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Nanoparticle-induced neuronal toxicity across placental barriers is mediated by autophagy and dependent on astrocytes

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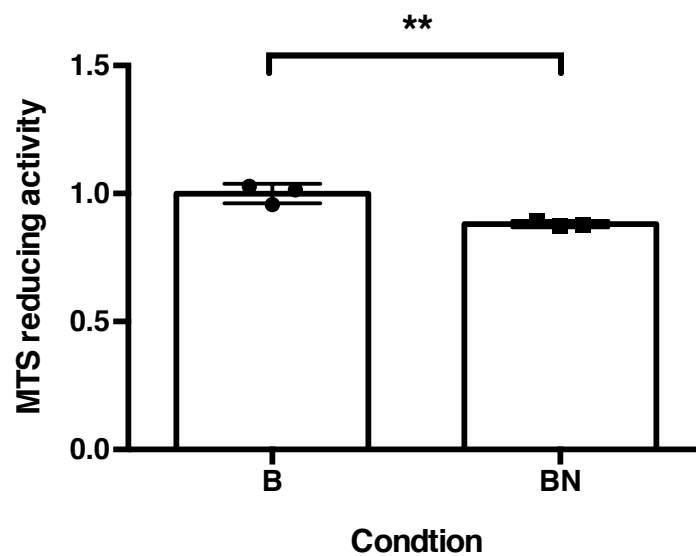
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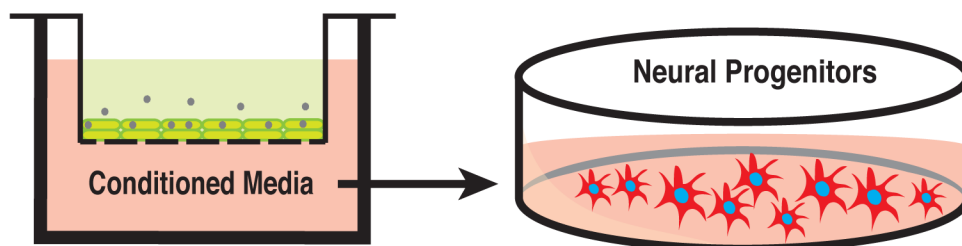
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Supplementary Figures

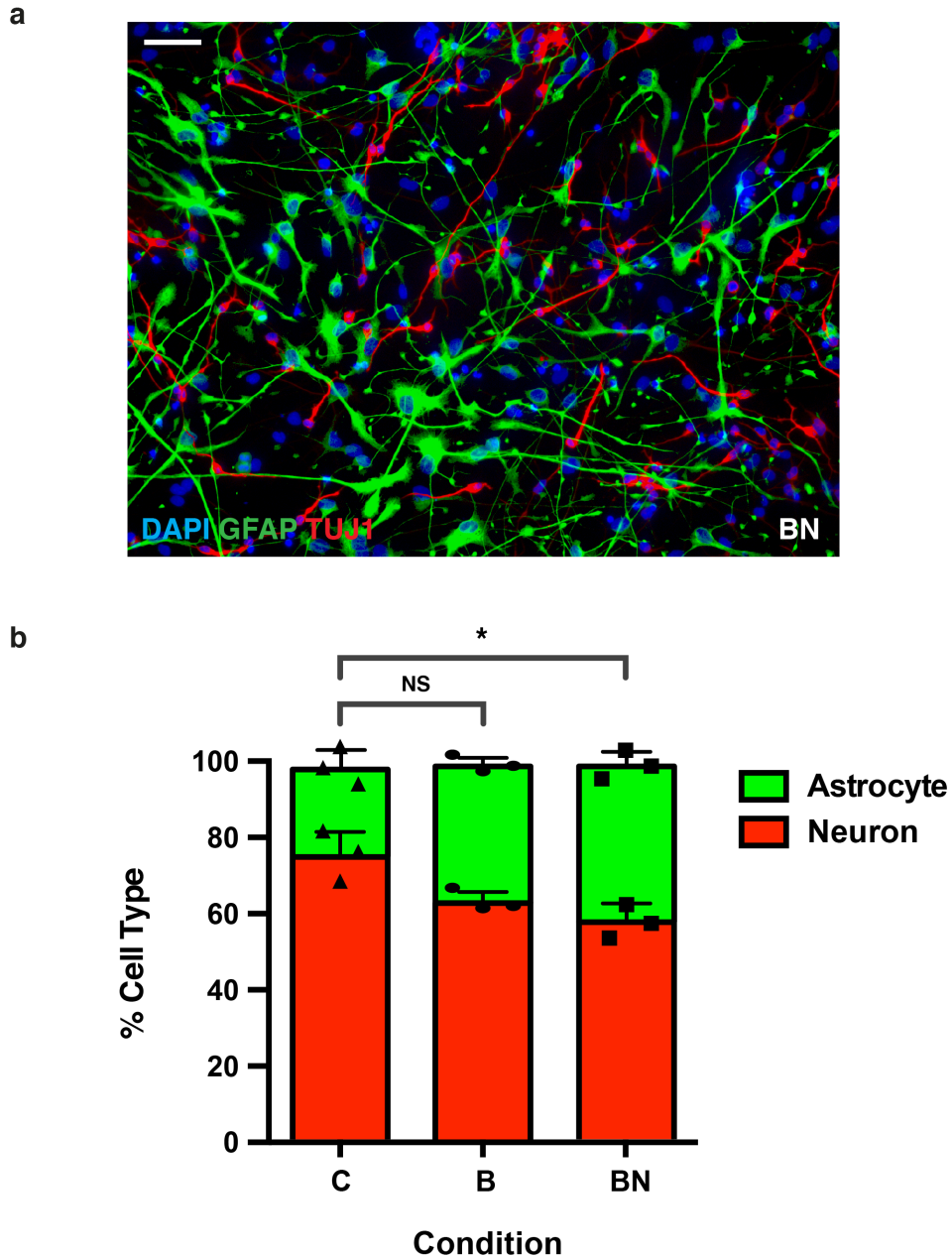


Supplementary Figure 1: Mitochondrial activity in the BeWo barrier following CoCr NP exposure. A decrease in MTS reducing activity was found following the exposure of BeWo barriers to CoCr NPs (BN) for 24 hours. Results are from three independent experiments and were analysed using an unpaired student's T test, two tailed. Data are normalised to the barrier only control (B). All values are: means $\pm$ SEM from three independent experiments. ** P = 0.0064.



Supplementary Figure 2: BeWo barrier media transfer model with neural progenitor cells

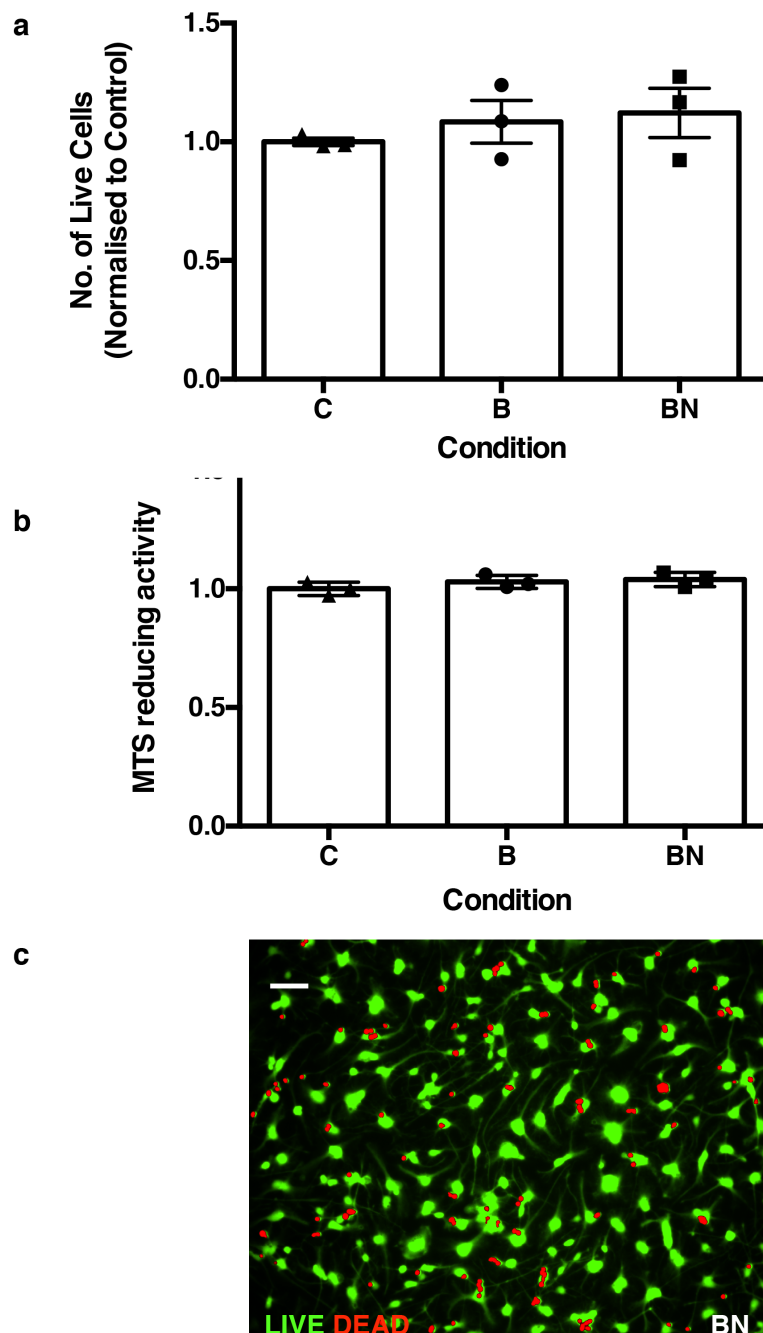
BeWo cells, a human trophoblast choriocarcinoma derived cell line, were grown on transwell inserts for 7 days so as to form confluent bilayered cell barriers used to model the placental barrier in vitro (Figure 1a) (Bhabra et al., 2009; Sood et al., 2011; Parry et al., 2010; Bode et al., 2006). After 7 days the barriers were checked for barrier integrity, TEER measurements shown in (Figure 1b), then 0.04mg/ml of CoCr NPs were applied to the upper layer. Neural progenitor cell media was placed beneath the barrier in the lower chamber and conditioned for 24 hours. This was then placed onto the dissociated neural progenitor cells. These had been dissociated and allowed to commence differentiating for 24 hours prior to exposure. These cells were then cultured in the transferred media for 6 days before the differentiated neural progenitor cells were assessed.



Supplementary Figure 3: The proportion of neurons and astrocytes formed from dissociated Neural Progenitor Cells (NPCs) following indirect exposure to CoCr NPs.

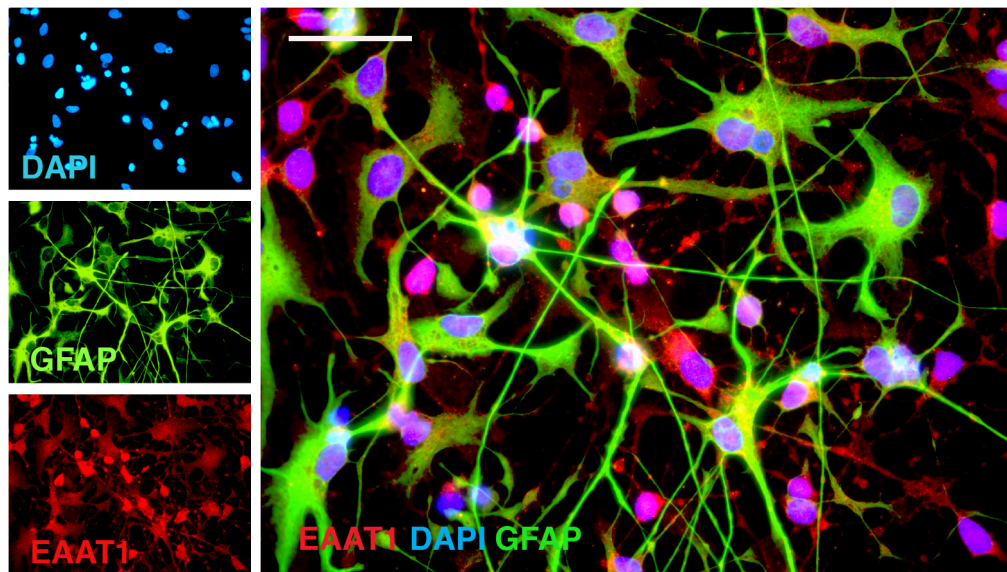
(a) Immunostaining of differentiated NPCs for GFAP and TUJ1 with scale bar 50 μ m. Representative image from three independent experiments (b) Graph of cell counts for GFAP⁺ astrocytes and TUJ1⁺ neurons showing no barrier control (C), Barrier only control (B) and Barrier with CoCr NP exposure (BN). All values are: means + SEM from three independent experiments. Statistical analysis compares percentage of

neurons by one way ANOVA with Newman Keuls post hoc test. C vs B, NS (non significant) $P \geq 0.05$, C v BN $P = 0.0426$.

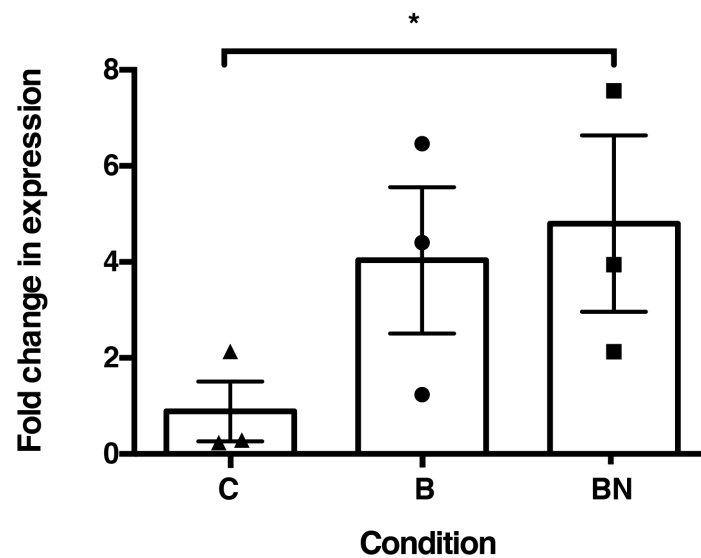


Supplementary Figure 4: Viability of neural progenitor cells following indirect exposure to CoCr NPs. (a) Graph of Live Dead cells counts across exposure conditions assessed on day 7 of differentiation. Data was normalised to control and analysis was performed using a one way ANOVA; $F = 0.6$, $P = 0.5751$, $n = 3$ independent experiments (b) Graph of MTS reducing activity also showing no difference between conditions. $F = 1.542$, $P = 0.2881$, $n = 3$ independent experiments. No barrier control (C), Barrier only control (B) and Barrier with CoCr NP exposure

(BN). (c) Live Dead cell image of BN exposed NPCs. Representative image of three independent experiments yielding similar results. Scale bar 50 μm .

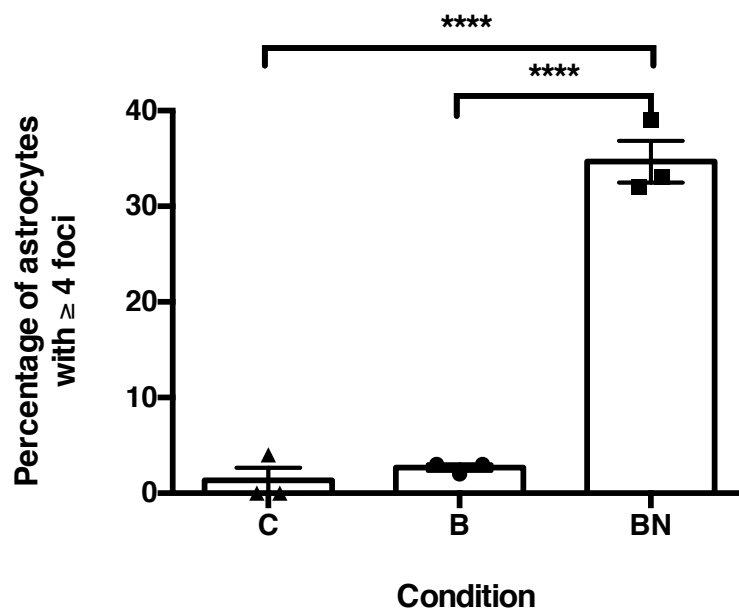


Supplementary Figure 5: Dissociated Neural progenitor cells differentiate to form neurons and astrocytes. Representative image of three independent experiments yielding similar results. These images demonstrate co-staining of GFAP⁺ cells with EAAT1. Scale bar is 50 μ m.



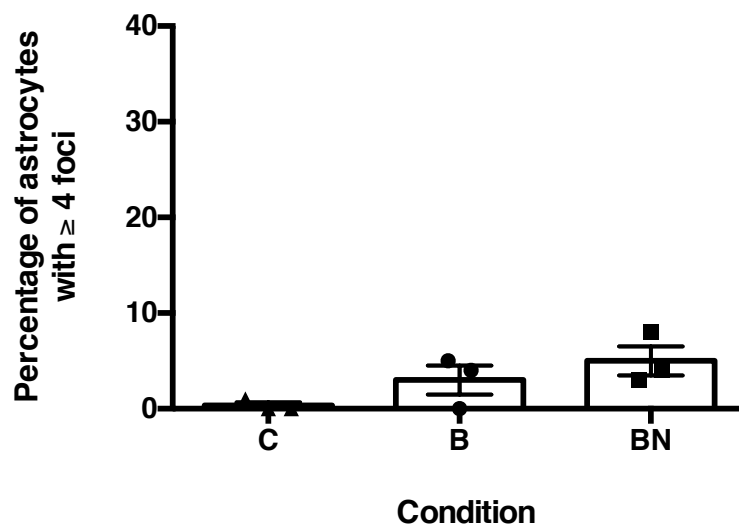
Supplementary Figure 6: Increased gene expression of *GFAP* in differentiated neural progenitors following CoCr NP exposure to the BeWo barrier compared to control.

Samples from differentiated neural progenitor cells were analyzed for *GFAP* expression by qPCR, using 50ng of cDNA. All values are: means $\pm$ SEM from three independent experiments. $F = 8$, $*P < 0.0408$ by one way ANOVA and Newman Keuls post hoc test.



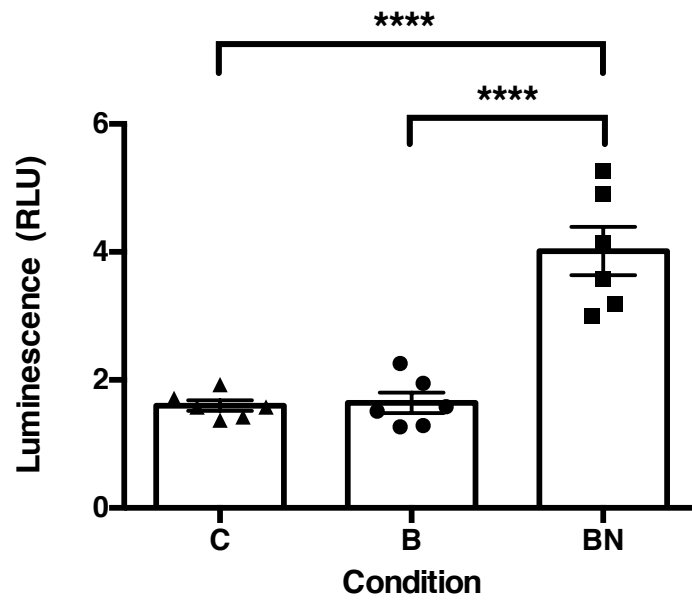
Supplementary Figure 7: TiO₂ NPs exposure to BeWo barriers results in an increase in the number of γ -H2AX foci in astrocytes.

Graph showing the percentage of astrocytes that contain ≥ 4 foci. All values are: means $\pm$ SEM from three independent experiments. $F = 160$, **** = $P < 0.0001$ by ANOVA and Newman Keuls post hoc test.

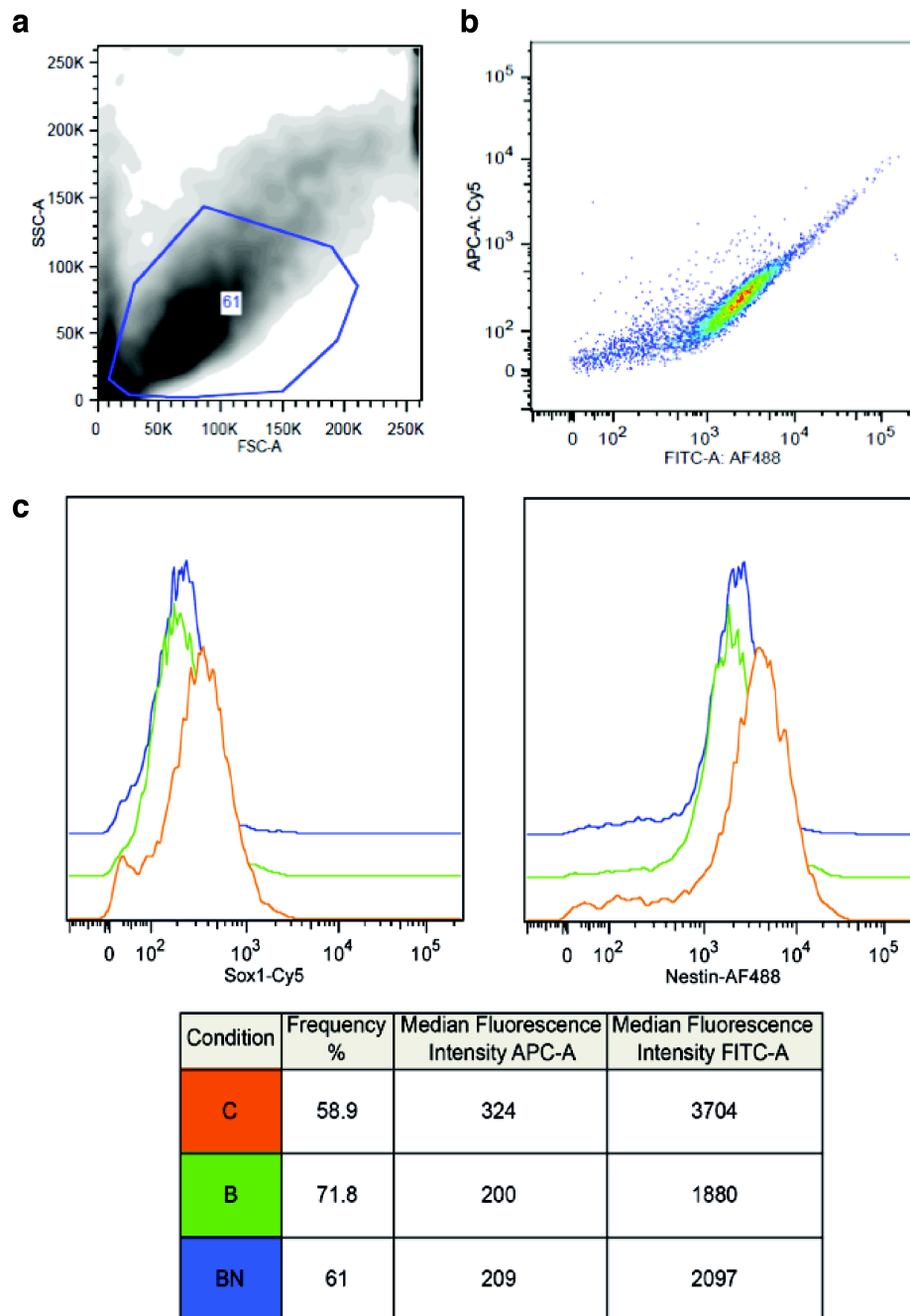


Supplementary Figure 8: F127 NP exposure to BeWo barriers does not result in an increase in the number of γ -H2AX foci in astrocytes.

Graph showing the percentage of astrocytes that contain ≥ 4 foci. All values are: means $\pm$ SEM from three independent experiments. $F = 3$, $P = 0.1010$; NS by One Way ANOVA.

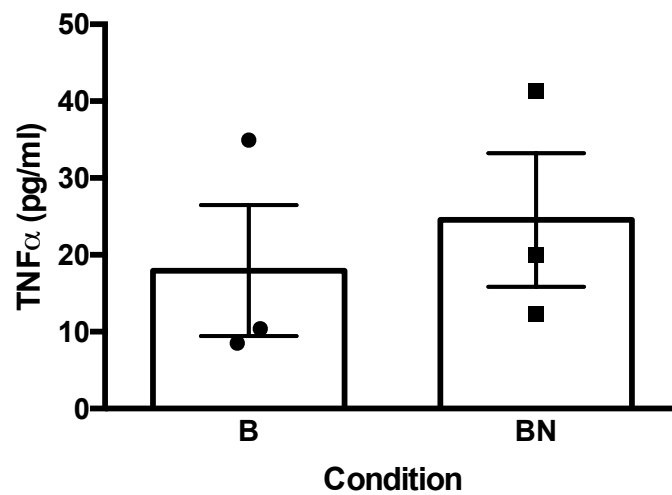


Supplementary Figure 9: Determination of glutathione levels in NPC cultures following indirect exposure to NPs. Graph of glutathione induced luminescence showing increased glutathione levels in NPCs differentiated in BN media. All values are: means $\pm$ sem from six independent experiments. $F = 33$, **** = $P < 0.0001$ by ANOVA and Newman Keuls post hoc test.



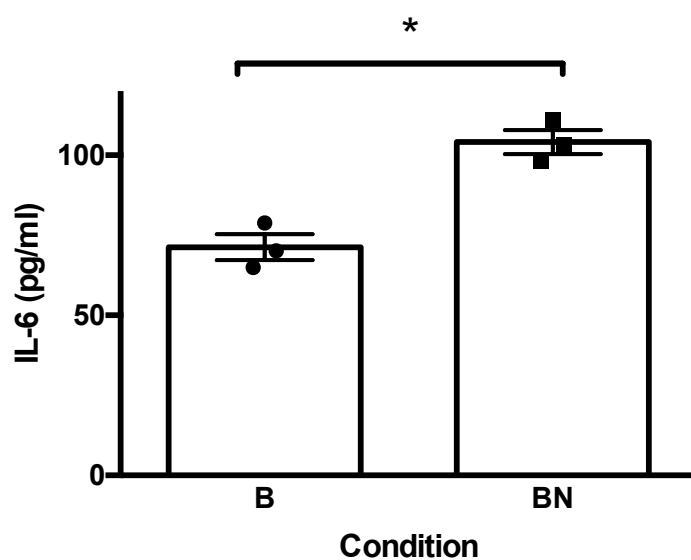
Supplementary Figure 10: Flow cytometry assessment of NSC markers Nestin and Sox1 in exposed embryoid bodies.

(a) The gating strategy was forward and side scatter (using a gate that was applied to all samples), followed by (b) analysis of median fluorescence for AF488 Nestin (green) and Cy5 Sox1 (blue). (c) Indirect NP toxicity does not affect the formation of neuroectoderm during the differentiation of embryoid bodies. Data shown is from one representative experiment from three independent experiments.



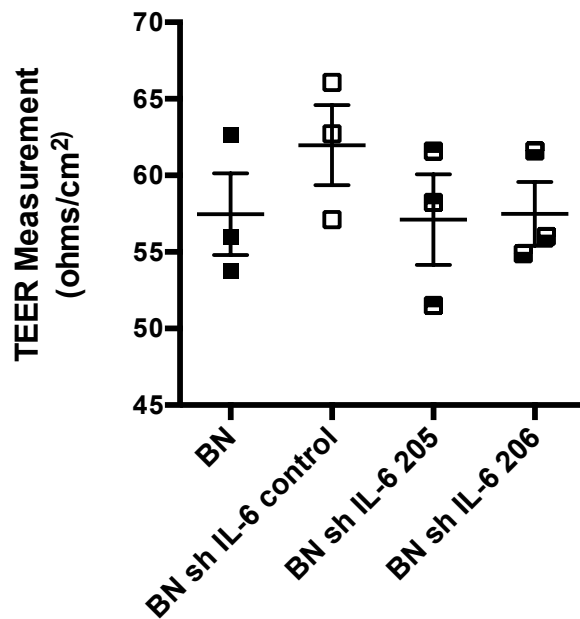
Supplementary Figure 11: TNF α levels in media conditioned beneath the BeWo Barrier

TNF α levels assessed by enzyme-linked immunosorbent assay found no increased expression following CoCr NP exposure. All values are: means $\pm$ SEM from three independent experiments. $P = 0.6163$; NS by unpaired T test, two tailed.



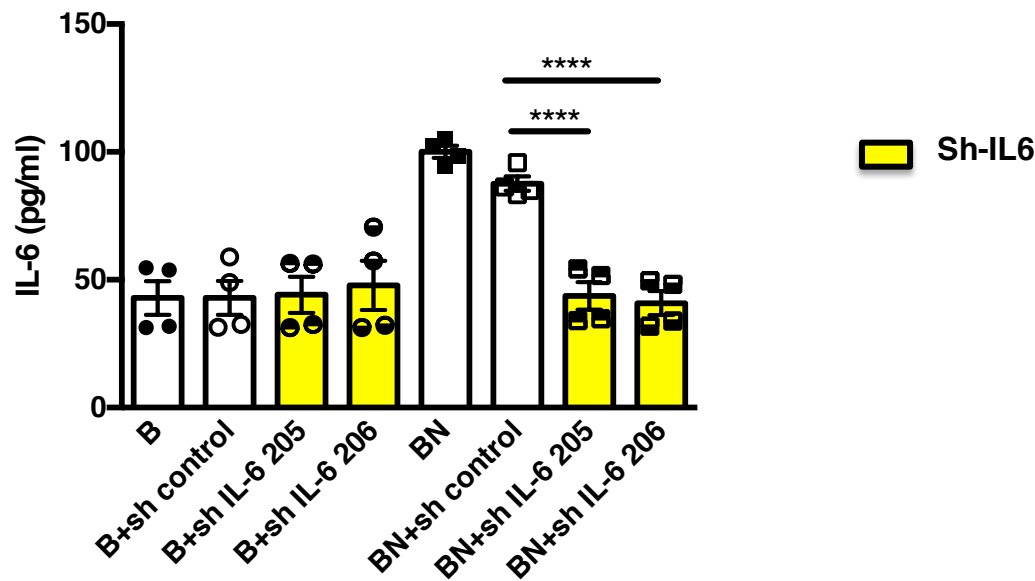
Supplementary Figure 12: IL-6 was released from BeWo barriers following the exposure of NPs.

IL-6 levels assessed by enzyme-linked immunosorbent assay were found to be significantly increased following CoCr NP exposure. All values are: means $\pm$ SEM from three independent experiments. * $P = 0.041$ by unpaired T test, two tailed.



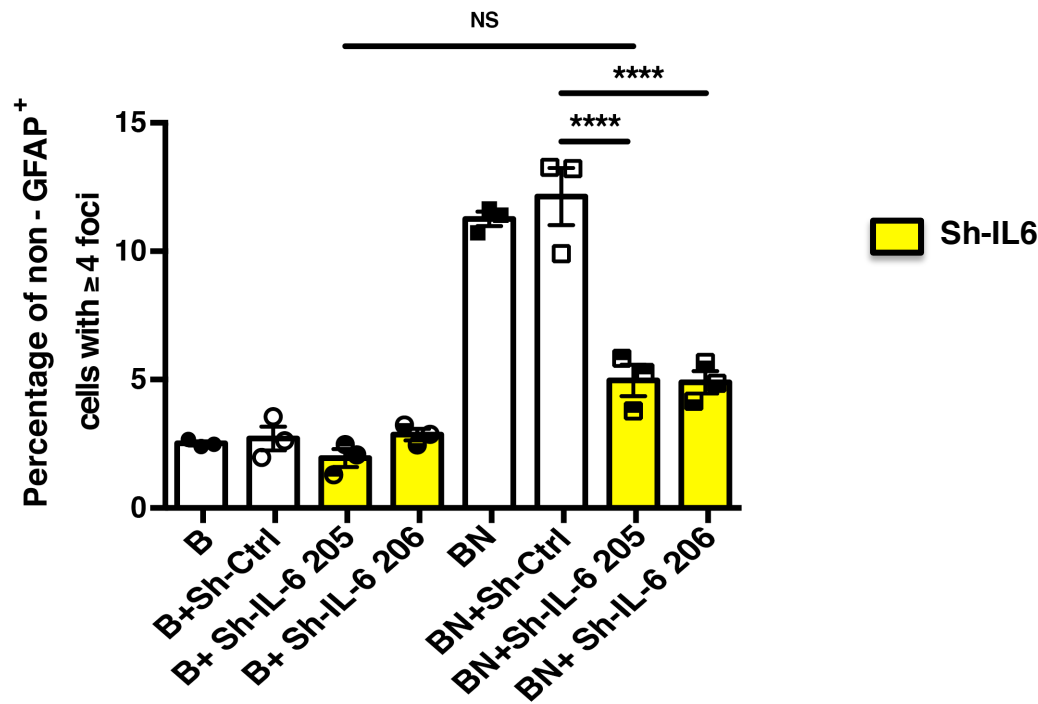
Supplementary Figure 13: TEER values of BeWo barriers following transduction of IL-6 sh-RNA using a lentiviral vector.

BeWo barriers were either transduced with shRNA lentiviral particles (or not transduced as a control). PLKO1 non-targeting shRNA (Sigma), or IL6 shRNAs TRCN0000059205 or TRCN0000059206 (Sigma), were added above and below the barrier BEWO media. TEER measurements were not found to be significantly altered following these exposures and similar TEER levels were achieved as in BeWo barriers not exposed to lentiviral vectors (See Figure 1b for comparison). All values are: means $\pm$ SEM from three independent experiments. $F = 0.8$, $P=0.5320$; NS by One way - ANOVA.



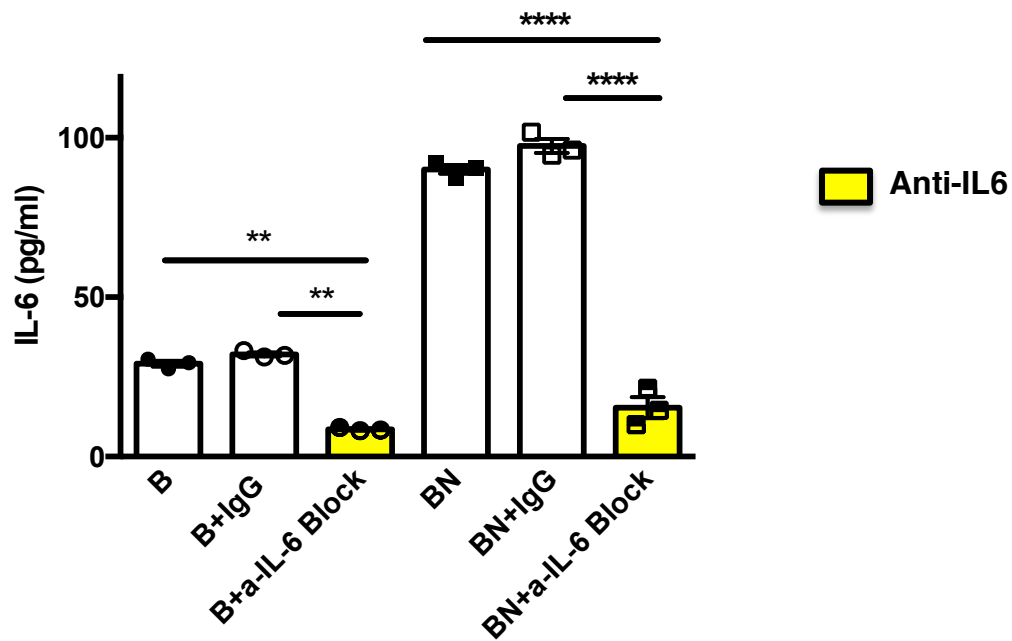
Supplementary Figure 14: Levels of IL-6 are increased following exposure of nanoparticles to the BeWo barrier, addition of an IL-6 shRNA reduces the level of IL-6 to control levels.

BeWo barriers were either transduced with shRNA lentiviral particles (or not transduced as a control). PLKO1 non-targeting shRNA (Sigma), or IL6 shRNAs TRCN0000059205 or TRCN0000059206 (Sigma), were added above and below the barrier BEWO media. Addition of an IL-6 shRNA reduces the level of IL-6; shRNA control has no effect (Data presented as mean + SEM of n=4 independent experiments). One way ANOVA plus Neuman Keuls post hoc test. $F = 15.06$, **** $P < 0.0001$ BN and BN+Sh control vs BN+Sh IL-6 205 and BN+Sh IL-6 206. B is barrier only; BN is barrier with nanoparticles.



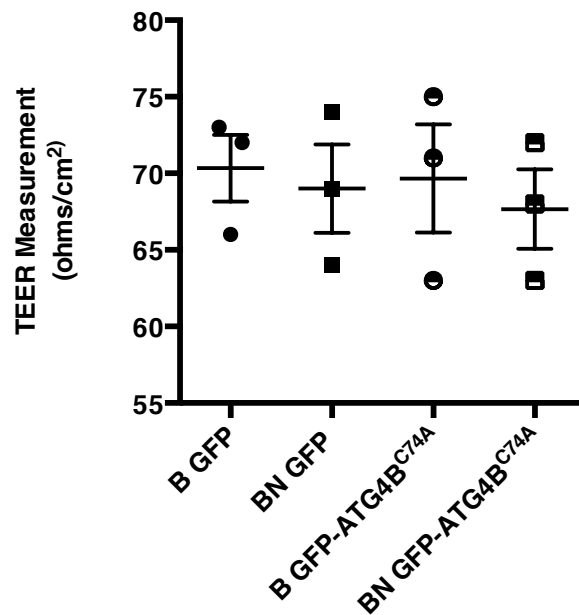
Supplementary Figure 15: Levels of γ -H2AX are increased in non-GFAP⁺ cells following exposure of nanoparticles to the BeWo barrier, then reduced to control levels following the addition of an IL-6 shRNA.

BeWo barriers were either transduced with shRNA lentiviral particles (or not transduced as a control). PLKO1 non-targeting shRNA (Sigma), or IL6 shRNAs TRCN0000059205 or TRCN0000059206 (Sigma), were added above and below the barrier BEWO media. This significantly reduced the number of γ -H2AX foci in non-GFAP positive cells; based on Suppl Figure 3 these are considered likely to be neurons (n=3). Data presented as mean + SEM of n=3 independent experiments. One way ANOVA plus Neuman Keuls post hoc test. F = 468. **** P < 0.0001 BN and BN+Sh ctrl vs BN+Sh IL-6 205 and BN+Sh IL-6 206. B is barrier only; BN is barrier with nanoparticles.



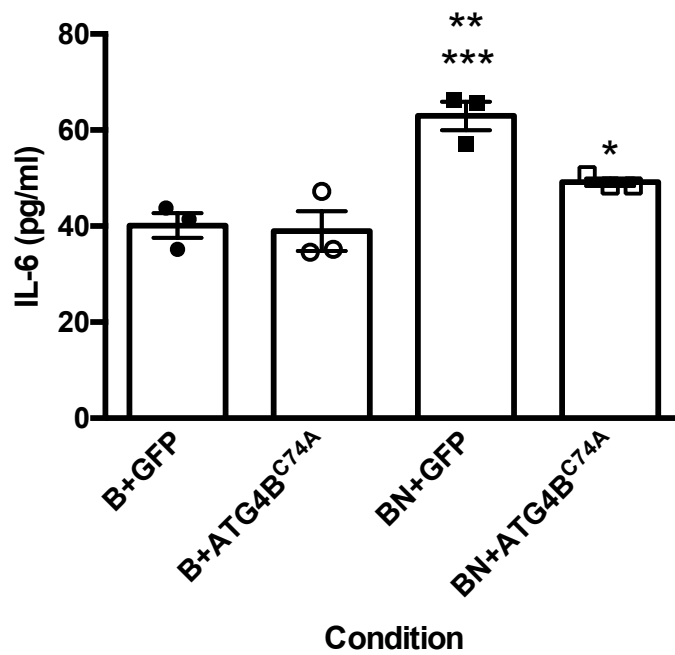
Supplementary Figure 16. Levels of IL-6 are increased following exposure of nanoparticles to the BeWo barrier, addition of an IL-6 blocking antibody reduces the level of IL-6.

IgG control has no effect (n=3 independent experiments). Data presented as mean + SEM of n=3 independent experiments). One way ANOVA plus Neuman Keuls post hoc test. F = 122, ** P = 0.0040 B vs B+a-IL-6 Block and P = 0.0082 B+IgG vs B+a-IL-6 Block. **** P < 0.0001 BN+a-IL-6 Block versus BN and BN + IgG control antibody. B is barrier only; BN is barrier with nanoparticles.



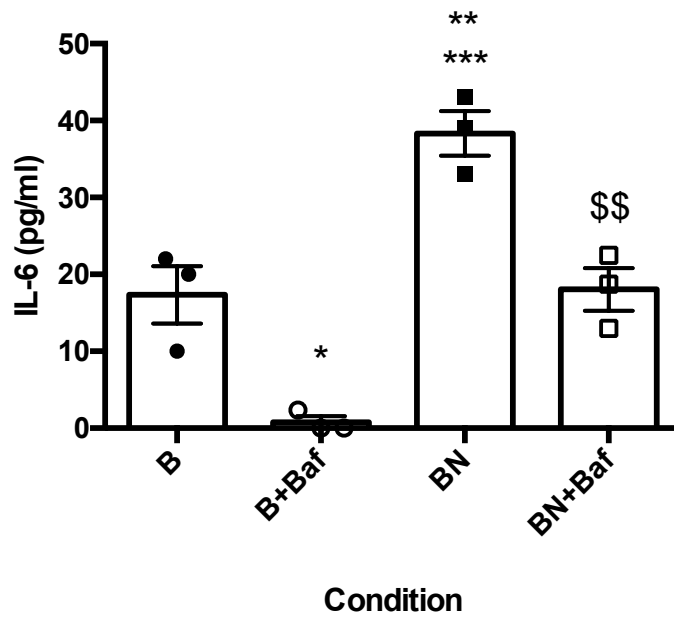
Supplementary Figure 17: TEER values of BeWo barriers following inhibition of autophagic degradation using a lentiviral vector containing human ATG4B^{C74A}

BeWo barriers were either transduced with lentiviral vectors containing GFP or GFP-ATG4B^{C74A}. These were then exposed to media containing CoCr NPs or media only. TEER measurements were not found to be significantly altered following these exposures and similar TEER levels were achieved as in BeWo barriers not exposed to lentiviral vectors (See Figure 1b for comparison). All values are: means ± SEM from three independent experiments. $F = 0.8$, $P = 0.9761$; NS by one way ANOVA.



Supplementary Figure 18: IL-6 secretion from the BeWo barriers exposed to NPs in the presence of ATG4B^{C74A} released less IL-6 than control GFP-expressing barriers.

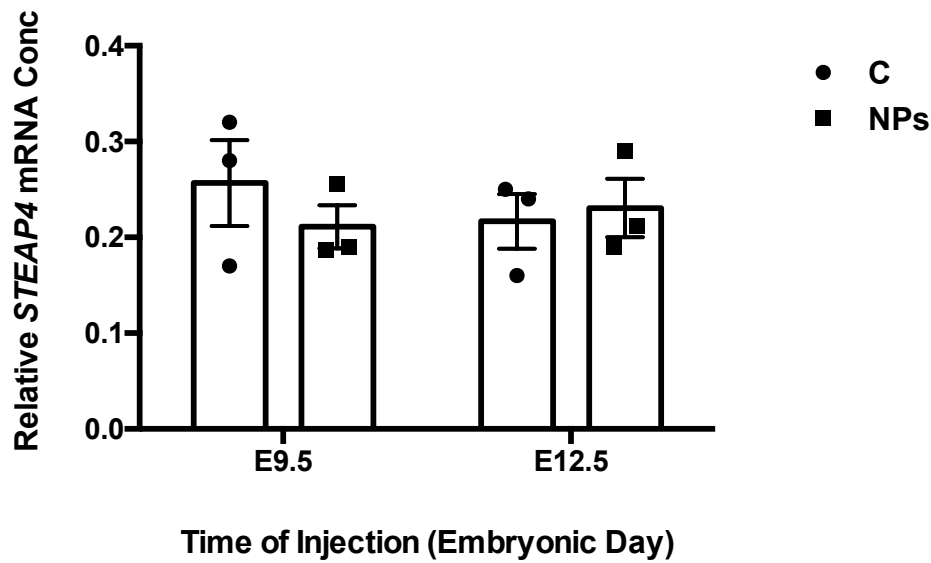
Measured by enzyme-linked immunosorbent assay. Means with SEM from three independent experiments assessed by One way ANOVA with Neuman Keuls post hoc test. Significance Values: F = 14.86, * P = 0.0370 versus BN+GFP; ** P = 0.0022 and *** P = 0.0001 versus B+GFP and B+ATG4B^{C74A}



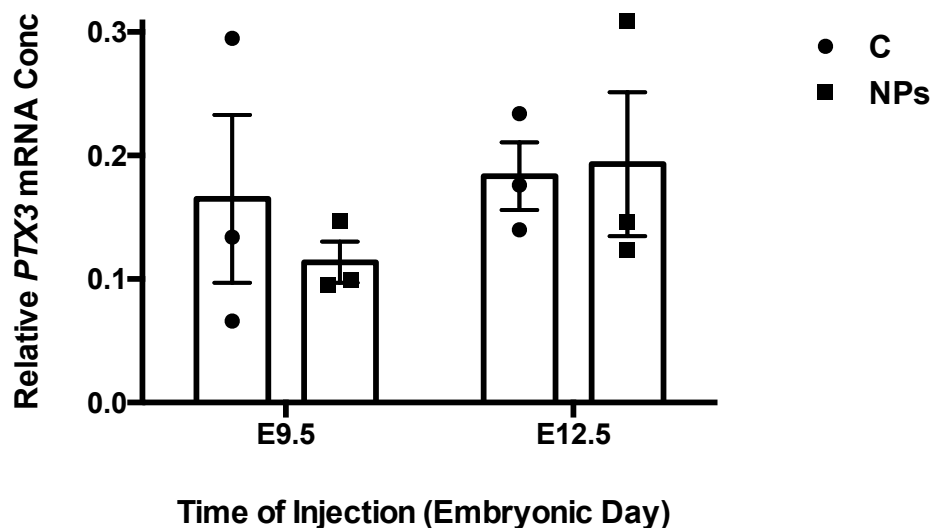
Supplementary Figure 19: IL-6 secretion from the BeWo barriers exposed to NPs in the presence of BafA1 also secreted significantly less IL-6 than barriers exposed to NPs in the absence of BafA1.

Measured by enzyme-linked immunosorbent assay. Means with SEM of three independent experiments assessed by One way ANOVA with Neuman Keuls post hoc test. Significance values: $F = 31.11$, * $P = 0.05$ versus B; \$\$ $P = 0.0058$ vs B+Baf and $P = 0.0036$ vs BN; ** $P = 0.0036$ versus B; *** $P \leq 0.001$ versus B+Baf.

a.



b.



Supplementary Figure 20: Expression of the astrocytic activation markers (a)

***STEAP4* and (b) *PTX3* did not alter significantly at either time-point of NP**

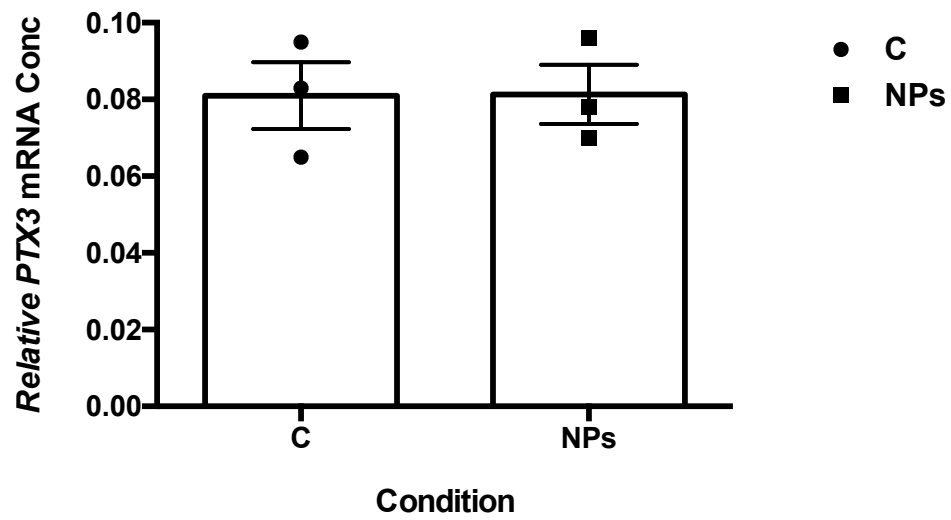
exposure in whole brain when compared to relevant control groups.

Gene expression assessment performed by qPCR relative to GAPDH expression. All

values are: means $\pm$ sem from three independent experiments. NS by Two way -

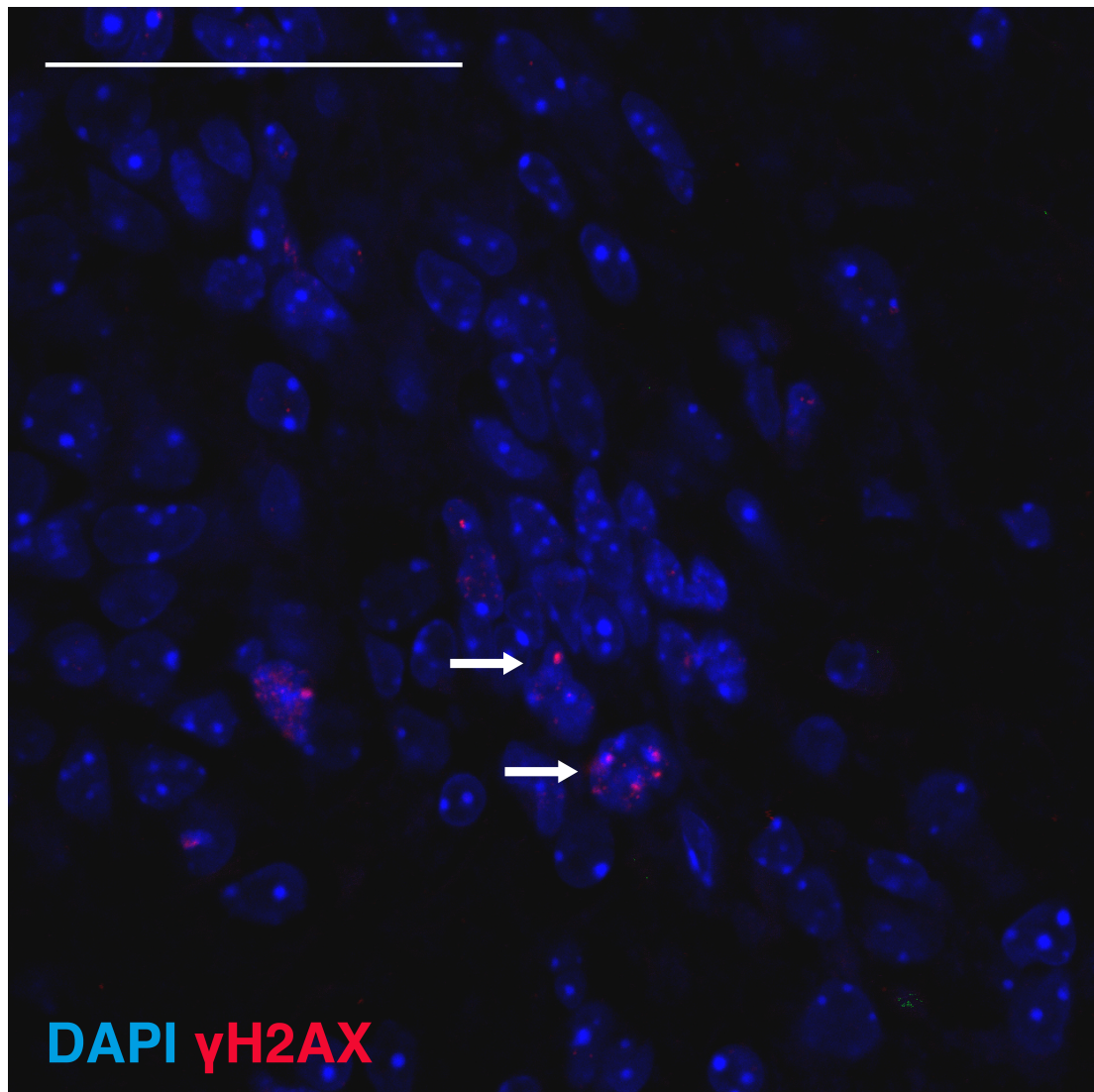
ANOVA. (a) $P = 0.3345$ for CoCr treatment and $P = 0.6729$ for E9.5 vs E12.5; (b) P

$= 0.7631$ for CoCr treatment and $P = 0.6402$ for E9.5 vs E12.5.



Supplementary Figure 21: Levels of PTX3 gene expression were not increased in the hippocampus following NP exposure at E12.5.

Gene expression assessment performed by qPCR. All values are: means $\pm$ sem from three independent experiments. $P = 0.9785$; NS by unpaired t test, two tailed.



Supplementary Figure 22: Immunohistological staining for γ -H2AX demonstrated significantly increased DNA damage in the hippocampus in E12.5 treated animals only compared to relevant controls

This demonstrate that increased γ -H2AX immunostaining (white arrows) was present in the hippocampus of mice at E12.5 following maternal exposure to CoCr NPs. Representative image from four independent animals yielding similar results. Scale bar is 50 μ m.

Supplementary methods

Preparation of nanoparticles

The 80 ± 14.6 nm CoCr NPs used were produced by a thermal plasma method (Raghunathan et al., 2013). They were polydisperse in size measuring 80 ± 14.6 nm with a smooth outline with some straight edges on transmission electron microscopy using a Jeol 1200EXmkII TEM operated at 120 keV. They had a mean electrophoretic mobility at 25°C of $-0.816 \mu\text{m}^2/\text{Vs}$ and a mean zeta potential of -13mV measured on Malvern Instruments Zetasizer Nano Z equipment. In media they have a tendency to aggregate demonstrated by a photon correlation spectroscopy measurement of 241nm in media carried out on a Brookhaven instrument and release predominantly Co ions (Raghunathan et al., 2013).

The NPs were prepared for use in tissue culture by being: placed in glass universals, weighed then washed with 100% ethanol. They were then heat sterilized at 180°C for 3 hours then resuspended in serum free growth media by sonication with 7 pulses each of 2 seconds long using a titanium probe and Sonics VibraCELL VC130PB sonicator (maximum output power: 130W). The metal NP exposures were expressed in mg/ml, this equates to 0.036 mg per cm^2 surface area of BeWo barrier. This was calculated from the mass of the NPs before addition of media in relation to the surface area of the Transwell insert on which the BeWo cells were grown. The surface area of the Transwell insert used in all the experiments was 1.12 cm^2 . This exposure level has previously been found not to cause transition of CoCr NPs across the BeWo barrier (Bhabra et al., 2009; Parry et al., 2010; Sood et al., 2011). TiO₂ NPs used for comparison to CoCr NPs were $50 \text{ nm} \pm 20 \text{ nm}$ (Sood et al., 2011). 5% F127 NPs were kindly donated by Dr Bienemann from the University of Bristol

and setup as previously described (Basak and Bandyopadhyay, 2013; Liu et al., 2016).

Supplementary Table 1: Composition of CoCr alloy (Raghunathan et al., 2013).

Element	C	Co	Cr	Fe	Mn	Mo	N	Ni	Si
Wt%	0.06	64.3	28.0	0.25	0.50	6.00	0.20	0.20	0.50
Atomic%	0.29	62.5	30.8	0.26	0.52	3.58	0.82	0.20	1.02

Transduction of BeWo barriers with IL-6 sh-RNA lentivirus.

Bewo cells were grown on transwell inserts for 7 days to form confluent bilayered layers of the cells. They were seeded at 10^5 cells/cm² on transwell inserts (Appleton Woods, Birmingham, UK) in Dulbecco's Modified Eagle's Nutrient Mixture (DMEM)/F12 Ham, supplemented with 1% L-Glutamine-Penicillin-Streptomycin, 1% amphotericin B solution and 10% FBS. On the fifth day after plating of BeWo cells onto the transwell, cells were transduced with shRNA lentiviral particles (or not transduced as a control). PLKO1 non-targeting shRNA (Sigma), or IL6 shRNAs TRCN0000059205 or TRCN0000059206 (Sigma), were added above and below the barrier BEWO media. After two days, media was removed and nanoparticles were added: pre-prepared nanoparticles were thawed and sonicated (bath sonicator) for 30 minutes. Media under the barrier was replaced with neural progenitor media comprising: 72% DMEM high glucose (Sigma), 24% DMEM/F12 + Glutamax (Thermo Fisher), 1% Glutamax (Thermo Fisher), 1% Penicillin/Streptomycin (Sigma) and 1% B27 (Thermo Fisher). 500µl of nanoparticles was added to the top of the barrier (or BEWO media without serum as a control). The following day, transepithelial/transendothelial electrical resistance (TEER) measurements were

taken in the media on top of the barrier. Then, the media under the barrier was collected and snap-frozen in liquid nitrogen.

To study the effects on NPC differentiation from the conditioned media neurospheres were dissociated using accutase (Sigma) then placed in media without mitogens. 1×10^5 cells were then seeded onto 13mm PDL/laminin coated glass coverslips and allowed to adhere for 24 hours after which their media was replaced with NPC differentiation media conditioned in the BeWo barrier model plus/minus IL-6 shRNAs then cultured until day 7 of differentiation.

IL-6 antibody blocking experiment.

In another set of experiments BeWo barrier conditioned media was added to the cells, together with an anti-IL-6 antibody or relevant IgG control, at a concentration of 0.15 ug/ml (R&D systems). The blocking antibody remained in the culture for 6 days prior to removal of the media for measurement of IL-6 or fixation with 4% PFA for immunohistochemistry.

BeWo barrier immunoblot

Cells were lysed in RIPA buffer plus protease inhibitor (Roche). Protein was quantified using a BCA assay (Thermo Scientific) and resolved on polyacrylamide SDS-Tris-Glycine gels. Proteins were transferred onto PVDF membrane (Roche) and blocked using 10% non-fat milk in 0.1% TBS-T (Sigma). Incubation with primary antibody (mouse anti-HSP70 (Stressgen #C92F3A) 1:1000, mouse anti-p62 (Abnova # H00008878-M01) 1:1000, mouse anti-p53 (Cell Signalling #2524) 1:1000, rabbit anti-LC3 (MBL #PM036) 1:1000, mouse anti- α -Tubulin (Sigma #T5168) 1:10,000) was carried out overnight at 4 °C in blocking buffer. Membranes were incubated with

the appropriate HRP-labelled secondary antibody for 2 h at room temperature.

Protein bands were visualized using Pierce Plus Substrate (Thermo).

Immunocytochemistry in NPCs

Cells were fixed in 4% paraformaldehyde (Fisher), with the exception of BeWo barrier staining where cells were fixed with ice cold 100% methanol. Cells were blocked at room temperature in PBS/10% goat serum/Triton X-100 0.1%. Primary antibodies were incubated overnight at 4 °C in PBS/2% goat serum/Triton X-100 0.1% using mouse anti-TUJ1 (Covance #MMS-435P) 1:1000, rabbit anti-GFAP (Dako #Z0334, Lot 20015408) 1:1000, chicken anti-GFAP (Abcam #Ab4674, Lot 2670366) 1:2000, rabbit anti-phosphorylated H2A.X (Ser139) (Cell Signalling Technology, #9718) rabbit anti-EAAT1 (Novus Biologicals NBP1-20135) 1:200, mouse anti-SQSTM1 (Abnova #H00008878-M01) 1:500 and rabbit anti-LC3-II (Viva #VB2930) 1:300. Alexa Fluor (Life Tech) secondary antibodies were incubated at room temperature for 2 hours in PBS/1% goat serum/Triton X-100. Cells were co-stained with DAPI (Sigma) and mounted in Vectorshield (Vectorlabs). Images were taken using fluorescence Leica DM RB upright microscope and processed using Adobe Photoshop and Image J software.

GSH-Glo Glutathione Assay in NPCs

The GSH-Glo luminescent assay (Promega) was used to detect the amount of GSH in exposed NPC cells and was measured on a Promax glomax multi detection system.

Live Dead Cell Count in NPCs

The Live/Dead viability/cytotoxicity kit (Life Technologies) was used to determine the number of live cells in the NPC cultures post media transfer experiments. The

number of live and dead NPC cells on coverslips was counted using a Leica DMRB (Leitz) fluorescence microscope and image J.

MTS Cell Proliferation Assay in NPCs

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used to determine the mitochondrial activity in NPC cells and was measured on a Promega glomax multi detection system.

Assessment of *GFAP* gene expression in differentiated NPCs

Total RNA was extracted from cells (RNeasy mini system, Qiagen) and treated with RNase-free DNase I (Roche). cDNA was generated using Multiscribe reverse transcriptase (Thermo) and then a pre-amplification step was carried out using Taqman PreAmp mastermix and gene specific primers (Thermo). qPCR was carried out using the Taqman Universal Master Mix and Taqman assays (Thermo). Applied Biosystems software was used for analysis and relative gene fold variations were compared to the values for *GAPDH* that acted as a housekeeping gene.

Assessment of gene expression in embryoid bodies by qPCR

Embryoid Bodies (EBs) were created from Shes3 hES cells and allowed to expand in culture for 4 days. Following this the EB media was removed and replaced with EB media conditioned under the BeWo barrier model. The EBs were removed from this on day 7 then total RNA was extracted. (RNeasy mini system, Qiagen), treated DNase I (Roche). cDNA was generated using Multiscribe reverse transcriptase (Thermo) and the qPCR was carried out using the Taqman Universal Master Mix and Taqman assays. Each 20µl a reaction contained 50 ng cDNA. Applied Biosystems software was used for analysis and relative gene fold variations were compared to the values for *GAPDH* that acted as a housekeeping gene.

Supplementary Table 2: Taqman gene expression assays

Gene	Taqman Assay ID
<i>EOMES</i>	Hs00172872_m1
<i>GAPDH</i>	Hs00266705_g1/Hs02758991_g1
<i>GATA4</i>	Hs00171403_m1
<i>GFAP</i>	Hs00909233_m1
<i>HAND1</i>	Hs02330376_s1
<i>NESTIN</i>	Hs00707120_s1
<i>OCT 4</i>	Hs03005111_g1
<i>P53</i>	Hs01034249_m1

Assessment of Embryoid Bodies by flow cytometry

Flow cytometry was used to assess the formation of the three germ layers from embryoid bodies (EBs). EBs were harvested following media transfer exposure on day 7 of expansion and dissociated using Accutase. Dissociated cells were then washed with DPBS then fixed in 4% PFA. 10% NGS diluted in 0.1% PBS-Triton X100 was used for blocking. Control samples were run for all primary and secondary antibodies prior to flow cytometry assessment to check for cross reactivity and to

determine levels of autofluorescence. Primary antibodies used were 1:4000 Goat anti chicken SOX1 (Millipore AB5934) and 1:20 Goat anti mouse Nestin (Abcam AB6320). Secondary antibodies used were 1:2000 Cy5 anti chicken (Abcam AB97147) and 1:500 Alexa fluor 488 anti mouse IgG (Life Tech A11001). Flow cytometry assessment was performed on a BD LSR Fortessa X20 using FlowJo software. The authors wish to acknowledge the assistance of Dr. Andrew Herman and Sally Chappell and the University of Bristol Faculty of Biomedical Sciences Flow Cytometry Facility.

ELISA

Analysis of IL-6, TNF α , RANTES and GM-CSF concentrations

Supernatant concentrations of IL-6, TNF α , RANTES and GM-CSF (all from R&D Systems), obtained from BeWo barriers +/- nanoparticles or from astrocyte cultures +/- media conditioned by the BeWo barrier were measured using ELISA. Cytokine / chemokine concentrations in the test samples were evaluated with reference to the standard curves prepared using recombinant cytokines of a known concentration.

Additional mouse in vivo methods

Immunofluorescence staining and analysis

Frozen sections were incubated in blocking solution containing 10% goat serum (Invitrogen) in 0.3% PBS-Triton X-100 (Sigma-Aldrich) at room temperature for 2 hours. Sections were stained with primary antibody, Polyclonal Chicken Anti-GFAP (1:500 Abcam #Ab4674) or rabbit anti-phosphorylated H2A.X (Ser139) (Cell Signalling Technology, #9718) (1:500) diluted in 2% goat serum in PBS-Triton X-100 and incubated at 4°C overnight. The sections were rinsed and washed (3x for 10min) in PBS and Triton X-100. Secondary antibody using Life Technology Alexa Fluor 488 Goat anti-chicken 1:500 dilution or Alexa Fluor 555 Goat anti-rabbit 1:500 dilution

was incubated for 2 hours at room temperature. Following wash steps, sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories) and coverslips applied.

GFAP immunofluorescence intensity was analysed using Image J software (NIH, Bethesda, US) in sagittal sections (20mm) in the hippocampal region of P0 mouse brains. Sections were imaged with a Zeiss Fluorescent microscope (Axio Imager Z1) using 20x/0.5Ph2 objective lens, connected to an AxioCam HRm camera powered by AxioVision software. Captured digital images were analysed using Image J software as described by (Karve et al., 2016; McCloy et al., 2014) whereby regions of interest (ROIs) containing GFAP positive fluorescing cells were traced, which measured selected areas and integrated densities. The corrected total cell fluorescence (CTCF) intensities were determined by subtracting the area of selected GFAP positive cells multiplied by the mean fluorescence of background readings from the integrated density of cells. $CTCF = Integrated\ Density - (Area\ of\ selected\ cell \times Mean\ fluorescence\ of\ background\ readings)$. Average CTCF values were calculated for 10 x GFAP positive cells and 5 x background levels in the hippocampal region of P0 mouse brains sections from each treatment group (3 sections per slide and 3 slides per animal). For quantification of γ -H2AX immunostained sections were viewed under a 63x objective lens on a Zeiss Aim-system confocal microscope. Within the area of the hippocampus each cell containing 4 or more foci was considered damaged and hence counted.

qPCR quantitation of STEAP4, GFAP and PTX3 mRNA expression in P0 whole mouse brain and hippocampus.

RNA was isolated using the NucleoSpin RNA II kit (Machery Nagel, UK) as per the manufacturer's instructions. Extracted RNA yield was quantified using a

NanoDrop'ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, UK) and cDNA was synthesised using a High Capacity cDNA archive kit (Applied Biosystems, UK). Analysis of gene expression was conducted using real time PCR with specific primer and probe sets (Applied Biosystems).

In order to quantify the expression of specific target genes of interest, samples were assayed in duplicate with either primers and probes for GAPDH and PTX3 with FastStart Universal Probe Master mix (Roche) or primers (STEAP4 and GFAP) with FastStart Universal Sybr Green Master mix (Roche). Quantitative real-time PCR reactions were performed in a StepOne Plus™ Real-Time PCR system (Applied Biosystems, UK) using the following cycling protocol: 95°C for 10min; 95°C for 10s and 60°C for 30s for 40 cycles.

Gene expression analysis was conducted by the relative quantitation method with cDNA synthesized from an LPS injected mouse brain, which was utilized as the standard that would induce expression of all target genes of interest. Following subsequent dilutions, a linear standard curve was created whereby cycle threshold (Ct) was relative to concentrations of transcripts to determine relative mRNA expression, as previously described by (Hennessy et al., 2015; Cunningham et al., 2005). GAPDH was used as the endogenous control gene and target genes of interest were standardised to GAPDH.

Supplementary Table 3: The sequences for the primers and probes for the qPCR in vivo mouse experiments.

Gene	Forward Primer	Reverse Primer
<i>GFAP</i>	CTCCAACCTCCAGATCCGAG	TCCACAGTCTTTACCAGATGT
<i>STEAP4</i>	TGCAAGCCGGCAGGTGTTTGT	TCCAGTGGGGTGAGCCCAAGA
<i>PTX3</i>	ACAACGAAATAGACAATGGACTTCAT	CTGGCGGCAGTCGCA; Probe, CCACCGAGGACCCCACGCC

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