Supplementary Information

Elucidating the mechanism of surface functionalization dependent neurotoxicity of graphene family nanomaterials

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Section1. MATERIALS AND METHODS

Materials. Reduced graphene oxide (RGO), and graphene functionalized with carboxylic acid (G-COOH), hydroxide (G-OH), and amine (G-NH_2) were procured from Hang Zhou Wan Jing New Material Co., Ltd. (Hangzhou, China). CCK-8 kit was obtained from Abcam (catalogue no. ab228554). Malondialdehyde (MDA) (catalogue no. A003-1) and ROS kits (catalogue no. E004-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MOPS (3-(N-morpholino)propanesulfonic acid) and 1,2-dioleoyl-snglycero-3-phosphocholine (DOPC) lyophilized powder was purchased from Sigma Aldrich (Germany). Ultraperformance liquid chromatography grade acetonitrile (Merck, Germany), methanol (Merck, Germany), ammonium acetate (Sigma-Aldrich, Germany), ammonium hydroxide (Sigma-Aldrich, Germany) and ultrapure water (18.2 MΩ-cm, Millipore, Billerica, MA) were used for solvent preparation in the metabolomics analysis.

Dye-leakage experiment

Liposomes were prepared using film rehydration method. Briefly, 20 mg DOPC was dissolved in chloroform and dried in a glass tube under a nitrogen stream. The tube was further dried in vaccum to form a dry layer of DOPC film. The dried film was rehydrated in MOPS buffer (50 mM, pH 7.4) containing 50 mM of carboxyfluorescein (CF). The mixture was passed through a polycarbonate membrane (100 nm) using an Avanti Polar Lipids mini-extruder. The obtained vesicle suspension was passed through a HiTrap Desalting column (GE Healthcare Life Sciences) equilibrated with 50 mM MOPS with 90 mM NaCl (pH 7.4) to remove excessive CF. The obtained lipid vesicles have a hydrodynamic diameter of 100 nm as measured by dynamic light scattering.

An aliquot of the liposome solution (20 mL) was added to a glass vial containing 10 mg/L graphene suspension to initiate the exposure experiment. The CF dye leaks into the extravesicular solution if the lipid membrane becomes damaged. After exposure, samples were centrifuged at 8000 g to remove aggregates and the intensity of the fluorescence was measured at excitation and emission wavelengths of 485 nm and 528 nm, respectively. After measurement, Triton X-100 (0.5 wt/vol%) was added to the vesicle solution to solubilize the lipid vesicles and release all entrapped CF fluorophore. The loss of membrane integrity can be calculated from $C/ C_m = (I_t - I_0) / (I_m - I_0)$ where C_m indicates the total CF concentration upon liposome solubilization; I_t indicates the

measured fluorescence intensity; I_0 indicates the initial CF intensity before exposure; and I_m is the total fluorescence intensity upon liposome solubilization.

Untargeted Metabolomics Analysis. The cells were seeded in 6-well-plates at 500,000 cells/well 24 h before exposure to GFNs. Four different GFNs were employed individually at a concentration of 10 mg/L. Seventy-two hours after treatment, cells were washed three times with phosphate buffered saline (1 x PBS), followed by addition of 1mL of methanol/acetonitrile/H_2O (2:2:1, v/v/v). Samples were ultrasonicated for 30 min at 4 °C twice, and then kept at -20 °C for 1 h for protein precipitation. The precipitates were removed by centrifugation at 13,000 rpm at 4 °C for 15 min. The supernatants were collected, lyophilized, and stored at -80 °C prior to metabolomics analysis. The supernatants prepared were transferred to the autosampler injection vial in an Agilent 1290. Infinity liquid chromatography system coupled to a Triple TOF 5600 System (AB SCIEX, Concord, ON) tuned to circa 30,000 mass resolution. The prepared sample solution was injected onto an ACQUITY UHPLC BEH Amide column (1.7 μm, 2.1 mm× 100 mm column, WATERS, Manchester, U.K). Column temperature was maintained at 25 °C, and flow rate was 300 μL/min. The mobile phase consisted of H_2O + 25 mM ammonium acetate + 25 mM ammonium hydroxide (mobile phase A) and acetonitrile (mobile phase B). A 23 minutes for the separation was utilized using the following gradient; 95% B 0-1 min, 95%-65% B from 1-14 min; 65%-40% B for 14-16 min; 40% B from 16-18 min before reverting to initial conditions between 18 and 18.1 min where it was left to reequilibraite for 5 min. The autosampler temperature was kept at 4°C throughout the analyses. Samples were analyzed in a randomised order, and QC samples were injected at regular intervals throughout the analytical run to monitor the stability and repeatability of the UPLC-QTOF system. Detection was performed both positive and negative ionization modes.

The original UHPLC-QTOF-MS data were converted to .mzXML format using ProteoWizard, and then exported to XCMS software for peak alignment, retention time adjustment, and extraction of peak intensities. The peak area normalized data was imported into SIMCA-P 14.1 software (Umetrics, Umea, Sweden), and pareto-scaled before multivariate statistical analysis and univariate analysis. A supervised clustering method, partial least-squares discriminant analysis (PLS-DA), was used to classify the samples and find the relevant variables related to the sample groupings. The model was validated using a 7-fold cross validation method and tested with 200 random permutations to assess the predictive variation of the model.

Variable importance in projections (VIP) scores indicate the importance of a variable to the entire model. VIP scores obtained from the PLS-DA model were used to assess the contribution a variable makes to the model. Metabolites that had VIP >1 drive the separation between groups and defined as discriminating metabolites. Based on the multivariate analysis and MS spectra, the discriminating metabolites were identified by comparison with the human metabolome database (http://www.hmdb.ca). The metabolite levels in the treated groups were compared with those in the control group using Student's *t test* statistical analysis. Metabolites that had VIP >1 and p <0.05 are defined as significantly changed metabolites. MetaboAnalyst (https://www.metaboanalyst.ca/) was used for the functional enrichment analysis of the disturbed metabolites.

Section 2. RESULTS AND DISCUSSION

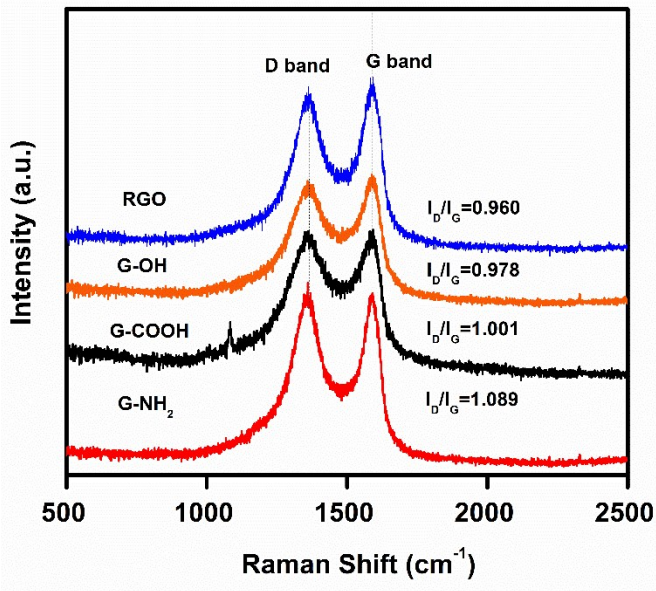

Figure S1. Raman spectra of RGO, G-OH, G-COOH and G-NH_2. The I_d/I_g ratios were 0.967, 0.978, 1.001 and 1.089 for RGO, G-OH, G-COOH and G-NH_2, respectively.

Table S1. Characterization of GFNs. Size and height were obtained by AFM. Hydrodynamic size and Zeta potential were obtained by measuring 10 mg/L graphene suspensions. Atomic percent was obtained by XPS analysis.

	Lateral size (μm)	Thickness (nm)	Hydrodynamic sizes (μm)	ζ potential (mV)	Atomic percent (%)		
					C	O	N
RGO	0.14±0.04	0.91±0.15	1108±182.3	-10.3±2.4	91.1	8.9	0
G-OH	0.16±0.06	0.89±0.23	1329±132.8	-19.2±1.9	88.8	11.2	0
G-COOH	0.14±0.04	0.90±0.16	1549±271.8	-18.7±2.1	87.7	12.3	0
G-NH_2	0.15±0.03	0.95±0.14	1301±122.3	10.5±2.2	91.2	6.2	2.6

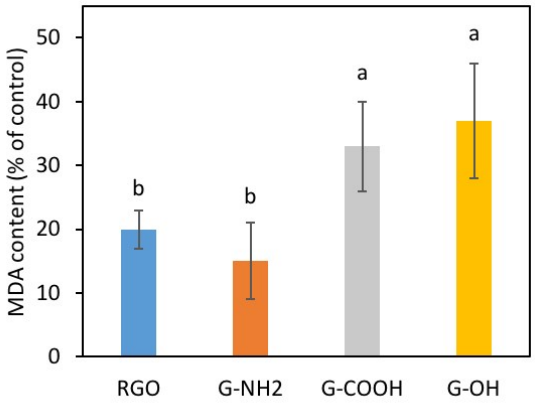

Figure S2. MDA content in SK-N-SH cells after exposure to GFNs for 72 h. Different lowcase letters indicate significant difference at $p < 0.05$. The over accumulation of MDA in cells suggested oxidative membrane damage. G-COOH and G-OH induced higher MDA content in cells than G-NH_2 and RGO, suggesting higher oxidative damage.

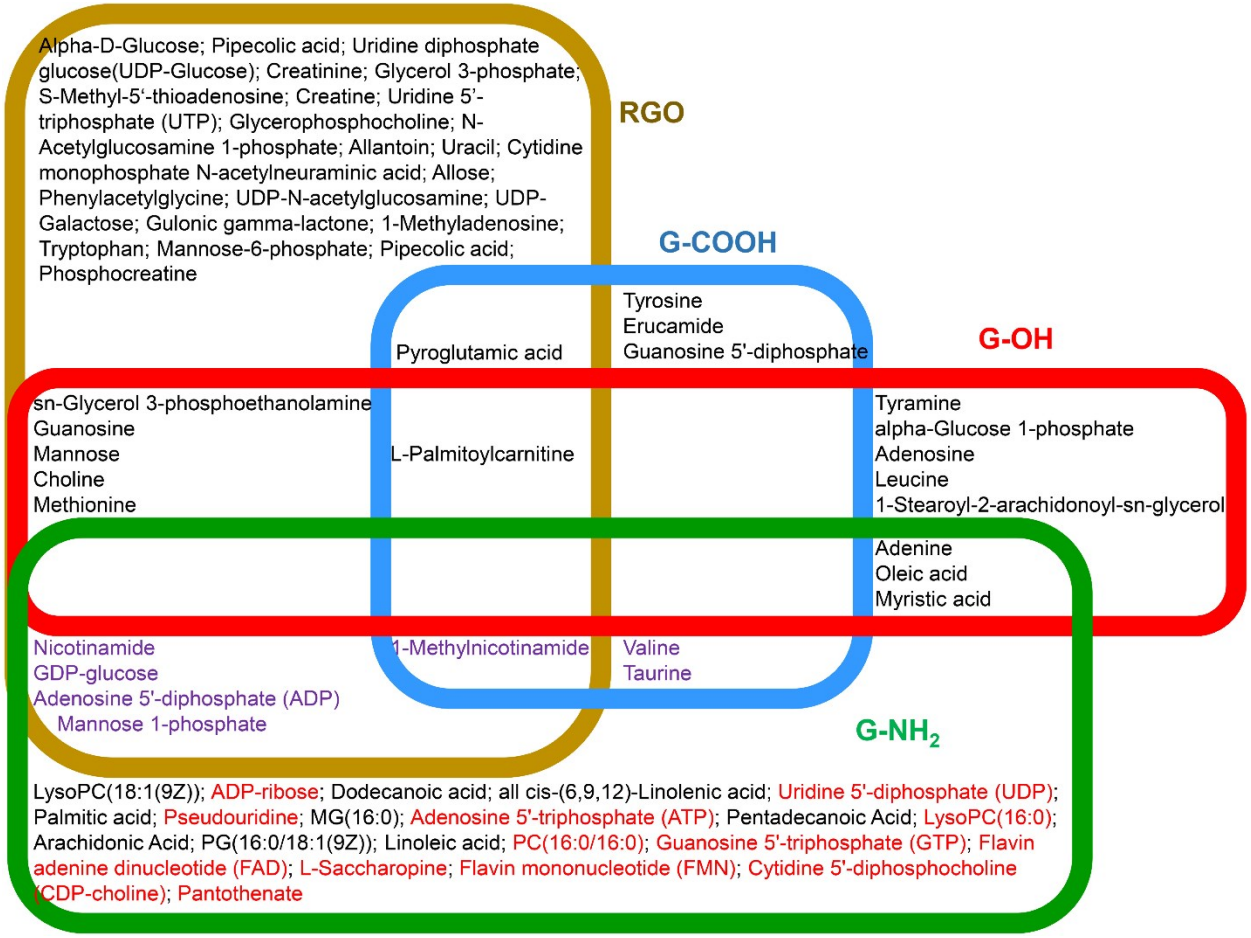

Figure S3. Up- (red text) and down- (black text) regulated metabolites in SK-N-SH cells after GFNs treatments. The results are the combination of PLS-DA analysis (VIP >1) and *t*-test ($p < 0.05$). Purple colored text indicates overlapping metabolites that exhibited different directionality with regard to their fold change in response to different treatments.

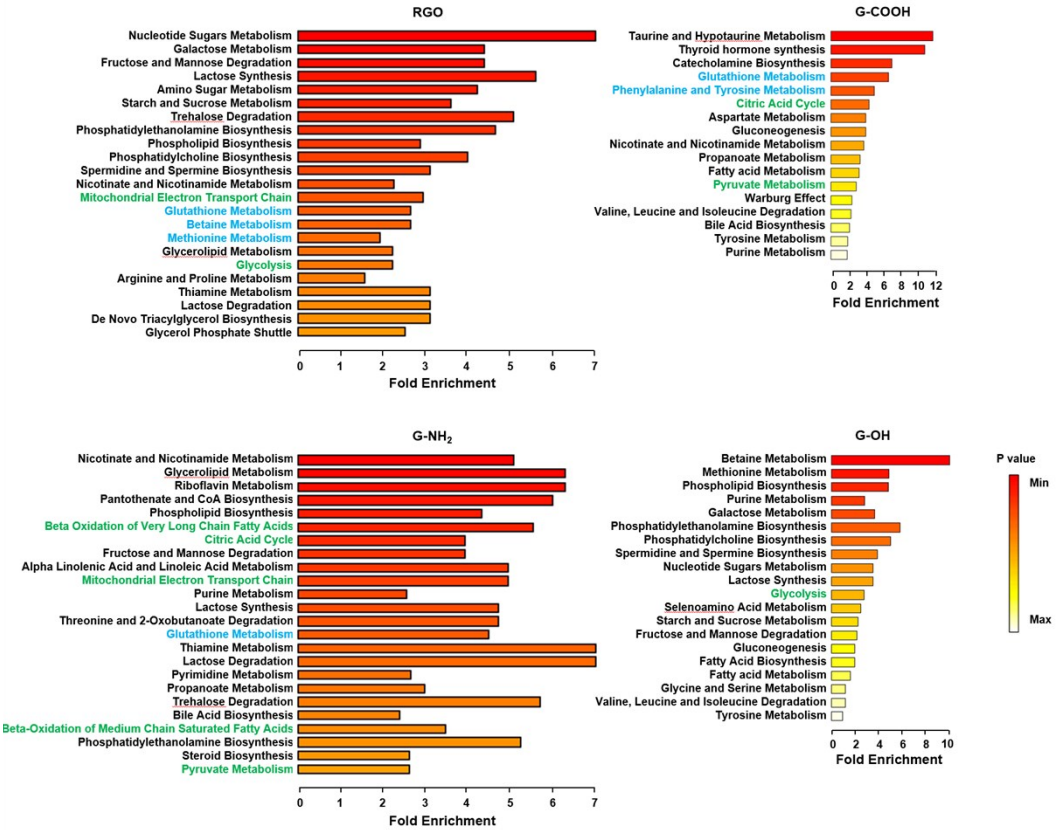

Figure S4. Metabolite set enrichment analysis of significantly changed metabolites after RGO (**A**), G-NH_2 (**B**), G-COOH (**C**), and G-OH (**D**) treatment of SK-N-SH cells.