Copper chloride dose-dependently alters spatial learning and memory, and glutamate levels, in the hippocampus of rats

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Abstract. Copper is a trace element which exerts an important role in neuronal functions. Excessive Cu exposure is associated with central nervous system dysfunction, including memory loss. The present study examined the effects of $CuCl_2$ exposure on the spatial learning and memory of rats, and on metabolites in the hippocampus. A total of 60 male Sprague-Dawley rats (10 rats/group) were intraperitoneally injected with various doses (0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/kg) of $CuCl_2$ three times every other day for 6 days. Rats administered with 1.0 ml/kg sterile saline were used as controls. A total of 2 days subsequent to the final injection, the rats were subjected to the Morris water maze (MWM) test, followed by proton magnetic resonance spectroscopy (^{1}H-MRS). The rats were subsequently sacrificed, and their hippocampal tissues were processed for high performance liquid chromatography (HPLC). The MWM test demonstrated that the high-dose groups exhibited worse spatial learning and memory compared with the controls; however, the rats that received a relatively low dose (2.0 mg/kg) exhibited a decreased impairment. The ^{1}H-MRS results revealed increased Glu, N-acetyl-L-aspartate (NAA)+N-acetylaspartylglutamate, phosphocreatine (PCr) and Cr+PCr levels in the hippocampus of the 2.0 mg/kg group. HPLC analysis revealed increased γ-aminobutyric acid and glutamate (Glu) levels in the 2.0 mg/kg group, and decreased Glu levels in the 6.0 mg/kg group. The results of the present study demonstrated a beneficial effect of short-term exposure to a relatively low dose of $CuCl_2$ on spatial learning and memory, and the association of this effect with increased NAA and Glu levels in the hippocampus.

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Abbreviations: CNS, central nervous system; GABA, γ-aminobutyric acid; Glu, glutamate; HPLC, high performance liquid chromatography; VOI, volume of interest; MWM, Morris water maze; TR, repetition time; TE, echo time; Cr, creatine; Gln, glutamine; GPC, glycerophosphorylcholine; mI, myo-inositol; NAA, N-acetyl-L-aspartate; NAAG, N-acetylaspartylglutamate; PCh, phosphorylcholine; PCr, phosphocreatine; Tau, taurine; MRS, magnetic resonance spectroscopy; AD, Alzheimer's disease; ROS, reactive oxygen species; GluNR, N-methyl-D-aspartic acid glutamate receptor; S-D, Sprague-Dawley; Tau, taurine; SOD1, superoxide dismutase; MDA, malondialdehyde; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Key words: copper, magnetic resonance spectroscopy, brain metabolites, cognitive function, hippocampus

Introduction

Copper is a trace element that is important for neuronal functions. Additionally, it is a cofactor of enzymes and proteins that are required for a number of physiological functions, including neural transmission, the scavenging of free radicals, the production of energy and the mobilization of iron (1,2). As an important cofactor of metalloproteins, Cu ions act as an active or structural site. However, excessive exposure to Cu has been associated with central nervous system (CNS) dysfunction, including memory loss, multiple neuritis and neurasthenia syndrome (3,4). Additionally, post-mortem studies have demonstrated that Cu levels are markedly elevated in the brains of patients with Alzheimer's disease (AD) (5). The neurotoxicity caused by excessive Cu is thought to be an important risk factor for cognitive impairment in the ageing population, particularly learning and memory impairment (6).

Numerous studies have associated chronic Cu intoxication with AD-like pathology (7). For example, treating primary hippocampal neurons (10-14 days *in vitro*) with $CuCl_2$ (up to 10 μM) for 3 h was observed to significantly increase the amplitude, frequency and time constants of synaptic events (8). In addition, Cu has been demonstrated to be involved in the

synthesis of phosphatidyl-L-serine and phosphatidyl inositide complexes through a process that required ATP-mediated regulation (9).

Mitochondria are an energy-producing organelle and are thus vital for cell survival. Cu is involved in a number of signalling cascades and has been hypothesised to serve important roles in neurodegenerative processes associated with respiratory chain dysfunction and the generation of reactive oxygen species (ROS). Cu is considered to be a co-factor for complex IV of the mitochondrial electron transport chain and for cytochrome c oxidase. Notably, Cu deficiency has been observed to impair brain development, as it impaired mitochondrial function and led to a disorder of brain energy metabolism (10). Conversely, previous studies have reported Cu concentrations as high as 200 or 400 μM in neurodegenerative diseases (11,12). As co-factors of certain enzymes, Cu ions are involved in the generation of ATP and the degradation of ROS in mitochondria.

γ-aminobutyric acid (GABA) is recognized as an important inhibitory neurotransmitter in the brain, while glutamate (Glu) functions as an excitatory neurotransmitter; in the brain, these transmitters serve a role in cognitive functions, including learning and memory. Cu has been observed to dampen $GABA_A$ and Glu receptor function (13-15). A previous study proposed a link between Cu and the activity of the N-methyl-D-aspartic acid subtype of Glu receptors (GluNRs), with a functional link between Cu homeostasis and GluNR activity (16). In the present study, neurometabolites in the hippocampus of rats acutely exposed to various doses of $CuCl_2$ were measured via proton magnetic resonance spectroscopy (^{1}H-MRS) and high-performance liquid chromatography (HPLC), and the spatial learning and memory of the animals were evaluated. The spatial learning and memory of the rats were affected in a dose-dependent manner and the beneficial effect of a relatively low dose of $CuCl_2$ was associated with a mild increase in Glu in the hippocampus of the rats.

Materials and methods

Animals and experimental design. A total of 60 male Sprague-Dawley (S-D) rats (age, 7 weeks; weight, 200±34 g) were used in the present study. The animals were purchased from the Experimental Animal Center of Shantou University Medical College (Shantou, China) and were group housed (7-8 rats/cage) under standard laboratory conditions (22±1˚C temperature and 5±4% humidity) on a 12-h light/dark cycle (7:00 a.m. on; 7:00 p.m. off). Standard rat diet and water were given *ad libitum*. Following 7 days of acclimatization, the rats were randomly and equally divided into six groups (10 rats/group). Various doses (0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/kg) of $CuCl_2$ (Yuanye Biotechnology, Co., Ltd., Shanghai, China) in sterilized saline were given to the rats by intraperitoneal injection three times every other day for a 6-day period. The injection volume was 1.0 ml/kg. A total of 2 days subsequent to the last injection, all rats were subjected to the Morris water maze (MWM) test to evaluate hippocampus-dependent spatial learning and memory abilities. One training test was performed each day for 4 consecutive days, followed by one probe test on the 5th day. A total of 24 h subsequently, the rats were subjected to magnetic resonance imaging (MRI) and the ^{1}H-MRS procedure. Subsequently, the rats were sacrificed and their brains were removed. The right hippocampus was dissected out on ice and stored at -80˚C for subsequent HPLC analysis. All animal procedures were performed in accordance with the guidelines set up by the Animal Care and Use Committee of Shantou University Medical College and were approved by the committee.

MWM test. The conventional MWM test was performed to evaluate the spatial learning and reference memory abilities of the rats (17). As previously described, the rats were placed in a circular white tank with a diameter of 120 cm and a depth of 50 cm. The tank was filled with opacified water (25±1˚C) to a height of 38 cm and was surrounded by dark geometric cues affixed to white curtains (18,19). The tank was divided into four imaginary quadrants (quadrants I, II, III and IV), and an escape platform was positioned 2 cm under the horizontal plane in the middle of quadrant II. The MWM test consisted of two phases: The place navigation test and the spatial probe test.

All rats were habituated to the maze 1 day prior to training. During the place navigation training session, each rat was placed into the tank at a randomly selected position and allowed to explore the pool using a systematic or random search strategy. The behaviour of the rats in the water maze was videotaped using a video camera suspended above the maze that interfaced with a computer-based video tracking system. One navigation test was completed each day for 4 consecutive days, followed by one spatial probe test on the 5th day.

The platform was submerged during the training. During the place navigation trial, the rats were placed in the water and were allowed to search for the submerged platform for 60 sec. If the rat failed to find the platform, the operator moved it to the platform, where it remained for 20 sec. The escape latency was measured as described previously (18).

On the first day following 4 days of training sessions, a probe trial was completed. The platform was removed, and the rat was placed into the water in the quadrant opposite the target quadrant (quadrant II) and allowed to swim for 120 sec. Rats that failed to locate the platform within 120 sec were manually guided to the platform and kept there for 20 sec. As the rats swam around the pool, various parameters, including the number of platform crossings, the time required to reach the platform, the ratio of distance travelled in the target quadrant, the percentage time spent in the target platform quadrant, the total distance and the average travel speed were recorded using the DigBehav-Morris Water Maze Video Analysis System (Jiliang Software Technology Co., Ltd., Shanghai, China). When all tasks had been completed, the rats were dried and placed back into the housing facility once their body temperature had returned to a normal level (36-37˚C).

MRI/MRS acquisition. The *in vivo* MRI/MRS experiments were performed using a horizontal bore (bore size, 160 mm) Agilent 7.0 Tesla animal MRI scanner (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a 20-mm standard one-channel ^{1}H volume coil for radio frequency transmission and reception. The rats were initially anaesthetised with 5% isoflurane (Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA) in oxygen and continuously anaesthetised with the 1-2% isoflurane

(for maintenance), which was delivered through a nasal mask for spontaneous respiration. Following anaesthetisation, each rat was placed in a prone position in the centre region of the horizontal bore with its head fixed on a palate holder equipped with an adjustable nose cone for MRI/MRS acquisition. Body temperature was recorded through the respiration system and was maintained at 36-37˚C using a plastic membrane.

In order to ensure high image quality, the target must be placed in the centre of the magnet. In order to achieve correct positioning, images of the head in three planes were acquired with a gradient echo sequence. Subsequently, T2-weighted images in coronal, sagittal and axial positions were obtained using a fast spin echo multi-slice pulse sequence with the following parameters: Field of view=23x19 mm^2; matrix size=256x256; repetition time (TR)=2,000 msec; effective echo time (TE)=31.58 msec; slice thickness=2 mm; slice gap=0.5 mm; and acquisition time=4 min 20 sec. The high-resolution T2-weighted images were used to position the ^{1}H-MRS voxels in the hippocampus.

Following morphological imaging, the volume of interest (VOI) was located by referring to a digital rat brain atlas (20). All MR spectra were acquired using a single-voxel, ultra-short echo-time stimulated echo acquisition mode pulse sequence, with TR=3,000 msec and TE =2.35 msec. The total number of acquisitions was 256. The 3x3x3 mm^3 VOI primarily contained the right dorsal hippocampus in addition to some adjacent tissue. Outer volume suppression was performed around the VOI to inhibit any non-hippocampal contaminant signals. Automated shimming was performed for the VOI to yield a water spectrum width of 15-20 Hz. The B0 field was shimmed using a 3D gradient shimming method. Shortly afterwards, a manual shim based on second-order 3D gradient shimming was adopted to reduce any signal in homogeneities in the B0 field. Localized voxel shimming was performed using the FASTMAP technique prior to spectral acquisition (21). Outer volume suppression and water suppression with variable pulse power and optimized relaxation delays were used to acquire proton spectra. In order to correct for small variations in coil sensitivity, the unsuppressed water signal from the prescribed voxel was used as a reference (22). The total scan duration of one MRS measurement was 13 min and 54 sec. The raw spectral data were exported to an external VnmrJ v4.0 workstation (Agilent Technologies, Inc.) for post-processing.

MRS data processing and analysis. The MRS-based quantification of metabolite concentrations was performed using LCModel software (version 6.2-4E; LCModel Inc., Oakville, ON, Canada) (23). The raw data were processed using LCModel. Quantitative metabolite concentrations were obtained from the raw data following processing using LCModel and scaled to the water signal. The quantitative analysis algorithm of LCModel, which is based on linear combinations, was used to calculate the optimal fitting of the objective spectra to the model spectra. The tissue water concentration was used as the internal standard. Only spectra with a full width at half maximum <20, metabolite concentration fitting results with Cramer-Rao lower bound <20% (24) and a signal-to-noise ratio ≥10 were used for data analysis.

The base set included the following 17 metabolites: Alanine, aspartate, Cr, GABA, glucose, Glu, glutamine (Gln), glutathione, glycerophosphorylcholine (GPC), lactate, myo-inositol (mI), N-acetyl-L-aspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphorylcholine (PCh), phosphocreatine (PCr), scyllo-inositol and taurine (Tau). The signal intensities were processed with water scaling for absolute quantification of the metabolic concentrations. Additionally, the sums of certain metabolites, including NAA+NAAG, Cr+PCr, Glu+Gln and GPC+PCh, were measured.

Preparation of tissue samples for HPLC analysis. The right hippocampus was homogenized (10% w/v) in ice-cold phosphate buffer solution (0.1 M; pH 7.2; Yuanye Biotechnology, Co., Ltd.). The homogenate was centrifuged at 12,000 x g for 20 min at 4˚C. The supernatant was mixed with an equivalent volume of methanol for overnight protein precipitation at 4˚C, and the resulting samples were frozen at -80˚C following membrane filtration.

Quantification of GABA and Glu in vitro. The HPLC procedure was performed according to a previously described method (25). Liquid chromatography was performed using an Agilent 1100 HPLC system (Agilent Technologies, Inc.). In order to detect the concentrations of GABA and Glu, a standard curve was obtained for each under standard chromatographic conditions, with concentration as the horizontal axis and the product peak area as the vertical axis. Chromatographic separation was obtained on a phase column with an Agilent C18 guard column (250x4.6 mm^2; 5 μm; Agilent Technologies, Inc.). The temperature of the column was maintained at 25˚C. The mobile phase consisted of a mixture of 0.1 mol/l sodium acetate (pH 6.8 with 2% tetrahydrofuran), methanol and water at a flow rate of 0.75 ml/min. The GABA and Glu levels were determined using a scanning fluorescence detector with the excitation and emission wavelengths set at 338 nm and 425 nm, respectively. The standard lines of GABA and Glu were y_{GABA}=9.94x+54.24 (R^2=0.99) and y_{Glu}=12.06x+122.2 (R^2=0.99) The HPLC system was connected to a computer to quantify the mixture of GABA and Glu by comparing the area under each peak with the corresponding measure of each reference standard, using the Agilent chemical workstation software (ChemStation for LC 3D, Rev. A.10.01; Agilent Technologies, Inc.). The Cu concentration in the brain tissue of the rats was not measured in the present study since Zhang *et al* (26) demonstrated that intranasal delivery of Cu nanoparticles at 1 and 10 mg/kg for 15 days did not change the Cu concentration in different tissues, including liver, lung, spleen, kidney and brain, in mice.

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA), and P<0.05 was considered to indicate a statistically significant difference. For the behavioural tests, the escape latency in the MWM test was analysed by repeated measures generalised linear model and multivariate analysis of variance (ANOVA) procedures, and one-way ANOVA with a Fisher's least significant difference post hoc test was performed for the other data. For the ^{1}H-MRS and HPLC brain metabolite data, the normality and homogeneity of the data were verified. The data were statistically analysed by one-way ANOVA as long as they satisfied the normality and homogeneity assumptions;

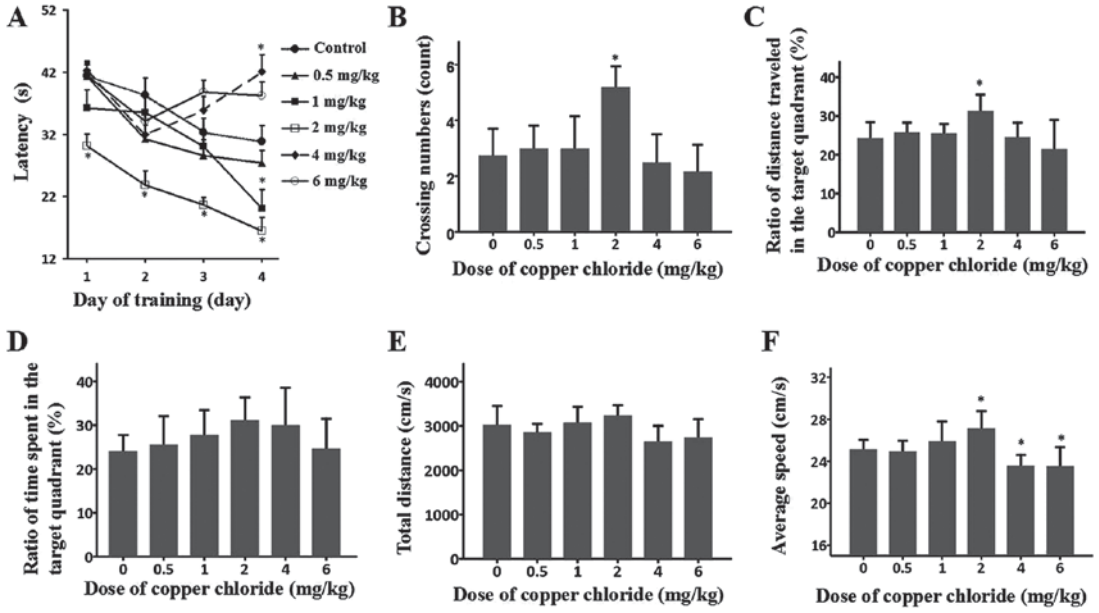

Figure 1. Copper chloride dose-dependently affects the spatial learning and memory of rats. (A) At 2.0 mg/kg, $CuCl_2$ facilitated the learning process of rats during the training trials of the Morris water maze test, whereas 4.0 and 6.0 mg/kg $CuCl_2$ exerted adverse effects. (B) Rats given 2.0 mg/kg $CuCl_2$ visited the target quadrant significantly more times compared with the control group. (C) Rats given 2.0 mg/kg $CuCl_2$ exhibited a significantly increased ratio of distance travelled in the target quadrant over the total distance in the water maze compared with the control rats. (D) Rats given 2 mg/kg $CuCl_2$ exhibited an increased ratio of time spent in the target quadrant, while the higher dose group (6.0 mg/kg) exhibited an adverse effect. (E) The total travelled distance of the rats in all groups was not significantly different. (F) Compared with the control rats, rats given 2.0 mg/kg $CuCl_2$ exhibited a significantly increased speed in the water maze, while those given 6.0 mg/kg $CuCl_2$ travelled more slowly. The data are presented as the mean ± standard deviation. *P<0.05 vs. control (1.0 ml/kg of sterilized saline with 0 mg/kg copper chloride).

otherwise, Mann-Whitney and Kruskal-Wallis non-parametric tests were performed. Correlations between the brain metabolite concentrations detected through ^{1}H-MRS and HPLC, and the behavioural test results, were investigated using Pearson and Spearman tests.

Results

Behavioural test. In order to examine the effects of exogenous Cu exposure on the spatial learning and memory of S-D rats, the MWM test was performed with subjects that were intraperitoneally injected with various doses of $CuCl_2$ (0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/kg). Overall, there was a significant effect of day and latency ($F_{(3, 236)}$=8.28; P<0.01), and an interaction between day and group was observed ($F_{(5, 234)}$=14.49; P<0.01). During the training phase, on the first day, all groups spent a comparable amount of time finding the submerged platform, except for the group treated with 2.0 mg/kg $CuCl_2$; this group spent less time finding the platform (P=0.002). On the following 3 days, the rats treated with 4.0 and 6.0 mg/kg $CuCl_2$ exhibited no progress in the learning phase in terms of the time spent finding the platform; by contrast, all of the other groups exhibited typical progress in the water maze test, as they spent less and less time finding the platform (Fig. 1A). During the probe test, the performance of the 0.5 and 1.0 mg/kg groups was comparable with that of the control group, although the 2.0 mg/kg group crossed the target quadrant more frequently than the control group (P<0.05). By contrast, the 4.0 and 6.0 mg/kg groups visited the target quadrant markedly fewer times (Fig. 1B). These results indicated deficient memory retrieval in the groups that received higher doses and a potential beneficial effect on memory retrieval in the 2.0 mg/kg $CuCl_2$ group. The ratios of distance travelled in the target quadrant (Fig. 1C), the ratios of time spent in the target quadrant (Fig. 1D), the total distance (Fig. 1E) and the average speed data (Fig. 1F) additionally suggested an adverse or beneficial effect of the different doses of $CuCl_2$ on the MWM performance of the various groups.

^{1}H-MRS analysis. The ^{1}H-MRS analysis was performed subsequent to the MWM test. A 3x3x3 mm^3 VOI was positioned in the right side of the dorsal hippocampus of the rat, as presented in the localization images (Fig. 2A). Representative spectra acquired from this VOI in each group are presented in Fig. 2B. The absolute concentrations of brain metabolites, including Cr, PCr, Cr+PCr, GABA, Glu, Gln, Glu+Gln, NAA, NAAG, GPC+PCh, mI and Tau, were calculated using LCModel. For Cr, all rats exposed to $CuCl_2$ were observed to have levels comparable to those of the control group. In terms of PCr, no difference was noted between the control group and any other group, except for the 2.0 mg/kg group, which exhibited a significantly increased level (P<0.01). Therefore, Cr + PCr levels exhibited the same pattern as PCr levels. The effects of $CuCl_2$ on the levels of NAA and NAA+NAAG exhibited typical bell-shaped curves; significantly increased levels of NAA were observed only in the 2.0 mg/kg group (P=0.018), while NAA+NAAG levels were increased in the 1.0 and 2.0 mg/kg groups (P=0.011 and P=0.01, respectively). The $CuCl_2$-induced alterations in GABA, Glu, Gln, and Glu+Gln produced similar bell curves, although the alterations were not significant. No differences in mI and GPC+PCh levels were observed between the control group and any other group. The

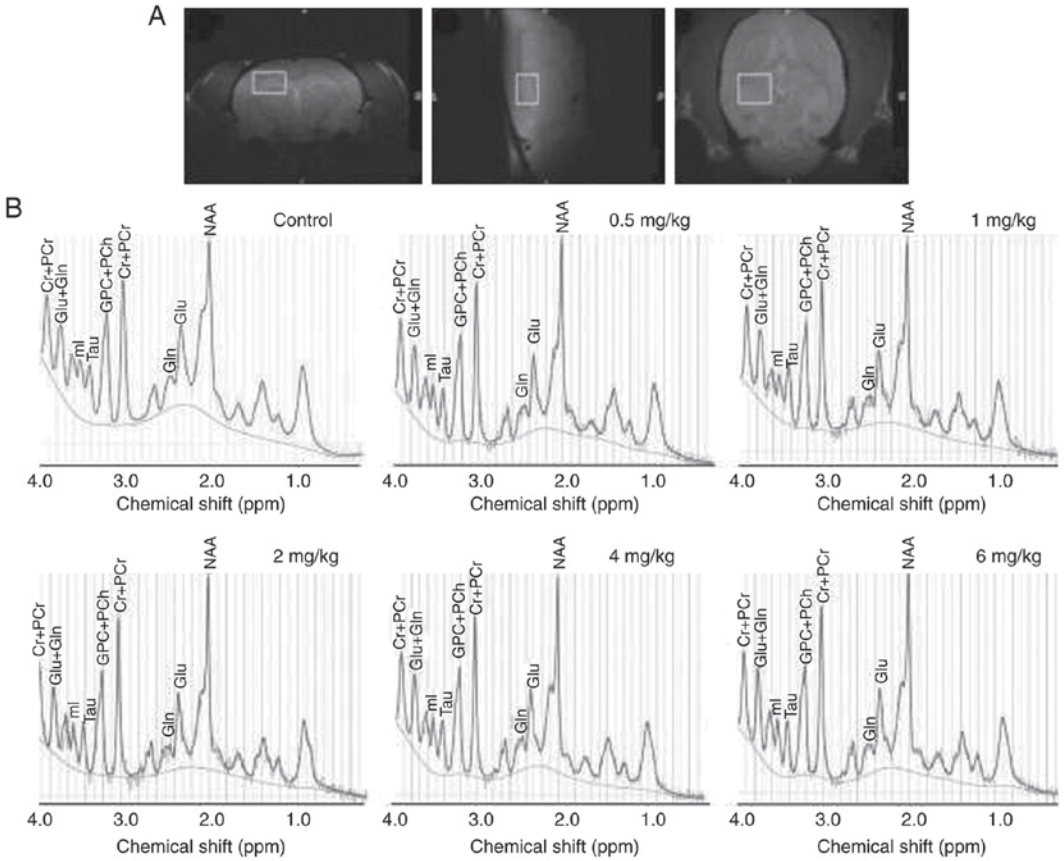

Figure 2. Location of the VOI and representative ^{1}H-MRS spectra for all groups. (A) The VOI was located in the right dorsal hippocampus of the rats, in the coronal, saggital and axial directions. (B) Representative ^{1}H-MRS spectra obtained from the control and 0.5, 1.0, 2.0, 4.0 and 6.0 mg/kg $CuCl_2$ exposure groups. Cr, creatine; PCr, phosphocreatine; Glu, glutamate; Gln, glutamine; mI, myo-inositol; Tau, taurine; PCh, phosphorylcholine; NAA, N-acetylaspartate; VOI, volume of interest; ^{1}H-MRS, proton magnetic resonance spectroscopy.

only significant difference in Tau levels was observed between the 2.0 mg/kg and control groups (P=0.021; Table I).

HPLC analysis. HPLC analysis, which may avoid certain confounding factors that may affect ^{1}H-MRS, including breathing, inhomogeneity of the B0 field and volume contamination, was performed to further evaluate the levels of two neurotransmitters, GABA and Glu, which were of interest. The absolute concentrations of these two neurotransmitters were determined subsequent to a standard curve being obtained for each. GABA and Glu peaks for a standard sample and a typical base peak chromatogram obtained for a hippocampus sample from a control rat are presented in Fig. 3. Similar to the ^{1}H-MRS results, the effects of $CuCl_2$ on GABA and Glu levels were shaped like bell curves. The highest GABA level was observed in the 2.0 mg/kg group, while comparable, although decreased, levels were observed in the other groups (P=0.028). However, the bell curve of the effect on Glu levels was unique. The curve increased until it reached the highest level for the 2.0 mg/kg group and subsequently declined (P=0.039). A remarkable decrease was observed for the 4.0 and 6.0 mg/kg groups (P=0.042 and P<0.01) compared with the control group (Table II).

Positive correlations between spatial memory and levels of Glu and NAA. In order to examine the association between the $CuCl_2$-induced effects on spatial memory and metabolite concentrations detected by ^{1}H-MRS and HPLC, the correlation between metabolite concentrations and crossing numbers was analysed using Pearson and Spearman correlation tests. This analysis revealed that the number of platform crossings on probe trials was positively correlated with the hippocampal levels of Glu (r=0.512; P=0.002; Fig. 4A) and NAA (r=0.69; P<0.001; Fig. 4B) detected by ^{1}H-MRS. In accordance with the ^{1}H-MRS results, a similar positive correlation was identified between the number of crossings and the Glu levels revealed by HPLC analysis (r=0.433; P=0.002; Fig. 4C).

Discussion

An increasing number of studies have reported on the effects of chronic Cu exposure on brain functions. However, the reported results have been inconsistent; Cu excess and deficiency have been demonstrated to impair the spatial memory capacity of the brain. For example, chronic exposure to Cu in drinking water has been demonstrated to impair spatial memory in mice (27), and chronic Cu exposure has been additionally observed to accelerate memory impairment in 3xTg-AD mice (28). In another study, compared with control rats, Cu-administered animals exhibited impaired spatial memory in addition to significantly decreased serum acetylcholinesterase activity (29). However, Cu deficiency is detrimental to the development of brain function. In support of this hypothesis, following water-mediated Zn supplementation for 4 months,

Table I. Brain metabolite concentrations determined by *in vivo* proton magnetic resonance spectroscopy in hippocampal tissue (mmol/l).

Metabolite	Control	$CuCl_2$ (0.5 mg/kg)	$CuCl_2$ (1.0 mg/kg)	$CuCl_2$ (2.0 mg/kg)	$CuCl_2$ (4.0 mg/kg)	$CuCl_2$ (6.0 mg/kg)
Cr	2.91±0.15	2.80±0.24	2.84±0.15	2.61±0.06	2.67±0.49	2.56±0.12
PCr	3.30±0.28	3.35±0.76	3.44±0.22	4.37±0.57[b]	3.28±0.20	3.23±0.35
Cr+PCr	5.65±0.50	5.87±0.76	6.12±0.24	6.73±0.27[b]	5.54±0.17	5.51±0.47
NAA	5.70±0.61	5.85±0.96	6.27±0.43	6.63±0.20[a]	6.18±0.44	5.80±0.64
NAA+NAAG	6.45±0.62	6.57±0.99	7.13±0.44[a]	7.49±0.51[b]	6.71±0.28	6.29±0.33
GABA	1.63±0.39	1.65±0.23	1.64±0.19	1.66±0.27	1.60±0.20	1.54±0.22
Glu	7.22±0.40	7.41±0.31	7.46±0.55	7.57±0.76	7.20±0.49	7.15±0.05
Gln	1.74±0.34	1.82±0.28	1.95±0.35	1.93±0.62	1.71±0.22	1.74±0.36
Glu+Gln	8.90±0.61	9.12±0.15	9.49±0.87	9.63±1.19	8.81±0.40	8.79±0.25
GPC+PCh	1.31±0.09	1.21±0.20	1.35±0.11	1.27±0.17	1.27±0.20	1.43±0.10
mI	4.22±0.68	4.33±0.87	4.51±0.28	4.65±0.28	4.34±0.46	3.99±0.38
Tau	3.97±0.43	4.09±0.65	4.41±0.45	4.65±0.32[a]	4.23±0.43	4.35±0.62

All data are expressed as the mean ± standard deviation. [a]P<0.05, [b]P<0.01 vs. control. Cr, creatine; PCr, phosphocreatine; Glu, glutamate; Gln, glutamine; Tau, taurine; PCh, phosphorylcholine; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; GABA, γ-aminobutyric acid; PCh, phosphorylcholine; GPC, glycerophosphorylcholine; mI, myo-inositol.

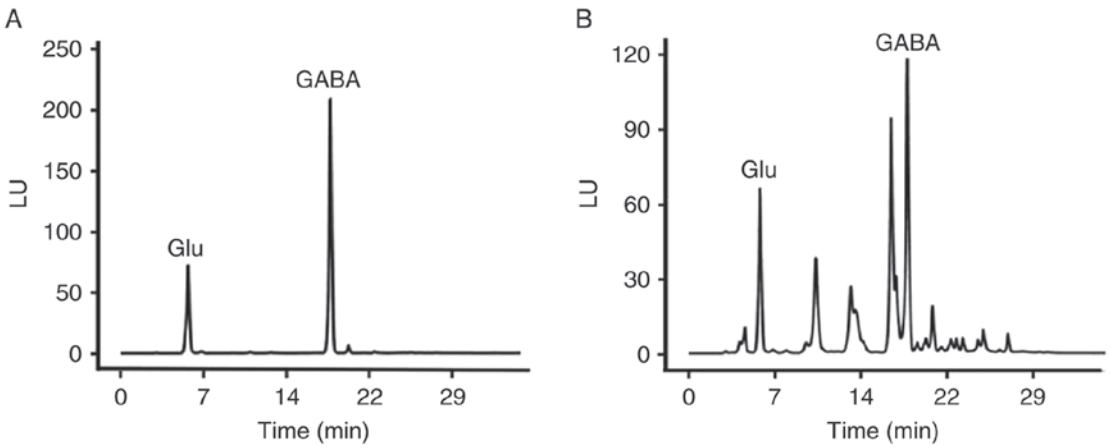

Figure 3. Standards and a representative high-performance liquid chromatography spectrum. (A) GABA and Glu peaks for a standard sample. (B) A typical base peak chromatogram obtained for a hippocampal sample from a control rat. GABA, γ-aminobutyric acid; Glu, glutamate.

S-D rats exhibited significantly increased levels of anxiety compared with controls raised on lab water. The Zn-treated rats took a markedly longer time to reach the platform in the MWM, suggesting a spatial memory deficiency. These behavioural changes are thought to be relevant to Cu deficiency as the addition of Cu to the Zn-supplemented water returned freezing and latency values closer to those of controls (30). The authors additionally examined the effect of increasing Zn levels in the drinking water in Tg2576 mice. Compared with mice raised on lab water, the Zn-supplemented mice exhibited a significant increase in latency and fewer platform crossings on probe trials in the MWM test. However, no significant differences were observed between the Zn+Cu group and the group raised on lab water. The authors suggested that the negative consequences of Zn may result in reduced Cu levels and that the effects may be due to an imbalance of these metal ions, rather than a direct effect of increased Zn (31). Similarly, a recent *in vitro* study observed biphasic effects of Cu on rat learning and memory in the MWM test. Free hippocampal Cu was demonstrated to increase at a lower concentration of Cu (II) acetate [$Cu(OAc)_2$] (0.2 mg/kg), which improved learning and memory. However, increased doses of Cu decreased superoxide dismutase-1 (SOD1) activity, increased malondialdehyde (MDA) levels and impaired spatial cognition (32).

Dysregulation of Cu metabolism contributes to an interruption of neuronal function, and further leads to neurodegeneration, necrosis and gliocyte hyperplasia (32). The effects of acute Cu exposure on the spatial learning and memory of rats *in vivo* were examined for the first time, to the best of our knowledge, in the present study. The following results were observed in the present study: i) 0.5 and 1.0 mg/kg $CuCl_2$ exerted no effects; ii) 2.0 mg/kg $CuCl_2$ promoted the

Table II. Concentrations of GABA and Glu detected by high-performance liquid chromatography (mmol/g).

Metabolite	Control	$CuCl_2$ (0.5 mg/kg)	$CuCl_2$ (1.0 mg/kg)	$CuCl_2$ (2.0 mg/kg)	$CuCl_2$ (4.0 mg/kg)	$CuCl_2$ (6.0 mg/kg)
GABA	2.83±0.17	3.01±0.34	3.35±0.39	3.53±0.83[b]	2.97±0.34	2.75±0.57
Glu	6.00±0.48	6.14±0.56	6.31±0.75	6.63±0.74[a]	5.42±0.72[a]	4.08±0.53[b]

All data are expressed as the mean ± standard deviation. [a]P<0.05, [b]P<0.01 vs. control. GABA, γ-aminobutyric acid; Glu, glutamate.

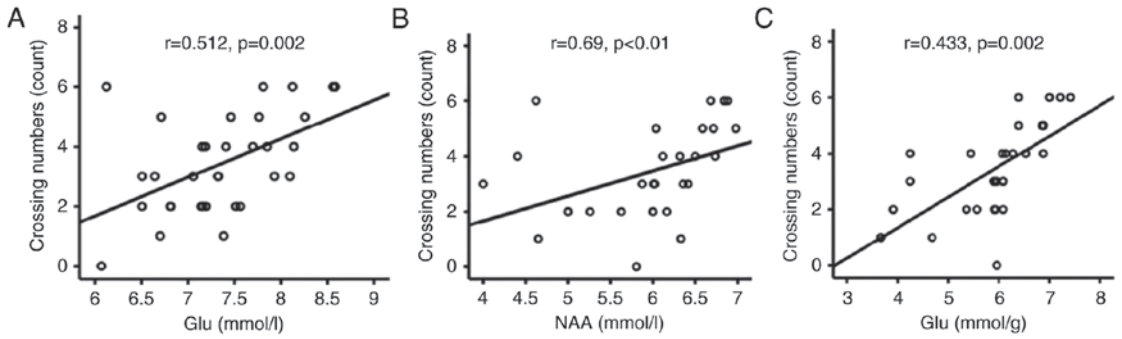

Figure 4. Positive correlations between spatial memory, and Glu and NAA levels. (A) The correlation between Glu concentrations (detected by proton magnetic resonance spectroscopy) in the hippocampus and the number of crossings of the target quadrant in the water maze. (B) The correlation between NAA concentrations in the hippocampus and the number of crossings of the target quadrant in the water maze. (C) The correlation between Glu concentrations (detected by high-performance liquid chromatography) in the hippocampus and the number of crossings of the target quadrant in the water maze. Glu, glutamate; NAA, N-acetyl-L-aspartate.

learning process in the training phase of the MWM test and benefited spatial learning and memory in the probe test; and iii) 4.0 and 6.0 mg/kg $CuCl_2$ impaired the learning process and the spatial memory of the subjects. These findings suggested that the spatial learning and memory functions of the brain may tolerate Cu exposure. In support of this hypothesis, a previous animal study demonstrated that Cu suppressed hippocampal long-term potentiation in rats, although it did not alter learning or memory in the MWM test (33). In a mouse study, intranasal administration of Cu nanoparticles at 1 and 10 mg/kg for 15 days did not alter the Cu concentrations in various tissues, including the liver, lung, spleen, kidney and brain, indicating that the animals were capable of removing or metabolizing the inhaled Cu nanoparticles (26). Although the $CuCl_2$ was intraperitoneally injected in the present study, the three lower doses (0.5, 1.0 and 2.0 mg/kg) were unlikely to be toxic to brain cells, based on the results of the studies referred to above. Additionally, 2.0 mg/kg $CuCl_2$ exerted a beneficial effect on the learning and spatial memory of the rats. The beneficial effect that was observed in the 2.0 mg/kg group is unlikely to be an artificial phenomenon, considering that the rats which received this dose of $CuCl_2$ performed better than all of the other groups in the training phase and probe test. The increased travel speed of the 2.0 mg/kg group during the test period of the water maze additionally indicates that the rats in this group were more energetic than the rats in the other groups. This energy may have contributed to the improved performance of these rats on the other indexes. The spatial memory impairments of the rats treated with 6.0 mg/kg $CuCl_2$ are consistent with the excess Cu-mediated cognitive function deficiency reported in previous animal studies. The result was correspondent with a previous *in vitro* study, which demonstrated that low concentrations of Cu (II) (1-100 nM) improved neuronal firing rate, although higher concentrations (1-5 μM) reduced the firing rate (32). It may be hypothesised that increased doses of $CuCl_2$ may decrease the activity of SOD1 and the levels of MDA, and impair spatial cognition. However, lower doses of $CuCl_2$ may have the opposite effect.

The second significant finding of the present study is that the effect of $CuCl_2$ on PCr, Cr+PCr, NAA and NAA+NAAG levels exhibited a typical bell curve, with a significantly increased level in the 2.0 mg/kg group. All of these indexes are associated with mitochondrial function. The increased levels in the 2.0 mg/kg $CuCl_2$ group suggested that this treatment had a beneficial effect on mitochondrial function; it may be suggested that this dose of $CuCl_2$ enhanced mitochondrial function in neurons. This hypothesis may account for the increased energetic status of the 2.0 mg/kg group, as illustrated in the MWM test. Notably, NAA levels in the brain impact the viability of neurons and are additionally associated with neuronal-oligodendroglial integrity, which is necessary for the normal function of neuronal circuits in the brain. In this context, the increased NAA level in the 2.0 mg/kg $CuCl_2$ group may explain the improved performance of this group of rats in the MWM test. In support of this explanation, 8 weeks of social isolation decreased the NAA and PCr levels in the dorsal hippocampus of rats and impaired spatial working memory (34).

Glu, the main excitatory neurotransmitter in the CNS, is not only associated with cognitive and emotional activities; it is also an excitotoxin. An overdose of extracellular Glu may cause the excessive accumulation of neurons, leading to cell death, and ultimately to learning and memory impairment. Dysfunction of

glutamatergic transmission is considered to be a predominant feature and fundamental pathological mechanism of neurodegenerative disorders that involve impaired spatial memory. Previous *in vivo* human ^{1}H-MRS studies reported decreased Glu levels in the hippocampus of patients with neurodegenerative diseases (35). In the present study, the ^{1}H-MRS results indicated a mild, although not statistically significant, increase in hippocampal Glu levels in rats that were exposed to 2.0 mg/kg $CuCl_2$ compared with those in the control group. This increase was confirmed via HPLC analysis; in this analysis, the difference reached significance. By contrast, the two higher doses (4.0 and 6.0 mg/kg) of $CuCl_2$ significantly decreased the levels of Glu. In the present study, the potential mechanisms of the Cu-induced changes in Glu levels were not studied. However, it may be hypothesised that the increase in Glu levels induced by treatment with 2.0 mg/kg $CuCl_2$ may reflect a compensatory effect to counteract the blockade of certain receptors of this neurotransmitter. In line with this speculation, CNS neurons possess the machinery to take up Cu and to subsequently release it at the synaptic cleft (36), where it may modulate excitatory and inhibitory neurotransmission. Indeed, Cu blocks GABAergic and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ergic neurotransmission when it is acutely applied to cultured rat olfactory bulb neurons (13). Cu was demonstrated to inhibit AMPAergic neurotransmission in rat cortical neurons (15) and GABAergic neurotransmission in acutely isolated rat cerebellar Purkinje cells (37). However, the compensatory response may reach its upper limit and even break down when the dose of $CuCl_2$ is increased, as observed in the rats that received 4.0 and 6.0 mg/kg $CuCl_2$ in the present study. In agreement with this interpretation, glutamic acid concentrations were decreased in certain brain regions, including the hippocampus, in mice that inhaled a high dose of Cu nanoparticles (38).

GABA is a principal inhibitory neurotransmitter in the CNS and is known to be associated with cognitive function (39). In a recent study, Cu was demonstrated to affect the holding current of neurons by affecting GABA-mediated signalling (40). In another study, extra-synaptic GABA receptors were observed to be susceptible to Cu modulation (41). In agreement with these previous studies, the results of the present study demonstrated that hippocampal GABA levels were increased in the group of rats treated with 2.0 mg/kg $CuCl_2$, while this treatment had a beneficial effect on the learning and spatial memory capacity of the subjects.

Notably, hippocampal Glu levels in the rats that received the various doses of $CuCl_2$ were positively correlated with the frequency of platform crossings in the target quadrant, suggesting a beneficial effect of higher Glu levels on the retention of spatial reference memory. This suggestion appears to argue against the commonly accepted notion that increased Glu levels in the hippocampus impair spatial memory. In order to interpret this apparent conflict, the following points are of importance: i) The increase in Glu observed in the rats that were given 2.0 mg/kg $CuCl_2$ was mild (approximately 10% higher compared with the level in the normal controls); ii) this mild increase reflected a compensatory response of hippocampal neurons to the blockade of AMPAergic neurotransmission, suggesting that the increase was unlikely to be toxic to the neurons, as mentioned above; and iii) the 2.0 mg/kg dose of $CuCl_2$ is relatively low and within the safe range for rats, as discussed above. In line with this claim, the WHO Provisional Maximum Tolerable Daily Intake upper limit for Cu was set to 0.5 mg/kg per day (42), based on the fact that Cu does not appear to be a cumulative toxic hazard for humans (International Programme on Chemical Safety, 1982) (43,44). In addition, in previous *in vivo* studies, a suspension of Cu nanoparticles was administered to animals at a dose of 30 mg/kg body weight, or at an even higher dose of 200 mg/kg to study the adverse effect of Cu nanoparticles (45,46). In a previous study, S-D rats were given once-daily intraperitoneal injections of $Cu(OAc)_2$ at doses of 0.2, 2, or 20 mg/kg for 5 days to examine the effects of hippocampal Cu concentration on learning and memory and observed biphasic dose-dependent effects of Cu on rat learning and memory (32). Considering all these factors, it is plausible to conclude that the 2.0 mg/kg dose of $CuCl_2$ that was administered to rats according to the procedure of the present study induced a mild increase in hippocampal Glu levels, and thus facilitated spatial learning and memory. In support of the beneficial effect of this dose of $CuCl_2$, hippocampal NAA levels were positively correlated with the number of times the rats crossed the target quadrant in the water maze.

Notably, the region of interest in the ^{1}H-MRS analysis was located in the right dorsal hippocampus of the rats, whereas the HPLC analysis measured metabolite levels in homogenates of the whole right hippocampus. In addition, ^{1}H-MRS results may be affected by a number of confounding factors, including breathing, inhomogeneity of the B0 field and volume contamination, which may decrease the sensitivity of the analysis and increase the variance of measurements. Indeed, HPLC, although not ^{1}H-MRS, revealed significant alterations in GABA and Glu levels in the hippocampal tissue of the rats that were treated with 2.0 and 6.0 mg/kg $CuCl_2$.

In conclusion, the present study provided the first report, to the best of our knowledge, of a beneficial effect of 2.0 mg/kg $CuCl_2$ on the spatial learning and memory of rats. The same treatment mildly increased Glu, GABA, NAA, PCr, and Cr+PCr levels in the hippocampus. The concurrent mild increases in these brain metabolites in the hippocampus suggested that the administration of $CuCl_2$ in accordance with the regimen used in the present study may cause a neurotrophin-like alteration. These results provided further evidence of the essential role of Cu in brain function.

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