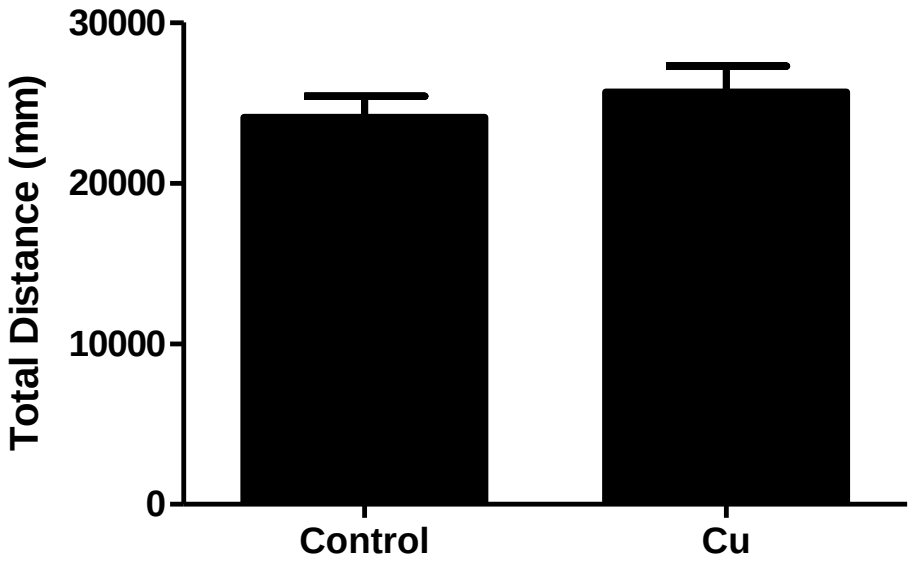

H

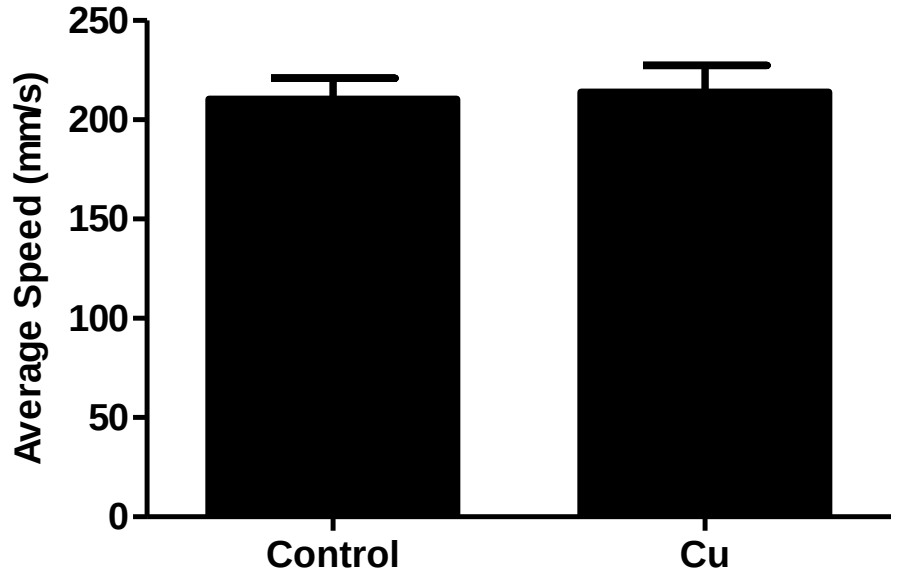

Table 1. Differentially expressed hippocampal proteins identified by 2D-DIGE/MALDI-TOF-MS/MS between Cu-treated mice and the control mice.

Spot No.	Accession No.	Protein Name	Function	Cu versus Control			
				Mascot score	MW (Da)	Ratio	p-Value
2	CPLX2_MOUSE	Complexin-2	Synapse-associated proteins	35	15499	-0.35	0.026
3	SRR_MOUSE	Serine racemase	Synapse-associated proteins	54	36735	-1.35	0.026
9	STMN1_MOUSE	Stathmin	Synapse-associated proteins	54	17264	-1.64	0.05
1	TBA1A_MOUSE	Tubulin alpha-1A chain	Cytoskeleton-associated proteins	152	50788	1.8	0.024
7	GFAP_MOUSE	Glial fibrillary acidic protein	Cytoskeleton-associated proteins	233	49927	1.86	0.039
4	ATPD_MOUSE	ATP synthase subunit delta	Energy-related signaling pathway proteins	131	17589	-1.59	0.031
5	HNRDL_MOUSE	Heterogeneous nuclear ribonucleoprotein D-like	Energy-related signaling pathway proteins	53	33709	2.34	0.033
8	GBG2_MOUSE	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2	Energy-related signaling pathway proteins	35	7959	1.9	0.042
6	HSP72_MOUSE	Heat shock-related 70 kDa protein 2	Molecular chaperones	138	69884	1.32	0.036

Table 2. The Analysis of KEGG pathway and KEGG diseases

Accession No	Protein Name	KEGG Pathway	KEGG Disease
CPLX2_MOUSE	Complexin-2	Synaptic vesicle cycle	
SRR_MOUSE	Serine racemase	Glycine, serine and threonine metabolism	
STMN1_MOUSE	Stathmin	MAPK signaling pathways; MicroRNAs in cancer	
TBA1A_MOUSE	Tubulin alpha-1A chain	Phagosome; Gap junction	
GFAP_MOUSE	Glial fibrillary acidic protein	Jak-STAT signaling pathway	Alexander disease
ATPD_MOUSE	ATP synthase subunit delta	Oxidative phosphorylation; Metabolic pathways;	Alzheimer's disease; Parkinson's disease; Huntington's disease
HNRDL_MOUSE	Heterogeneous nuclear ribonucleprotein D-like	Spliceosome	
GBG2_MOUSE	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2	Ras signaling pathways; Chemokine signaling pathways;PI3K-Akt signaling pathways	Glutamatergic synapse; Serotonergic synapse; GABAergic synapse
HSP72_MOUSE	Heat shock-related 70 kDa protein 2	Spliceosome; MAPK signaling pathways; Estrogen signaling pathways	