SUPPLEMENTARY INFORMATION

Manganese Neurotoxicity: Nano-oxide Compensates the Ion-damage in Mammals

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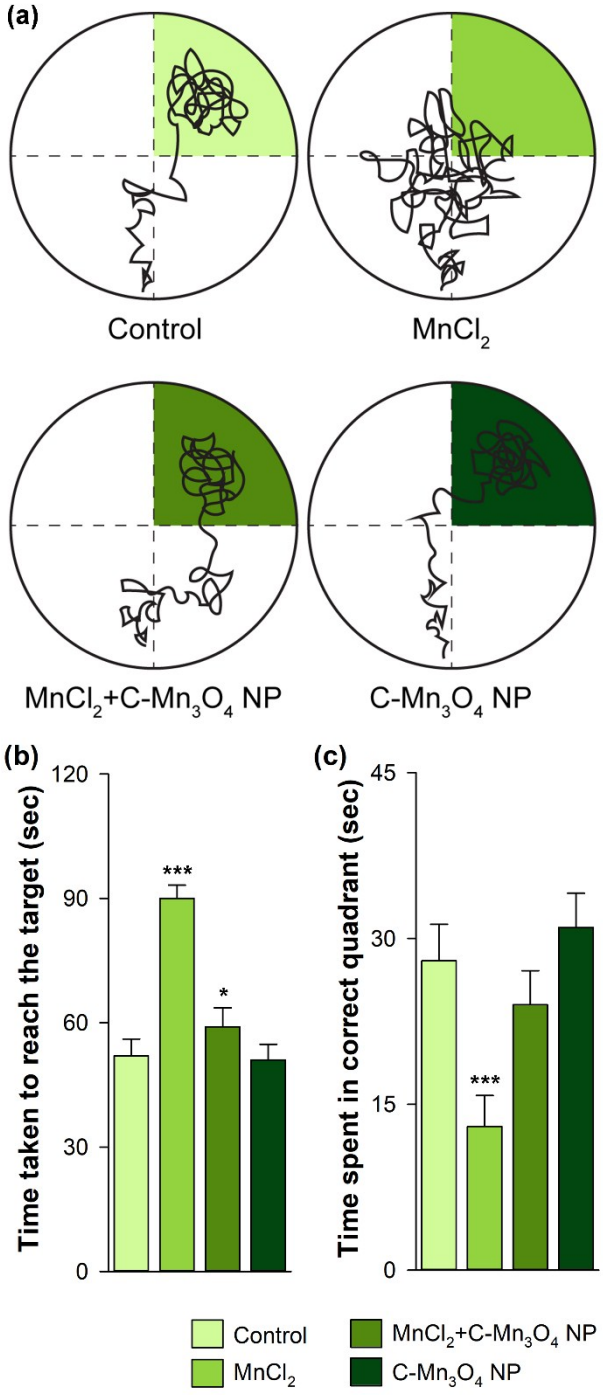
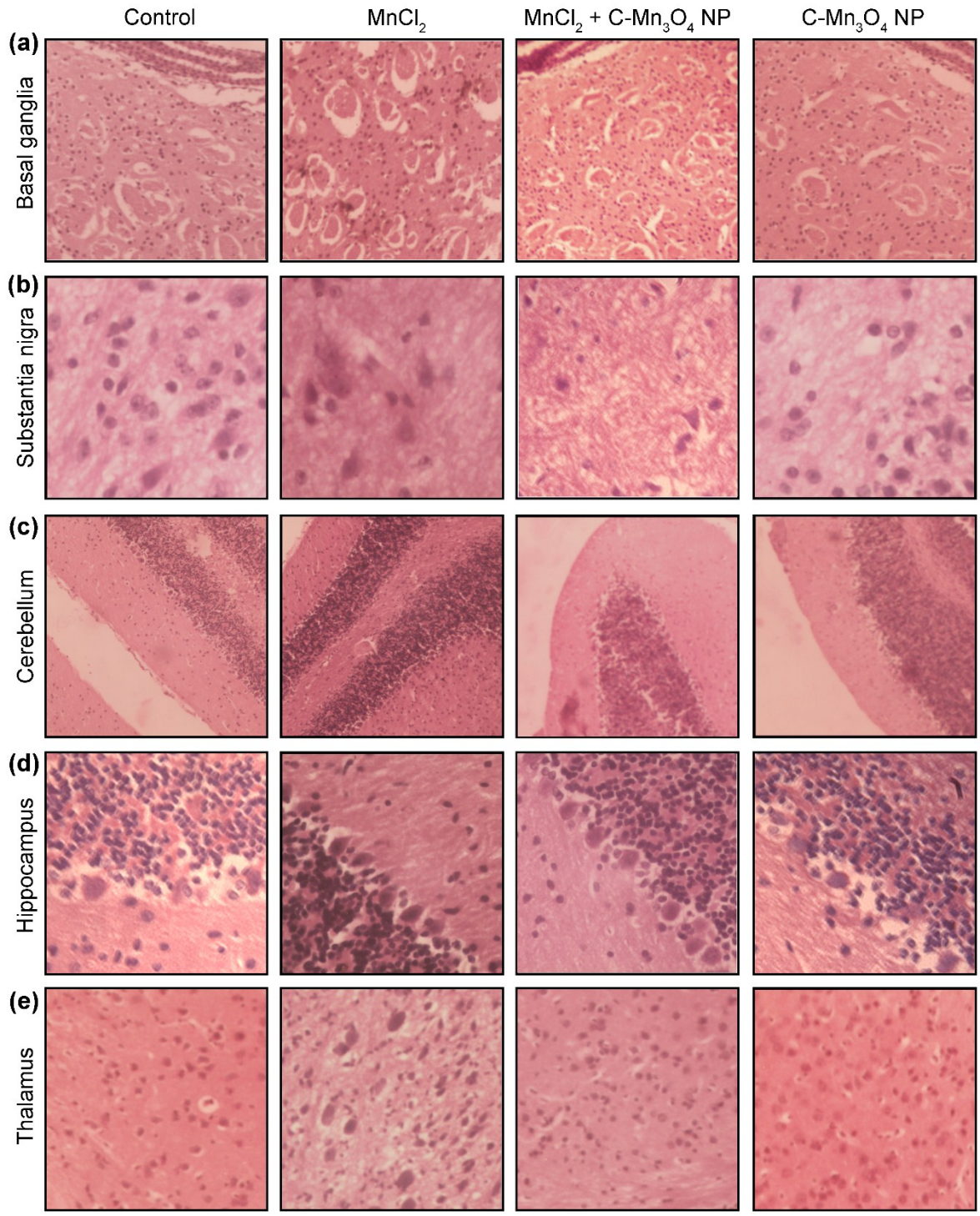
SUPPLEMENTARY FIGURES



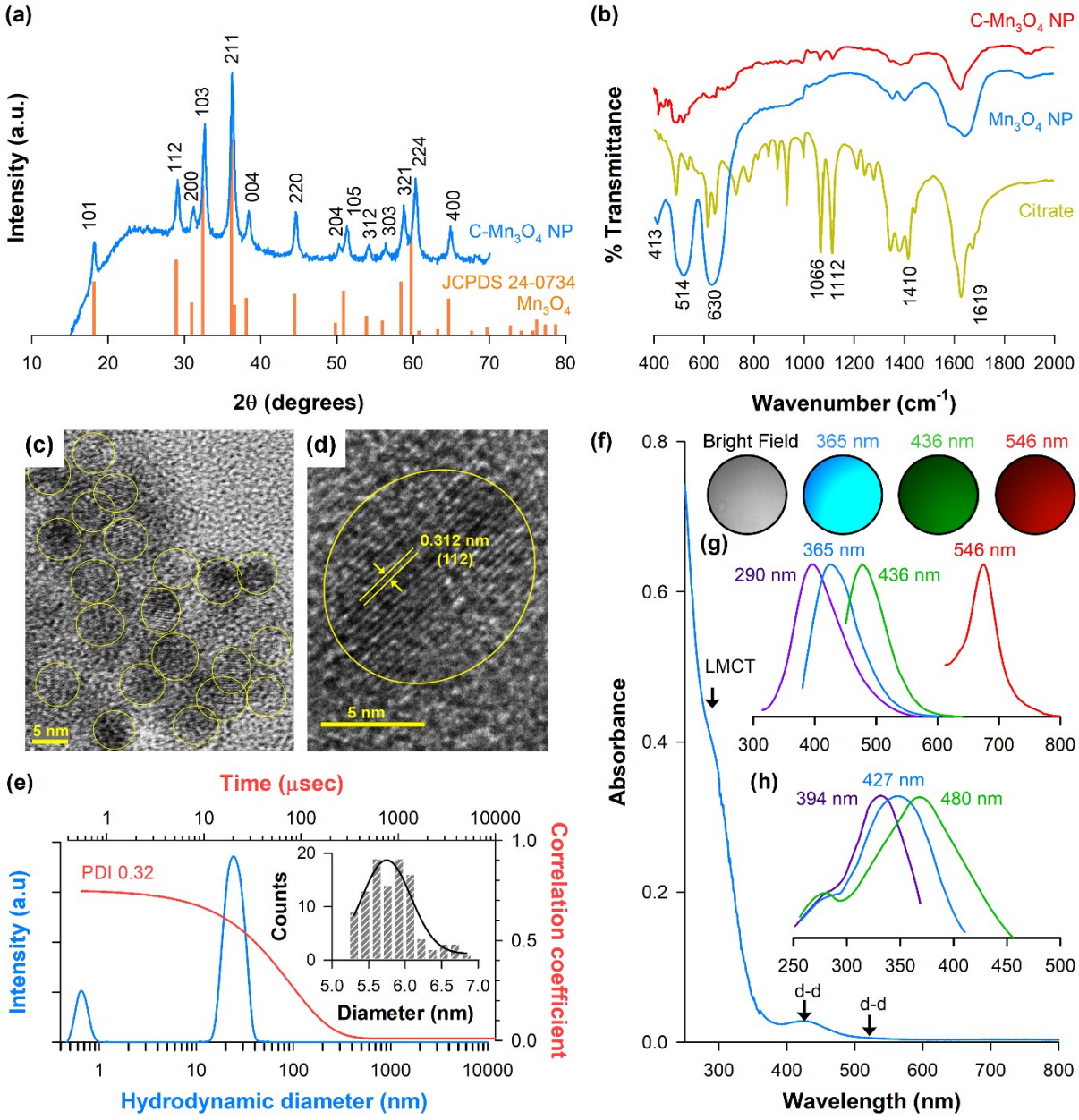
Supplementary Figure S1. Effect of C-Mn_3O_4 NPs on hippocampus dependent spatial learning as measured by Morris water maze test. (a) Representative swimming paths in the Morris water maze. The colored part depicts the target quadrant. (b) Time taken to reach the target. (c) Time spent in the target quadrant.Data are expressed as Mean ± SD. *N*=6.

*, **, *** Values differ significantly from control group (without treatment) (***p < 0.001; **p < 0.01; *p < 0.05).

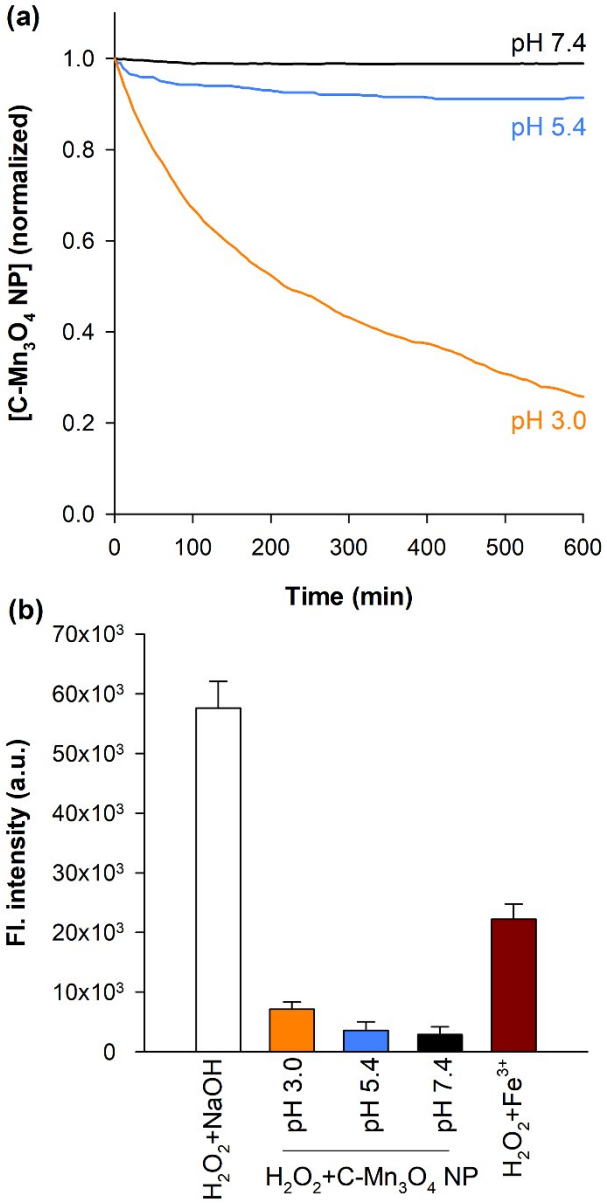


Supplementary Figure S2. Effect of C-Mn_3O_4 NPs on histological changes at different brain regions caused by $MnCl_2$. Control and C-Mn_3O_4 NP treated groups showed normal cellular architecture. $MnCl_2$ treated sections (particularly the basal ganglia region) showed marked apoptosis with degenerated and pyknotic nuclei. Sections from the cerebellum region shows cortical atrophy, degeneration of Purkinje neurones and small shrunken cells. Co-treatment with C-Mn_3O_4 NPs ameliorated the changes.

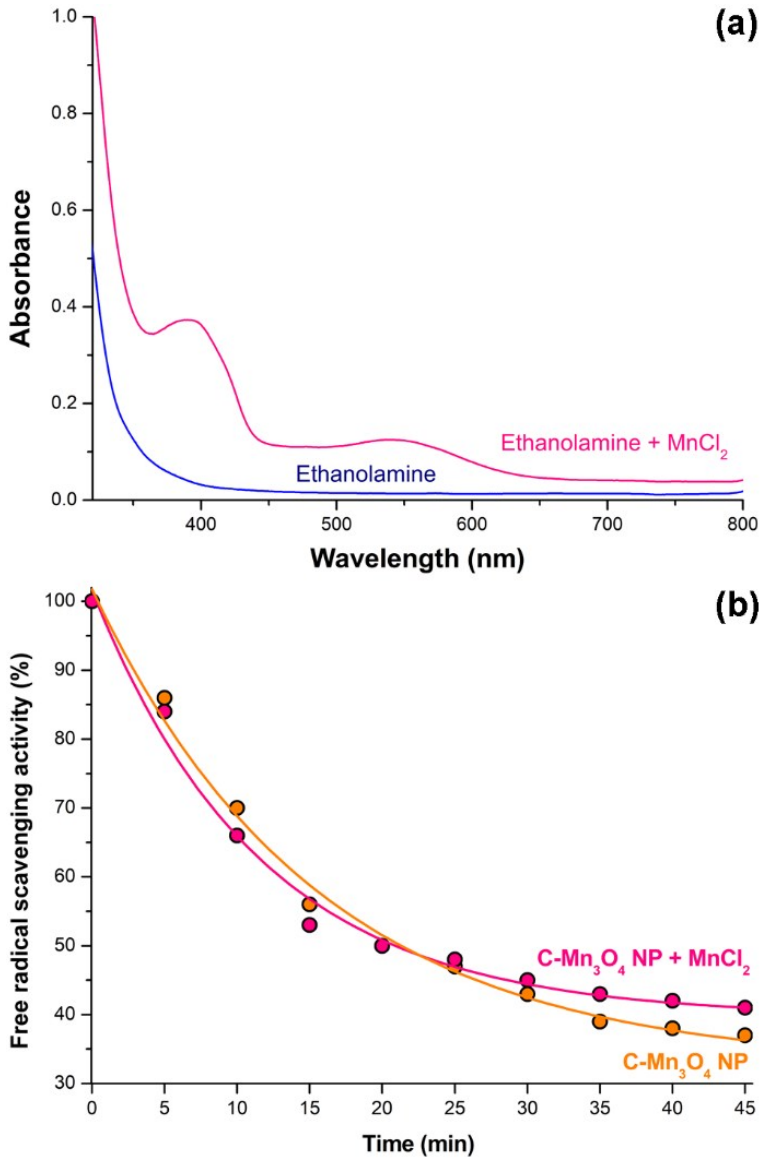
All sections are stained with haematoxylin and eosin.



Supplementary Figure S3. Characterization of C-Mn_3O_4 NPs. (a) XRD spectra of C-Mn_3O_4 NPs. (b) FTIR spectra shows covalent binding of ligand citrate to as prepared Mn_3O_4 NPs. (c) TEM of C-Mn_3O_4 NPs. (d) HRTEM reveals the fringe distance to be 0.312 nm corresponding to (112) crystal lattice of the C-Mn_3O_4 NP. (e) Hydrodynamic diameter as measured by DLS. Inset shows the size distribution of C-Mn_3O_4 NPs as calculated from TEM analysis. (f) UV-visible absorbance spectra of C-Mn_3O_4 NPs. (g) Fluorescence emission and (h) excitation spectra of C-Mn_3O_4 NPs. The images were acquired using fluorescence microscope at different excitation wavelengths.



Supplementary Figure S4. Effect of pH on dissolution of Mn ions from C-Mn_3O_4 NPs and their effect on *in vitro* Fenton like reaction. (a) Dissolution profile of Mn_3O_4 NPs at different pH. (b) Chemiluminiscence of luminol as a marker of OH• free radical.



Supplementary Figure S5. Effect of Mn-chelation on antioxidant activity of the nanoparticle. (a) Absorbance spectra of $MnCl_2$ in ethanolamine displays prominent d-d transition bands. (b) No change in free radical scavenging activity was observed after formation of C-Mn_3O_4 NP-Mn^{2+} chelator complex, as evident from DPPH radical scavenging experiments.

SUPPLEMENTARY MATERIAL AND METHODS

Synthesis of citrate functionalized Mn_3O_4 nanoparticles. For synthesis of bulk Mn_3O_4 nanoparticles at standard temperature and pressure we followed a bottom up approach. In a typical procedure, 0.598 g of $MnCl_2$, $4H_2O$ (3 mmol) was added to 30 ml of ethanol amine (EA) in a beaker, and ultrasonicated at 56 kHz operating frequency for 15 mins. This dissolves $MnCl_2$ to form a clear brown solution. Then equal amount of Mili-Q (from Millipore) water (30 ml) was added and the resultant mixture was stirred at room temperature for 6 hrs. Then the suspension was centrifuged at 3000 rpm for 15 minutes, and the black precipitate was subsequently washed three times using ethanol in order to remove excess EA. After that it was dried in an incubator at 60°C to get a glossy black powder, the as prepared Mn_3O_4 NPs.

To functionalize the as-prepared Mn_3O_4 NPs with ligand citrate, at first, 0.5 M citrate solution (pH 7.0) was prepared in Milli-Q water. In the ligand solution, as-prepared Mn_3O_4 NPs (~150 mg of powder Mn_3O_4 NPs in 6 ml ligand solution) were added and extensively mixed for 10 hrs in a cyclomixer. Finally, we filtered out the nonfunctionalized large NPs using a syringe filter (0.22 μm diameter). The resulting filtrated solutions (after proper dilution) were used in further experiments.

Characterization of C-Mn_3O_4 NPs. For physicochemical characterization of the NPs various microscopic and spectroscopic studies were performed. For preparation of TEM samples, C-Mn_3O_4 NPs were drop casted onto a 300-mesh carbon-coated copper grid and airdried for 12 hrs. TEM micrographs were documented using a FEI TecnaiTF-20 field emission high-resolution transmission electron microscope (FEG-HRTEM) operating at 200 kV. XRD patterns was obtained by employing a scanning rate of 0.02° s^{-1} in the 2θ range from 10° to 80° by PAN analytical XPERT-PRO diffractometer equipped with Cu Kα radiation (at 40 mA and 40 kV). Optical absorbance spectra of the solutions were recorded using a quartz cuvette of 1 cm path length in Shimadzu Model UV-2600 spectrophotometer. For the fluorescence imaging study of liquid NPs, Olympus BX51 fluorescence microscope was used. Jobin Yvon Model Fluoromax-3 Fluorimeter was used to study the characteristic fluorescence excitation and PL spectra of Mn_3O_4 NP solutions. XPS measurements were carried out using an Omicron Nanotechnology instrument equipped with seven channeltrons with a binding energy resolution of 0.1 eV. XPS was performed using monochromatic Al Ka X-rays (1486.6 eV). The X-ray source was at 15 keV with the emission current of the filament of 20 mA. All measurements were done under ultrahigh vacuum conditions of 5×10^{-10} mbar. Electron flooding was employed for sample charging compensation during the measurements. The binding energies were calibrated with respect to an adventitious C 1s feature at 284.6 eV and the internal oxygen peak. XPS spectra were deconvoluted to their individual components using a Gaussian Lorentzian function after background subtraction with a Shirley function using Origin Pro software (v8.5, OriginLab Corporation, MA, USA).

Animals. C57BL/6j mice of both sexes (6–8 weeks, weighing 19–21 g) were used for the current study. Four or five mice were housed together in polypropylene cage under a 12:12 light-dark cycle. During the time, food and water were accessible *ad libitum*. Mice were procured from National Institute of Nutrition, Hyderabad, India. All animal studies and experimental procedures were performed at animal house of Uluberia College, West Bengal,

India (Reg. No.- 2057/GO/ReRcBi/S/19/CPCSEA) following the protocol approved by the Institutional Animal Ethics Committee (Ethical Clearance No. - 02/S/UC-IAEC/01/2019) as per standard guideline of CPCSEA, New Delhi, India.

Treatment. The treatments were as follows,

Group 1: Control (Normal saline 28 days)
Group 2: $MnCl_2$ treated (10 mg Mn kg^{-1} body weight (BW) for 28 days)
Group 3: $MnCl_2$ (10 mg Mn kg^{-1} body weight (BW) for 28 days) + C-Mn_3O_4 NPs (5 mg kg^{-1} BW for 21 days)
Group 4: C-Mn_3O_4 NPs (5 mg kg^{-1} BW for 28 days)

Serum isolation. At the end of the experimental period, the animals were euthanized and decapitated after being fasted. Blood was collected from retro orbital plexus just before sacrifice, kept in sterile non-heparinized tubes in slanting position for 45 min and centrifuged at 3500×g for 20 min. The clear serum was obtained and used in subsequent biochemical analysis.

Histopathological examination. For microscopic evaluation, a conventional technique of paraffin wax sectioning and differential staining was used [1]. Brains were removed following incision, fixed in 10% neutral buffered formalin saline for 72 h, dehydrated in graduated ethanol (50–100%), cleared in xylene, and embedded in paraffin. Microtome was used to prepare ultrathin sections (4–5 μm), followed by staining with hematoxylin and eosin (H&E) and silver stain. Histopathological changes were examined under the microscope (Olympus BX51) equipped with a CCD based camera.

Tissue homogenate preparation. Tissue samples were collected, homogenized in cold 0.1 mM phosphate buffered saline (PBS; pH 7.4), and centrifuged at 10,000 rpm at 4°C for 15 min. The supernatants were collected to determine the activity of SOD, CAT, GPx and GSH as well as the content of malondialdehyde (MDA).

Assessment of lipid peroxidation & antioxidant enzyme activity. The supernatants were used to determine the activity of SOD, CAT, GPx and GSH as well as the content of MDA. SOD, CAT and GSH activities were estimated using commercially available test kits (Sigma-Aldrich, MO, USA) following the protocols described by the manufacturer. To assess the extent of lipid peroxidation, the level of malonyldialdehyde (MDA), a substance that reacts with thiobarbituric acid, was determined in the homogenates of organs and in serum according to the method of Buege [2].

Behavioral assay: The selected animals were transferred to a single housed room for acclimatization 4 weeks prior to tests. All mice were subjected to sequential tests started with open field test then followed by forced swim test, nasal bridge removal, tail-flick, hind limb clasping reflex, beam walking, elevated plus maze, light preference, sucrose preference, novel object recognition and three chambered social approach. The tests were performed over a period of 6 weeks by the same observer who was not aware of the existing groups. In between two consecutive tests the animals were left undisturbed for 5-6 days. All tests were performed following the guideline of CPCSEA, India.

Open field test (OFT): This test measures thigmotaxis behavior of the subject which is the indicator of anxiety of the subject. For this test a box, divided into a center and peripheral zone, was used (36 cm X 34 cm X 26 cm). Each animal was placed into the box and allowed to explore the box for 5 minutes and an automated video camera was used to record its behavior. Time spent by the animal in the central zone and peripheral zone was measured to evaluate its anxiety level.

Forced Swim test: Individual mouse was placed into a glass beaker of diameter 16 cm and height of 26 cm. The apparatus was filled up to 15 cm with water of temperature 25ºC. Time spent by the animal in the water was recorded and immobility (refers to the minimal movement of the animal to keep its head over the water surface) of the animal was measured. An increased in immobility reflects increase in depression. After test all animals were dried properly and put into new cages.

Tail flick test: Tail-flick test is a nociceptive essay based on the measurement of the latency of the avoidance response to thermal stimulus in rodents. Basically, a thermal stimulus is applied to the tail; when the animal feels discomfort, it reacts by a sudden tail movement. The tail flick reaction time is then measured and used as an index of animal pain sensitivity [3].

Beam walking: This test is a useful way to access fine motor coordination and balance of rodents. Each mouse was placed at the end of a narrow beam of diameter 2.5 cm and 50 cm long which it had to cross. At the starting end an aversive stimuli, i.e. a brightly lit blub, was given and at the opposite end a platform was placed into darkness which was the goal for the animal. Each animal was evaluated by measuring the delay period before starting, the time to reach into the platform or fall or false start, the distance covered by the animal before falling from the beam and the number of paw slip. 3 additional trails were given to the successful mice in the interval of one minutes.

Elevated plus maze: Elevated plus maze is widely used for evaluating anxiety like behavior in the rodents. The test apparatus is made up of 4 arms two of which is closed and the other two is open. The apparatus is placed 50 cm above the ground level and the closed and open arms were perpendicularly crossed each other in a central area. All the arms were brightly lit. Closed arms were covered by wall of 15 cm and open arms had a barrier of 3 cm that prevent slipping of animal. Animals were scored depending on the entry to the open arm and time spent in it. This score reflect the measurement of anxiety that is induced in the animal by the open space.

Sucrose preference test. Consumption of a 2% sucrose solution between 08:00 am and 09:00 pm was recorded in all mice to measure sucrose anhedonia [4]. Before presentation of the sucrose solution, animals were administered water in modified water bottles for 3 consecutive days, to control for novelty of the bottles. The bottles were weighed before and after the 5-h sample time; the next day, animals were provided a choice between a 2% sucrose solution and water. Sucrose consumption was normalized to water consumption.

Novel object recognition. Two variations of the novel-object task were run. Both were conducted in an arena 36 cm long, 34 cm wide and 26 cm high. For both tests, the snouts of the mice were tracked and object interaction was measured as time spent with snout within 2

cm of the object. The objects (a plastic ball, a small rubber box and a small plastic cube) were secured to the arena with neodymium magnets to render them immovable. In the first variation, mice were habituated to the arena and objects 1 and 2 over the course of four 5-min trials separated by an inter-trial interval of 10 min. Mice were then tested for object recognition memory 1 h after the fourth trial during the 5-min-long fifth trial. Either object 1 or object 2 (counterbalanced) was swapped for object 3 during the fifth trial. In the second variation of this test, the mice were habituated to the empty arena for 10 min each day for three consecutive days. On day 4, the mice were exposed to a pair of either object 1 or object 2 for 5 min. Object recognition memory was tested 1 h after this trial by exposure to objects 1 and 2 for 5 min. In both protocols, object recognition memory was measured as the increased time spent investigating the novel object.

Morris water maze test. After acclimatization, animals from each of the groups were taken for the Morris water maze study to evaluate their spatial learning ability. The complete study consists of training period of 5 days and then test period. This training was done in a pool with a platform (target) whose top was lifted above the water surface. Distal cues were clearly visible for the animals at the time of training. The animals were placed in maze in a random fashions from 4 different start points. 10 days after training session animals were evaluate for their spatial learning ability. All the animals were placed from a fixed point and the platform were hidden beneath the water surface in this session. All the animals were given 90 sec for exploration. Time taken to reach the platform and no of time the animal entered in the target quadrant was analysis for accessing their spatial learning and memory that give insight to hippocampal damage.

Sociability test. This test was performed as described previously [5]. In brief, mice were placed in an arena divided into three equal-sized compartments by plastic mesh. On day 1 a 5-min sociability trial was conducted. A littermate was placed in the left or right compartment (systematically alternated) and the test subject was placed into the centre compartment. The time that the test subject spent investigating each compartment (snout within 2 cm of the mesh barrier) was measured, and a difference score was computed.

Mitochondria Isolation. Mitochondria isolation was done from mouse brain following the method of Graham[6] with some modifications. In brief, brains were excised and homogenized in brain homogenization medium that contains 250 mM D-mannitol, 125 mM sucrose, 0.05 mM EGTA, 0.01% BSA, 10 mM HEPES (pH 7.2), 1x protease inhibitors. Then the homogenates were centrifuged for 15 minutes at 700 x g and the supernatants were again centrifuged as the previous step. Then supernatant were washed collected and centrifuged at 10000 x g for 15 minutes. The resultant pellets were dissolved in ice cold buffer with added digitonin. Again it was centrifuges at 10000 x g for 15 minutes and discared the supernatant while re-suspended the pellet in extraction buffer. For all procedure temperature was maintained at 4ºC. Commercially available kit (Autospan Liquid Gold, Span Diagnostics Ltd., India) was used for determining protein concentration following the protocol described by the manufacturer.

Complex IV (of respiratory chain) activity. Total complex IV activity was measured spectrophotometrically using isolated mitochondria [7]. Briefly, reduced cytochrome c was

prepared by mixing cytochrome c and ascorbic acid in potassium phosphate buffer. Complex IV activity was taken as the rate of ferrocytochrome c oxidation to ferricytochrome c, detected as the decrease in absorbance at 550 nm.

Measurement of mitochondrial membrane permeability transition. Opening of the pore causes mitochondrial swelling, which results in reduction of absorbance at 540 nm. Mitochondrial permeability transition (swelling assay) was monitored as changes at 540 nm at 10 s intervals over 10 min time with 250 µg mitochondrial protein in the swelling buffer, which contained 120 mM KCl (pH 7.4) and 5 mM KH_2PO_4 .

Measurement of mitochondrial membrane potential. The mitochondrial membrane potential (ΔΨm) was measured using the fluorescent probe rhodamine 123 (Sigma) [8,9]. Because rhodamine 123 is a cationic dye, it accumulates in the mitochondria driven by ΔΨm. Under appropriate loading conditions, the concentration of rhodamine 123 within the mitochondria reaches sufficiently high levels that it quenches its own fluorescence (λex = 503 nm, λem = 527 nm). If the mitochondria depolarize, rhodamine 123 leaks out into the cytoplasm and is associated with a reduction in the amount of quenching. Thus the changes in ΔΨm are revealed as changes in total fluorescence intensity following the method of Chen [10].

Mitochondrial dehydrogenases activity. The methyl tetrazolium (MTT) assay was used as a colorimetric method for the estimation of mitochondrial dehydrogenases activity in isolated brain mitochondria [11, 12]. Briefly, mitochondrial suspension (1 mg protein/mL) in a buffer containing 320 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, and pH= 7.4, was incubated with 40 µL of theMTT solution (0.4% w: v) at 37°C (30 min, in the dark). The product of purple formazan crystals was dissolved in DMSO (1 ml). Then, 100 µl of dissolved formazan product was added to a 96 well plate, and the optical density (OD) was measured at λ= 570 nm using an EPOCH plate reader (BioTek Instruments, USA).

Mitochondrial ATP level. A luciferase-luciferin-based kit from Promega (ENLITEN, Madison, WI, USA) was used to assess brainmitochondrial ATP content [12]. Samples and buffer solutions were prepared based on the kit instructions. Briefly, mitochondria fractions (1 mg protein/ml) were treated with 100 µL of trichloroacetic acid (0.5% w: v). Samples were centrifuged (15,000 g, 15 min, 4°C). Then, 100 µL of the supernatant was added to 100 µL of the kit content, and the luminescence intensity was measured at λ= 560 nm.

Statistical analysis. All quantitative data are expressed as mean ± SD unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software (CA, USA). $p < 0.05$ was considered significant.

Data availability. The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials. Any other related data that support the findings of this study are available from the corresponding author, [SKP], upon reasonable request.

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