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Supplemental Information

Identification of 14-3-3 epsilon as a regulator of the neural apoptotic pathway for chronic-stress-induced depression

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Supplementary Figures

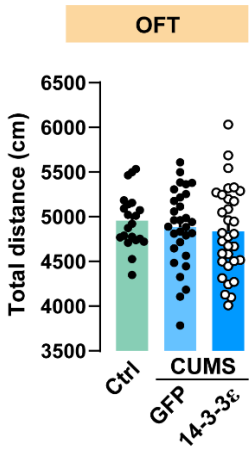

Figure S1. Total distance in open-field test (related to Figure 3).

No significant change in total distance travelled was found in the GFP and 14-3-3ε overexpressing mice, suggesting unchanged locomotion activity. Data represents mean±SEM.

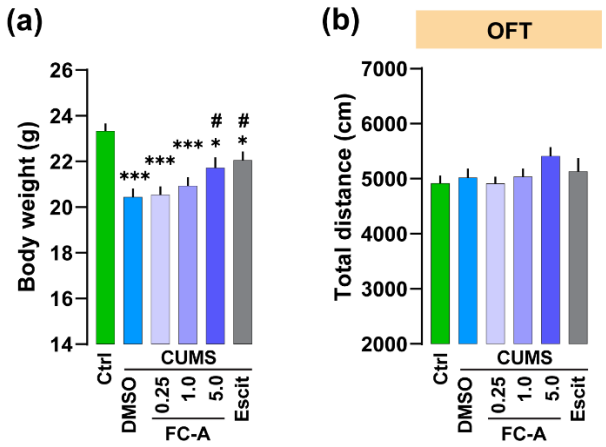

Figure S2. Effect of FC-A treatment on body weight and locomotion in CUMS-exposed mice (related to Figure 4).

(**a**) CUMS-induced weight loss was significantly rescued by 5.0 mg/kg FC-A treatment (post hoc, FC-A 5.0 mg/kg vs. DMSO, $t_{136} = 3.11$, $P < 0.05$). (**b**) No difference in total distance travelled was found between FC-A treatment groups. Data represents mean±SEM. *$P < 0.05$, ***$P < 0.0001$ compared with the Ctrl group; #$P < 0.05$ compared with the DMSO group.

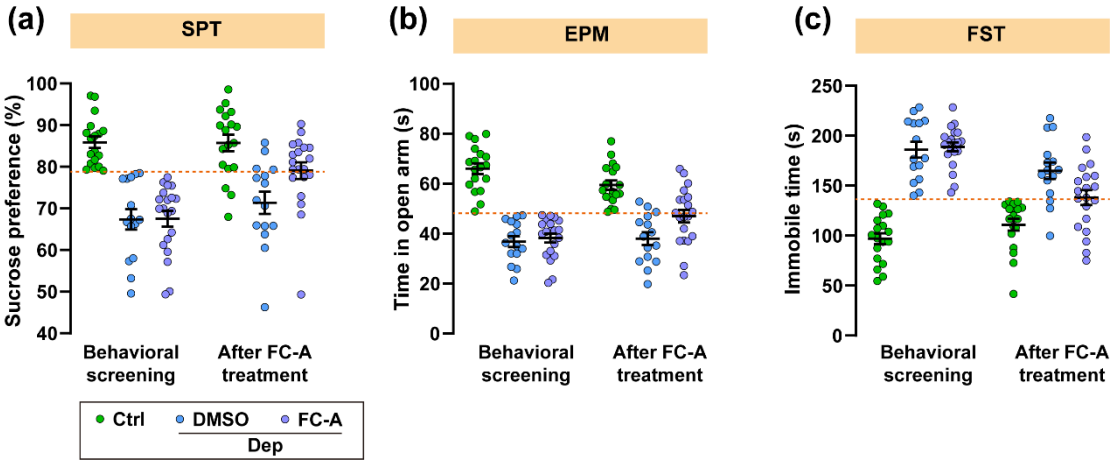

Figure S3. No learning effect of the testing was found between the behavioral screening and FC-A treatment (related to Figure 4).

(**a**) Sucrose preference test. Post hoc test, screening vs. post-treatment, Ctrl: $P = 0.9998$, DMSO: $P = 0.4908$, FC-A: $P = 0.0002$. (**b**) Time in open arms in elevated plus-maze. Post hoc test, screening vs. post-treatment, Ctrl: $P = 0.1267$, DMSO: $P = 0.9804$, FC-A: $P = 0.0163$. (**c**) Immobile time in forced swim test. Post hoc test, screening vs. post-treatment, Ctrl: $P = 0.291$, DMSO: $P = 0.0908$, FC-A: $P < 0.0001$. Data were analyzed by two-way RM ANOVA followed by Sidak's multiple comparisons test. Data represent means±SEM.

Supplementary Tables

Table S2 (related to Figure 2)

Pathway enrichment for the differentially expressed proteins.

	Pathway ID	Pathway name	Proteins	Protein number	*P*-value	Enrichment score
1	ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	Q3TYT1	1	0.00354	27.805
2	ko04060	Cytokine-cytokine receptor interaction	Q9EPU5	1	0.00613	15.889
3	ko04614	Renin-angiotensin system	Q9CYN9	1	0.00613	15.888
4	ko05010	Alzheimer's disease	P0DN34, Q61337, Q547J4	3	0.01314	2.648
5	ko04722	Neurotrophin signalling pathway	Q61337, Q3UHE3	2	0.01654	3.587
6	ko05034	Alcoholism	Q3UVW5, Q3UHE3	2	0.02416	3.271
7	ko04610	Complement and coagulation cascades	Q00898	1	0.04054	5.561
8	ko05030	Cocaine addiction	Q3UVW5	1	0.04194	4.835
9	ko04010	MAPK signalling pathway	Q547J4, Q3UHE3	2	0.04330	2.118

Transparent Methods

Animals

Adult C57BL/6J mice (male, 20-22 g, 8 weeks) were obtained from the animal centre at Xi'an Jiaotong University and habituated for 1 week. Mice were housed individually in a humidity- (50 ± 5%) and temperature-controlled (22 ± 3°C) room, with access to food and water *ad libitum*, except when animals were subjected to light disturbance or deprivation stressors during the CUMS procedure. They were allowed to acclimatize for 5 days before the experiments. All training and testing sessions were conducted during the light phase (lights on from 7:00 to 19:00). The experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. All efforts were made to minimize the number of animals used and their suffering.

CUMS protocol

Mice in the CUMS group was exposed to 8 weeks of consecutive CUMS, while mice in the control group received only regular handling. A mice CUMS protocol was used as an animal model of depression on the basis of well-established methods with minor modifications (Wang et al., 2010; Willner et al., 1987). Briefly, after 5 days of initial habituation, mice were subjected to various unpredictable mild stressors for a period of 8 weeks. Weekly stress consisted of the following stressors in a random order: 45° cage tilting, wet bedding, fasting, water deprivation, swimming in 4°C cold water, swimming in 40°C hot water, nip tail, shaking, paired housing and inversion of the light/dark cycle. On average, two different stressors were applied per day. At the end of every week, the body weight and sucrose preference of all mice was assessed. The behavioral tests were carried out in the final week. The control group was left unchallenged except for 14 hr food and water deprivation before sucrose preference test.

Behavioral tests

All behavioral tests were conducted in a testing room which was dimly lit by a lamp with luminosity between 20 and 30 lux. Each animal was allowed 15-min acclimation before the behavioral tests. The behavioral tests including sucrose preference (SPT), elevated plus maze (EPM), forced swim test (FST) and open-field test. The testing order was alternated across the CUMS and control group.

Sucrose preference test (SPT)

Each animal was provided with two drinking tubes in their home cages during the 24 hr training phase. After training, mice were deprived of water for 24 hr, then the mice were given the choice to drink from two bottles for 12 hr: one was filled with a sucrose solution (1% w/v), and the other was filled with water. The positions of the bottles in the cage were switched after 6 hr. Sucrose and water consumptions were recorded before and after the test. Sucrose preference% = (sucrose intake/total intake) × 100%.

Elevated plus maze (EPM)

The EPM test consisted of a plus shaped platform with four 33 × 6 cm plates connected to a central platform (6 × 6 cm). Two opposing close arms were enclosed by 14 cm-high walls, while two open arms were not enclosed. Mice were placed individually in the center of the apparatus for 10 min, and the time spend in the open arm were recorded to detect the anxiety of mice by the Anymaze video tracking software.

Forced swim test (FST)

Mice were individually placed in a transparent acrylic cylinder (height 30 cm, diameter 15 cm) for 6 min, which was filled with tap water (temperature 24 ± 1°C) to a depth of 20 cm. The water was replaced for each mouse. Only the last 4 minutes were analyzed due to the fact that most mice are very active in the first 2 minutes of the test. Mice performances were recorded and analysed using the Anymaze video tracking software. Immobility time was calculated as floating or no active movements except that necessary for the mouse to keep its head above water.

Open-field test (OFT)

A black square arena (45 × 45 × 30 cm) was used to examine locomotor activity. Mice were placed in the arena and allowed to explore the apparatus freely for 15 min. Total distance was analysed by the Anymaze video tracking software (Ver. 5.2, Stoelting Co., Wood Dale, IL, USA).

iTRAQ labelling and high pH reversed-phase (RP) fractionation

The mice of the depressed group (Dep, n = 9) and the control group (Ctrl, n = 9) were randomly chosen and sacrificed by decapitation immediately after the final behavioral testing. The VLO tissues from 9 mice of each group were dissected and pooled into 3 different biological replicates. The liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed in GeneChem Co. (Shanghai, China).

The mice were sacrificed by decapitation immediately after the final behavioral testing. The whole brain of each animal was rapidly removed. VLO tissue was dissected and stored in liquid nitrogen before storing frozen at -80°C. Samples from the CUMS group (n = 9) and the control group (n = 9) were homogenized in SDT buffer (4% SDS, 100 mM Tris-HCl, pH 7.6) and then boiled for 10 min. After centrifugation at 14,000 × *g* at 4°C for 15 min, the supernatant was obtained through a 0.22 μm filter. The proteins were quantified with the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA). For iTRAQ labelling, 3 pooled samples were obtained corresponding to 9 mice in each group, then each pooled sample was divided into two equal parts as biological replicates afterward.

Pooled samples was digested using the filter-aided sample preparation method (FASP) in a 30-kDa molecular weight cut-off centrifugation filter (Sartorius, Göttingen, Germany) (Wisniewski et al., 2009). The protein was diluted with 200 μL of UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.5) and then centrifuged at 12,500 × *g* for 25 min. After a repetition of this step, 100 μL of 100 mM iodoacetamide (IAA) in UA buffer was added to the filters, and the samples were incubated in darkness for 30 min. Filters were washed twice with 100 μL of UA buffer followed by two washes with 100 μL of dissolution buffer (50 mM

triethylammonium bicarbonate, pH 8.5). Proteins were digested for 18 hr in a 40 µL dissolution buffer (containing 4 µL trypsin) (Promega Co., Madison, WI, USA) at an enzyme-to-protein ratio of 1:50 at 37°C. The released peptides were collected by centrifugation at 12,500 × *g* for 15 min followed by two washes with dissolution buffer and then and quantified by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Next, 100 µg of peptides from each pooled sample were labelled using the iTRAQ Reagent-8plex Multiplex Kit (AB Sciex, Redwood city, CA, USA), according to the manufacturer's instructions. Labelled peptides were mixed at equal ratios and fractionated by the Agilent 1260 infinity II HPLC system. Afterward, approximately 36 fractions were collected from each test.

LC-MS/MS and data analysis

The liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed in GeneChem Co. (Shanghai, China). A Thermo Easy-nLC binary buffer system (Thermo Fisher Scientific, Waltham, MA, USA) with buffer A (0.1% formic acid) and buffer B (0.1% formic acid in 80% acetonitrile) was performed for this experiment. Samples were first loaded to C18-RP columns (4.6 µm × 100 mm, 5 µm) before preparing analytical columns (50 µm ×150 mm, 3 µm). Peptide mixtures were isolated at a flow rate of 300 nL/min. Related liquid phase gradient as follows: 0-5 min, buffer B from 0 to 6%; 5-45 min, buffer B from 6 to 28%; 45-50 min, buffer B from 28 to 38%; 50-55 min, buffer B from 38 to 100%; and 55-60 min, buffer B remained 100%. Peptides eluted by HPLC were directly injected into the Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The mass range of positive ion mode was 350-1800 m/z. Survey scans were obtained at 70,000 (200 m/z) and 17,500 (200 m/z), with the resolution for higher-energy collisional dissociation (HCD) spectra and maximum ion injection times fixed at 50 and 45 ms, respectively. The top ten abundant precursor ions were used for MS/MS analysis with a normalized collision energy at 30 eV, and the underfill ratio defined as 0.1%.

Protein identifications were performed using the MASCOT search engine (version 2.6. Matrix Science, Boston, MA, USA) embedded into Proteome Discoverer 2.1 (Thermo Fisher Scientific, Waltham, MA, USA). A publicly available Uniprot_MusMusculus_84433_20180123 database was used for the search. The searching parameters were: enzyme, trypsin; max missed cleavages, 2; precursor mass tolerance, ± 20 ppm; fragment mass tolerance, 0.1 Da; modification groups, iTRAQ 8 plex; dynamic modification, oxidation (M), acetyl (protein N-term) and deamidated (NQ); static modifications, carbamidomethyl (C); database pattern, decoy. Furthermore, parameters of proteins quantification were set as follows: normalize on total peptide amount, use of only unique peptides and the median protein ratio should be 1 after normalization.

Bioinformatics analysis

A strict cutoff for protein identification was applied with false discovery rate (FDR) < 1% to minimize false positive results. Protein identification was supported by all peptide matches with 95% confidence (Han et al., 2015). Since iTRAQ is fold change sensitive, we filtered the results by the fold change (> 1.20 or < 0.84) in comparison between depressed and control groups and manually examined all proteins (Ren et al., 2013), accepting the final

list of differentially expressed proteins (122 out of 4953 identified proteins). Student's t-test *P* values of the differentially expressed proteins was corrected and ranked by using the approach reported by Benjamini, Krieger and Yekutieli (Benjamini, 2006). To estimate the final FDR of the accepted proteins, we performed permutation analysis (n = 1,000 permutations) following a reported procedure (Xie et al., 2005) and found that the estimated FDR below 12%. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was subsequently performed using the KEGG automatic annotation server (KAAS) (Moriya et al., 2007). Fisher's exact test *P* value < 0.05 was performed to analyze enrichment of the pathways. The capability of the resulting feature proteins in differentiating both sample groups was evaluated by hierarchical clustering analysis using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and Java Treeview (http://jtreeview.sourceforge.net) software (Pan et al., 2015). Additionally, the differentially expressed proteins were sent to the IntAct (http://www.ebi.ac.uk/intact/main.xhtml) to build functional protein association networks, which were further analysed by Cytoscape software (version 3.2.1).

Immunoblotting

The VLO tissues were then processed for protein extraction as described before (Wang et al., 2017). For verification of the proteomic results, Western blotting was performed with the same protein samples that used for iTRAQ quantitative analysis. Protein samples were separated by 10% or 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% BSA and then incubated with different primary antibodies (see supplementary materials) overnight at 4°C. Then the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. An enhanced chemiluminescence kit (Merck Millipore, Billerica, MA, USA) was used to detect immunoreactive protein bands. Band intensities were analyzed using ImageLab software (Bio-Rad Laboratories, Hercules, CA, USA). Protein expression levels were normalized to the GAPDH expression levels and the data were presented as relative quantifications.

Stereotaxic surgery and virus injection

The recombinant adenovirus associated virus (rAAV)-enhanced green fluorescent protein (eGFP) constructs containing full-length human 14-3-3ε expressing were generated by OBio Technology (Shanghai, China). To create the rAAV-14-3-3ε vector, the DNA sequence corresponding to wildtype human 14-3-3ε at the C-terminal end was subcloned from the pcDNA3.1-14-3-3ε vector into the multiple cloning site (MCS) of the AAV9-CaMKIIa-bGlobin-MCS-eGFP-3FLAG vector. The integrity of the rAAV-14-3-3ε construct was verified by DNA sequencing analysis, and the 14-3-3 constructs were identical to NCBI sequences U28936 (14-3-3ε). The titer of the virus was approximately 10^{12}, measured by quantitative PCR. Twenty-eight days after virus injection, the in vivo efficacy of rAAV-mediated 14-3-3ε overexpression was approximately 2.1-fold relative to the vector control group. For the virus injection, mice were anesthetized with isoflurane (4% in O_2 for induction, 1% in O_2 for maintenance) and secured in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Pulled capillary tubes were inserted into the bilateral VLO (AP: +2.4,

ML: ± 1.2, DV: −2.6). Then, 1 μL of virus was infused bilaterally at a flow rate of 0.5 μL/min. The injector was left in place for an additional 15 min to minimize diffusion up the injector tract.

Co-immunoprecipitation (co-IP)

VLO samples were homogenized in lysis buffer (50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 10 mM Tris, 5 mM EDTA, 0.1 mM Na_3VO_4, 1 mM PMSF, with 1% Triton X-100 and protease inhibitor tablet). Lysates were centrifuged (12,000 g) at 4°C for 15 min. Supernatant fraction was incubated with anti-14-3-3ε mAb (Abcam, Cambridge, MA, USA) (negative controls used the antibody against mouse IgG or no antibody), followed by incubation with 50 μL of protein A/G plus agarose (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hr at 4°C. The beads were washed three times with lysis buffer, then boiled in 2×SDS loading buffer for 5 min. Samples were resolved by SDS-PAGE and transferred to PVDF membrane followed by immunoblotting using anti-14-3-3ε (1:500) and anti-Bad (1:1000), respectively.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and DAPI staining

Frozen brain tissues were embedded with OCT compound (Leica, Wetzlar, Germany). Tissues were cut into 20-μm thick sections and analyzed with the TUNEL apoptosis assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol with minor modifications. Briefly, sections were fixed with 4% paraformaldehyde for 20 min at room temperature. Then, they were washed twice for 10 min in PBS and permeabilized in PBS with 0.3% Triton X-100 for 5 min in 37°C. Sections were then incubated with TUNEL mixed detection solution at 37°C in a dark and humid environment for 1 hr followed by three 10-min rinses with PBS. Finally, sections were incubated with DAPI staining solution for 5 min at room temperature (Beyotime Biotechnology, Shanghai, China). DAPI staining was detected with a DAPI filter, and TUNEL staining was detected with a FITC filter. The sections were observed under a fluorescence microscope (Nikon, Chiyoda, Japan) and the results were calculated by Image-Pro Plus 6.0 software.

Experimental design

Experiment 1: Mice in the CUMS group (n= 60) was exposed to 8 weeks of consecutive CUMS, while mice in the control group (Ctrl, n= 20) received only regular handling. At the end of CUMS regime, behavioral tests including SPT, EPM and FST were carried out to assess the depressive phenotypes in these mice. Mice in the CUMS group were further classified as depressed (Dep) or undepressed according to the cutoff values based on ROC curves. The undepressed mice were excluded from subsequent study. The Dep mice and Ctrl mice were sacrificed immediately after the final behavioral testing. The VLO tissue was extracted for subsequent use.

Experiment 2: For iTRAQ proteomics, we randomly selected VLO samples from Dep and Ctrl group (n = 9/group) to pool into 3 different biological replicates. Following the bioinformatics analysis, we performed Western blots to verify the expression levels of the differentially expressed proteins and other proteins of interest by using the same samples

from Experiment 1.

Experiment 3: A new cohort of mice were stereotaxically injected with virus that overexpressed 14-3-3ε (n = 32) or the GFP virus (n = 30) into bilateral VLO. After 2 weeks of recovery, these mice were exposed to CUMS regime, while mice in the Ctrl group (n= 21) received only regular handling. At the end of CUMS, the behavioral tests were carried out to assess the depressive phenotype. Molecular changes in VLO were determined by Western blot and co-IP.

Experiment 4: A new cohort of mice were treated with different dose of FC-A (0.25, 1.0 and 5.0 mg/kg body weight, i.p., once per 2 days; n = 22-27 per group) during the whole CUMS period. Escitalopram (Escit) was given once daily (20 mg/kg, i.p.; n = 22) as the positive control. An equal volume of 1% DMSO was given i.p. as vehicle control (n = 24). Ctrl group received only regular handling (n= 25). At the end of CUMS regime, the behavioral tests include FST, EPM and SPT were carried out to assess the changes in depressive phenotypes. The apoptosis in VLO were determined by TUNEL staining. The apoptotic markers were determined by Western blot. In addition, another cohort of mice were exposed to CUMS regime and were screened for depressive phenotype (Dep: n = 35/92; Ctrl: n = 18/40). Then, Dep mice were treated with FC-A (5.0 mg/kg/day, i.p.; n = 20 per group) or 1% DMSO (n = 15) for 7 days. After the treatment, depressive-like behaviours were assessed by SPT, EPM and FST. Moreover, SPT were assessed at the 7, 14, 21 and 28 days after the FC-A treatment.

Statistical analysis

To determine whether individual animals were positive or negative for a specific behavioral phenotype as measured in the SPT, EPM and FST, we used ROC curves(Zou et al., 2007). Youden J Index was calculated from ROC curves in order to identify the optimal cutoff value that gives the lowest false positive rate (FPR) and the highest true positive rate (TPR). Youden J index maximizes the difference between TPR (sensitivity) and FPR (1−specificity): Youden J Index = TPR−FPR = sensitivity+specificity−1. Thus, by maximizing (sensitivity + specificity) across various cutoff points, the optimal cutoff point was calculated.

In order to determine statistical differences for behavioral, histological and immunoblot data, we performed unpaired Student's t-test, Fisher's exact test, and one- and two-way ANOVA (ordinary and repeated measures) using Graphpad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). Tukey's or Sidak's post hoc analysis was applied, when applicable, to correct for multiple comparisons. Statistical significance was $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. All data are presented as means ± SEM. Investigators were blinded to allocation of groups and outcome assessment for all experiments. Statistical details can be found in the supplementary materials.

Supplementary references

Benjamini, Y., Krieger, A. M., Yekutieli, D. (2006). Adaptive linear step-up procedures that control the false discovery rate. Biometrika *93*, 491–507.

Han, X., Shao, W., Liu, Z., Fan, S., Yu, J., Chen, J., Qiao, R., Zhou, J., and Xie, P. (2015). iTRAQ-based quantitative analysis of hippocampal postsynaptic density-associated proteins in a rat chronic mild stress model of depression. Neuroscience *298*, 220-292.

Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A.C., and Kanehisa, M. (2007). KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res *35*, W182-185.

Pan, H.T., Guo, M.X., Xiong, Y.M., Ren, J., Zhang, J.Y., Gao, Q., Ke, Z.H., Xu, G.F., Tan, Y.J., Sheng, J.Z*., et al.* (2015). Differential proteomic analysis of umbilical artery tissue from preeclampsia patients, using iTRAQ isobaric tags and 2D nano LC-MS/MS. J Proteomics *112*, 262-273.

Ren, Y., Hao, P., Dutta, B., Cheow, E.S., Sim, K.H., Gan, C.S., Lim, S.K., and Sze, S.K. (2013). Hypoxia modulates A431 cellular pathways association to tumor radioresistance and enhanced migration revealed by comprehensive proteomic and functional studies. Mol Cell Proteomics *12*, 485-498.

Wang, W., Sun, D., Pan, B., Roberts, C.J., Sun, X., Hillard, C.J., and Liu, Q.S. (2010). Deficiency in endocannabinoid signaling in the nucleus accumbens induced by chronic unpredictable stress. Neuropsychopharmacology *35*, 2249-2261.

Wang, Y., Yin, F., Guo, H., Zhang, J., Yan, P., and Lai, J. (2017). The Role of Dopamine D1 and D3 Receptors in N-Methyl-D-Aspartate (NMDA)/GlycineB Site-Regulated Complex Cognitive Behaviors following Repeated Morphine Administration. Int J Neuropsychopharmacol *20*, 562-574.

Willner, P., Towell, A., Sampson, D., Sophokleous, S., and Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology (Berl) *93*, 358-364.

Wisniewski, J.R., Zougman, A., and Mann, M. (2009). Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. J Proteome Res *8*, 5674-5678.

Xie, Y., Pan, W., and Khodursky, A.B. (2005). A note on using permutation-based false discovery rate estimates to compare different analysis methods for microarray data. Bioinformatics *21*, 4280-4288.

Zou, K.H., O'Malley, A.J., and Mauri, L. (2007). Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. Circulation *115*, 654-657.