Connexin 43 mediated mitochondrial transfer prevents cisplatin induced sensory neurodegeneration.

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Keywords: dorsal root ganglia, mitochondria, chemotherapy, cisplatin, neurodegeneration, inflammation, monocyte, macrophage, neuropathy, mitochondria, gap junction, connexin 43

Abstract

Platinum based chemotherapeutics cisplatin are front-line treatments for paediatric and adult cancer. Despite advancements in medical interventions chemotherapy-induced peripheral sensory neuropathy is a common adverse health related complication that can persist for the long-term and impacts upon individual's quality of life. Recently, the causes of chemotherapy induced sensory neurodegeneration has been linked to sensory neuronal mitochondrial dysfunction. Here this study investigated cisplatin induced sensory neurodegeneration and how donation of monocytic mitochondria to recipient cisplatin damaged dorsal root ganglia (DRG) sensory neurons prevent platinum-based chemotherapy-induced sensory neurotoxicity. Neuronal cell line, SH-SY5Y, or mouse DRG sensory neurons were treated with either vehicle or cisplatin, and co-cultured with mitotracker-labelled THP1 monocytes. Cisplatin induced dysmorphic mitochondria and shifted to a glycolytic dependent energy production, with diminishment in oxidative phosphorylation in cisplatin treated dorsal root ganglia sensory neurons. DRG sensory neurons exposed to cisplatin were recipients of monocyte mitochondria indicated by increased intracellular mitotracker fluorescent labelling. Mitochondrial transfer to sensory neurons was neuroprotective by preventing neurite loss and neuronal apoptosis. Vehicle treated DRG sensory neurons did not demonstrate significant mitochondrial uptake. Furthermore, cisplatin induced mitochondrial transfer was prevented by pharmacological inhibition of gap junction protein, connexin 43. Connexin 43 inhibition led to reduced neuroprotective capacity via mitochondrial transfer. These findings demonstrate that monocytic mitochondria transfer to DRG sensory neurons damaged by cisplatin is dependent upon gap junction intercellular communication to promote sensory neuronal survival. This novel process in sensory neuronal protection is a potential novel therapeutic intervention for alleviating neuropathic pain in individuals treated for cancer.

Introduction

Cisplatin is a front-line platinum-based chemotherapeutic used for the treatment of adult and paediatric cancer. Recent improvements in chemotherapy regimens have resulted in an increase in 5 year-survival rate from paediatric malignancies to 86% in 2018[7]. However, chemotherapy also impacts non-cancerous host cells, such as immune cells and sensory neurons, causing adverse health related complications[1]. Consequently, cisplatin is causative of a myriad of development complications as paediatric chemotherapy treatment disrupts the development and functionality of crucial physiological systems into adulthood. Symptoms of CIPN commonly manifest years after the termination of chemotherapy[11], with CIPN affects 70% of childhood cancer survivors[3] and is unfortunately set to increase with survival rates of paediatric cancers improving. As a consequence, a large number of adult survivors of paediatric cancers commonly suffer from chemotherapy-induced peripheral sensory neuropathy (CIPN)[2]. This CIPN commonly manifests as dysesthesia and paresthesia in the extremities[9] that can persist for the long-term[10] and is often accompanied by other complications such as anxiety, depression, deficits in the auditory system, cognitive impairments, and fatigue, impinging on the individuals' quality of life.

Cisplatin induced sensory neuropathy arises due to sensory neurodegeneration typified by damage to sensory neuronal atrophy described as reductions in intraepidermal nerve fibre density and reduced sensory nerve fibre conduction velocity inconjunction with nociceptor sensitization. Dorsal root ganglia sensory neurons place high energy demands upon their own cellular respiration processes compared to other cell types. Consequently, sensory neuronal mitochondrial dysfunction has been heavily implicated in the underlying mechanisms of CIPN, due to the increased susceptibility to oxidative stress and fluctuations in energy provision. Chemotherapy induced sensory neurodegeneration and nociceptor sensitisation is attributable to the generation of reactive oxygen species that can activate ion channels, with cisplatin forming adducts with mtDNA, impairing mitochondrial function and induces morphological abnormalities. Mitochondria are dynamic organelles that are essential for providing the energy provision to support neuronal function and responders to neuronal stress. An impairment in neuronal mitochondrial function leading to dysregulated oxidative phosphorylation and a disrupted neuronal mitochondrial distribution, subsequently impairing neuronal ATP levels and engagement of alternative energy sources. Together, these changes induce atrophy and loss of DRG neurons and altered excitability of peripheral neurons contributing to chronic neuropathic pain.

This study will investigate if the uptake of monocyte- derived mitochondria recovers neuronal architecture and function. Finally, this study will elucidate new neuro-modulatory pathways involved in the transfer of mitochondria from monocytes to cisplatin- damaged sensory neurons, in order to identify a novel therapeutic target for alleviating chronic neuropathic pain in adult survivors of paediatric chemotherapy treatment.

Methods

Ethical approval and animals used

All experimental studies involving animals were performed following consultation with local Animal Welfare and Ethics Review Board at Nottingham Trent University and in accordance with UK Home Office animals (Scientific procedures) Act 1986 and ARRIVE guidelines. Animals had ad libitum access to standard chow and were housed under 12:12h light:dark conditions.

Immortalised Cell culture

Neuroblastoma cell line SH-SY5Y were maintained in Dulbecco's Modified Eagle Medium (DMEM), substituted with Fetal Bovine Serum (FBS) (10%), Penicillin and Streptomycin (Pen-Strep) (1%), and Sodium Pyruvate (1%), at 37°C and 5% CO_2. SH-SY5Y cells were cultured until 100% confluent, and plated in a 24-well plate at 5,000 per well. Monocyte Thp-1 cells were maintained in Rodwell Park Memorial Institute (RMPI) media, substituted with FBS (10%) and Pen-Strep (1%), at 37°C and 5% CO_2.

Primary Neuronal Cell Culture

Mouse Dorsal Root Ganglia (DRG) neurons were maintained in Modified Bottenstain & Sato's (BS) culture media, consisting of Ham's F12 (Gibco) medium, substituted with N2 (1%) (Gibco 17502-048), Bovine Serum Albumin (BSA) (1%) (Sigma A9576), and Pen-Strep (2%) at 37°C and 5% CO_2. Following dissection, DRG neurons were enzymatically dissociated with collagenase type XI (Sigma C9407) (0.0125%) for 2 hours at 37°C and 5% CO_2. Using either a 24-well plate or 8 well removable chambers (Ibidi), wells were coated in Poly-L-Lysine (Sigma P4707) overnight at 37°C and 5% CO_2. Poly-L- Lysine was removed, and Laminin (Sigma L2020) (1μM) was added to all wells for 2 hours. DRG neurons were centrifuged with two 15% BSA cushions, consisting of 1ml BSA, 1ml BS media, and 1ml DRG suspension, at 1000 relative centrifugal force (rcf) for 16 minutes, and pellets were resuspended in BS medium. Cells were counted and plated in either a 24-well plate or removable glass chambers at 7,000, and 5,000 DRG neurons per well, respectively. DRG neurons were allowed to grow at 37°C and 5% CO_2 for one week.

Drug Preparation and Experimental Treatments

Either DRG neurons or SH-SY5Y cells were treated with either vehicle or cisplatin (5μg/ml) (TOCIS 2251 5A/256231) for either 2 or 24 hours. Thp-1 cells previously labelled with Mitotracker Red CMXRos (1:10,000) were added to either DRG sensory neurons or SH-SY5Y cultures at 5,000 per well. In some instances, Thp-1 cells treated with cisplatin (5μg/ml) for 2 hours were added to DRG cultures. SH-SY5Y and DRG sensory neurons were treated with increasing concentrations of TAT Gap19 (TOCRIS 2267 9A) (0, 0.1, 1, 7, and 10μM) were pretreated for either 2 or 24 hours. THP1s were labelled with Mitotracker Red CMXRos (1:10,000, Thermofisher) were added to either DRG or SH- SY5Y cultures at 5,000 per well.

Immunohistochemistry

DRG neurons on glass coverslips in a 24- well plate were washed with Phosphate Buffered Saline (PBS) solution for 5 minutes and fixed using Paraformaldehyde (1%) for 15 minutes at room temperature. DRG neurons were then washed with PBS (twice for 5 minutes), and then permeabilised using PBS 0.2% Tritonx-100 for 5 minutes Coverslips were incubated in blocking solution (PBS 0.2% Triton x-100, 5% BSA) for 30 minutes at room temperature. Phalloidin (AlexaFlor®488 A12379) (1μM) was added to coverslips for 30 minutes at room temperature, and coverslips were washed twice with PBS for 5 minutes. DRG neurons were incubated at 4ºC overnight with β3 tubulin (Abcam Ab18207) (1μM). Coverslips were washed twice for PBS for 5 minutes. Coverslips were incubated with Alexafluor 647 donkey anti-mouse IgG (A31571) (2μM) for 2 hours at room temperature. Coverslips were washed twice with PBS for 5 minutes, and then incubated with DAPI (Abcam Ab228549) (1μM) for 5 minutes. Each coverslip was mounted onto glass slides using Vectasheild Antifade Mounting Medium. The LEICA Thunder microscope was used to visualise brightfield, β3 tubulin, phalloidin, and DAPI staining of DRG neurons, magnification X40. 3 images were acquired per coverslip.

Live cell imaging

The LEICA Thunder microscope was used to visualise Mitotracker Red CMXRos and Brightfield in either SH-SY5Y or DRG neurons. Images of brightfield and Mitotracker fluorescence (555nm) were acquired over 30 minutes, 1 hour, or 15 hours. Either magnification X20 or X63 oil immersion was used. 3 regions of interest were acquired per well of either a 24-well plate or removable glass slides.

Statistical Analysis

Analysis was carried out using image J (Fiji), Microsoft Excel, Imaris software, and GraphPad Prism 8 software. All data are presented as mean ± SD. Mitotracker functionality; Image J was used to quantify Mitotracker functionality in either SH-SY5Y or DRG neurons. 3 images were acquired per well of either a 24-well plate or removable glass chambers, and 3 regions of interest were analysed per image. The integrated density of Mitotracker fluorescence was measured in either SH-SY5Y or DRG neurons by using the freehand tool to draw around the circumference of either SH-SY5Y or DRG neurons in the Mitotracker fluorescence channel. Results were normalized to that of the vehicle for each experiment. Neurite outgrowth; Neurite outgrowth was measured using Image J. Parameters for neuronal identification were determined by the presence of β3 tubulin staining, and neurite outgrowth was determined as the length of phalloidin staining from the cell body. This was achieved by using the freehand line draw tool to draw from the phalloidin staining of the DRG cell body to the end of the neurite. A two-way ANOVA and a Dunnett's post hoc test was used to analyse change in Mitotracker fluorescence. A one-way ANOVA was used to determine significance in

neurite outgrowth in DRG neurons co-cultured with Thp-1 following cisplatin treatment compared to vehicle. A logarithmic scale was used to calculate the EC50 of Gap-19. P values are represented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and NS not significant.

Results

Cisplatin causes mitochondrial dysfunction in SH-SY5Y cells

To characterise the effects of cisplatin on neuronal mitochondrial function, the neuroblastoma cell line, SH-SY5Y, were fluorescently labelled with Mitotracker and subsequently treated with cisplatin (5µg/ml) or vehicle for 2 hours. Mitotracker CMXRos Red binds to complex IV of the mitochondrial electron transport chain (ETC) and facilitates the visualization of functional mitochondia. A reduction in Mitotracker fluroescence was observed in SH-SY5Y treated with cisplatin compared to vehicle (Figure 1A&B). To investigate the ability of monocytes to donate functional mitochondria to cisplatin damaged sensory neurons, SH-SY5Y cells were treated with either vehicle or cisplatin (5µg/ml). Consequently co-cultured with monocyte Thp-1 cells, which were either treated with vehicle or cisplatin (5µg/ml), and fluorescently labelled with Mitotracker. Mitotracker functionality was measured in SH-SY5Y for 30 minutes. An increase in Mitotracker functionality was observed in SH-SY5Y cells when monocytes were treated with vehicle. No increase in Mitotracker functionality was observed in SH-SY5Y cells when monocytes were treated with cisplatin (Figure 2A&B). To determine the transfer of mitochondria from monocytes to cisplatin-damaged sensory neurons, postnatal P7 mice Dorsal Root Ganglia (DRG) neurons were isolated, enzymatically dissociated, seeded, and cultured for 7 days. DRG neurons were treated with either vehicle or cisplatin (5µg/ml), and co-cultured with Thp-1 cells fluorescently labelled with Mitotracker. An increase in Mitotracker fluorescence was observed in cisplatin-treated DRG neurons compared to vehicle (Figure 3A&B). Additionally, cisplatin induced sensory neurodegeneration (Figure 4A-D) was prevented by coculture with THP1s as well as rescuing OCR values (Figure 4E). To identify a novel therapeutic pathway involving mitochondrial transfer from monocytes monocytes to cisplatin-damaged sensory neurons, connexin 43 was identified in DRG sensory neurons (Figure 5A&B). Furthermore, following cisplatin treatment there was increased expression of connexin 43 was induced in sensory neurons that presented increased neurodegeneration (Figure 5C&D). Therefore pharmacological inhibition of connexin 43 was adopted to investigate the effects on the transfer of mitochondria from monocytes to cisplatin-damaged sensory neurons. SH-SY5Y were treated with vehicle or cisplatin (5µg/ml) as well as TAT Gap-19 (connexin 43 inhibitor; 0, 0.1, 1, 7, and 10µM). A decrease in mitotracker visualisation in cisplatin treated SH-SY5Y was observed in a dose-dependent manner by Gap-19 (Figure 6A&B). Furthermore, connexin 43 inhibition also prevented monocyte mitochondrial donation to cisplatin damaged DRG sensory neurons (Figure 7A&B). To determine whether the transfer of mitochondria from monocytes to cisplatin damaged sensory neurons results in the recovery of neuronal architecture and was dependent upon connexin 43, DRG neurons were treated with either vehicle or cisplatin (5µg/ml) alongside TATGap19, and co- cultured with monocytes. DRG neurons were stained with β3 tubulin for neuronal identification, and phalloidin to determine neurite outgrowth (Figure 8A). An increase in neurite outgrowth was observed in cisplatin-treated DRG neurons following the uptake of monocyte donated mitochondria, which was diminished with increasing concentrations of TATGap19 (Figure

8B). In addition, monocyte donated mitochondria prevented cisplatin induced DRG sensory neuronal cell death was prevented by connexin 43 inhibition (Figure 8B).

Discussion

Systemic treatment with cisplatin invokes a general inflammatory response, causing the influx of immune cells into peripheral tissue. The monocytes that infiltrate this tissue release a cascade of pro-inflammatory mediators that influence the functionality of cells within the affected areas [15]. Importantly, neurons are a cell type that are profoundly affected by inflammation induced by cisplatin resulting in neuronal damage leading to peripheral neuropathies[6,12]. Although acute neuropathic pain may be attributed to local mediator release, this is less likely to result in progressively elevated long-term nociception as mediators released are often transitory with traditional analgesics lacking clinical efficacy. This suggests an alternative mechanism driving chronic pain pathology[8]. This has led to a current focus on sensory neuronal mitochondrial dysfunction having a key role in CIPN[17], through dysfunctional oxidative phosphorylation in neuronal mitochondria exposed to cisplatin[13]. This arises as neurons are highly metabolically active with greater ATP demands resulting in a higher density of mitochondrial than normal cells[5]. Therefore, damage to mitochondria following cisplatin treatment will significant impact sensory neuronal functionality and nociception through an oxidative stress driven mechanism. Recent evidence suggests that the drive for macrophages into neuronal tissue such as the dorsal root ganglia is a supportive one, with macrophages donating healthy mitochondria to neuronal cells resulting in an amelioration of pain[16].

This study demonstrates that monocytes transfer mitochondria to cisplatin-damaged sensory neurons to recover neuronal mitochondrial function, with the aim to stabilize neuronal energy balance and alleviate dysfunction that can lead to chronic neuropathic pain. The mechanism that monocytes transfer mitochondria to damaged neurons is through a gap junction mediated mechanism driving a recovery in the architecture of cisplatin- damaged sensory neurons. There is a dynamic balance between mitochondrial fission and fusion that is crucial for maintaining neuronal mitochondrial function and integrity. In the pathophysiology of both inflammatory and neuropathic pain, an essential role of Drp1 in driving mitochondrial fission was uncovered[18]. Here we showed that cisplatin caused an upregulated neuronal mitochondrial fission in cisplatin-damaged sensory neurons producing fragmented mitochondria. This has been associated with impairment in neuronal mitochondrial Ca^{2+} channels, leading to decreased nociceptor excitability, and the potentiation of chronic neuropathic pain.

We also observed damage to the neuronal mitochondrial network and decreased mitochondrial localization in the neurites of cisplatin-treated DRG neurons in comparison to vehicle treated controls, with mitochondria primarily localized within the cell body. This is a result of the inhibition of microtubule assembly induced by cisplatin, causing the disruption of neuronal mitochondrial trafficking[14]. Additionally, cisplatin exposure resulted in dysmorphic neuronal mitochondria which had a decreased surface area which is consistent with the pathophysiological features of mitochondrial damage and are indicative of a compromised neuronal bioenergetic state.

Cell-cell coupling, and the intercellular exchange of ions, metabolites, and cellular organelles, including mitochondria, critically depend on the involvement of Cx43 in the formation of gap junctions (GJs) and hemichannels (HCs)[4]. A model for the transfer of mitochondria from cells to cells has been proposed to involve the internalization of gap junctions containing Cx43.

Mitochondria present within cellular protrusions that connect to other cells by invaginating gap junctions can become fully enclosed within internalized gap junctions in a receiving cell facilitating mitochondrial transfer. To assess the involvement of Cx43 in mitochondrial transfer between monocytes and cisplatin-damaged neurons, we inhibited the functional properties with a Cx43 inhibitor, Gap-19. This showed a dose-dependent inhibition of mitochondrial transfer from monocytes to cisplatin- damaged sensory neurons confirming Cx43 involvement in the process. These observations provide a promising mechanism for therapeutic target of chronic pain in adult survivors of paediatric cancers.

Author contribution statement: RPH, MPC, BO, JC performed the experimental work and contributed to the conception or design of the work in addition to acquisition, analysis or interpretation of data for the work. All authors drafted the article or revised it critically for important intellectual content. All authors approved the final version of the manuscript.

Acknowledgements:
This work was supported by the Medical Research Foundation (MRF-RGM-CACP-23-104 to RH and MPC) and Nottingham Trent University. The authors would like to thank Graham Hickman of the Imaging Suite at Nottingham Trent University for their support and assistance in this work.

Data availability statement: All data is presented in this article and is available by reasonable request from the corresponding author.

Conflict of interest disclosure: Authors have nothing to disclose.

Ethics approval statement: This work has not been published or under review for publication elsewhere. All authors have seen and approved the final version of the abstract. All studies involving rodents were approved by local ethical review board at host institutions and in accordance with UK Home Office Legislation.

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Figure Legends

Figure 1:

Cisplatin causes mitochondrial dysfunction in SH-SY5Y cells. [A] SH-SY5Y cells were either treated with either vehicle or cisplatin (5µg/ml) for 2 hours, and Mitotracker functionality was measured for 15 hours. [B] A decrease in Mitotracker- labelled fluorescence was observed in cisplatin-treated SH-SY5Y cells compared to vehicle. **** $p<0.0001$, one-way ANOVA and a Dunnett's post-hoc test for multiple comparisons. Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. Error bars are mean + SD.

Figure 2: Monocytes transfer mitochondria to cisplatin-treated SH-SY5Y cells. [A] SH-SY5Y cells were treated with either vehicle or cisplatin (5µg/ml) and co-cultured with Thp-1 cells fluorescently labelled with Mitotracker. Mitotracker functionality was measured for 15 hours. [B] When co-cultured with Thp-1 cells, an increased uptake of Mitotracker-labelled mitochondria was observed in cisplatin-treated SH-SY5Y cells compared to vehicle. **** $p<0.0001$, one-way ANOVA and a Dunnett's post-hoc test for multiple comparisons. Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. Error bars are mean + SD.

Figure 3: Monocytes transfer mitochondria to cisplatin-treated DRG neurons. [A] Neonatal P7 mice Dorsal Root Ganglia (DRG) neurons were dissected and isolated, enzymatically dissociated, seeded, and cultured for 7 days. DRG neurons were treated with either vehicle or cisplatin (5µg/ml) and co-cultured with Thp-1 cells fluorescently labelled with Mitotracker. [B] When co-cultured with Thp-1 cells, an increased uptake of Mitotracker-labelled mitochondria was observed in cisplatin-treated DRG neurons compared to vehicle. **** $p<0.0001$ at t=12 mins, two-way ANOVA and a Dunnett's post-hoc test for multiple comparisons. Images were acquired using a Leica Thunder Microscope, Magnification X20. N = 12. Scale bar; 50µm. Error bars are mean + SD.

Figure 4: Mitochondrial transfer from monocytes to DRG neurons recovers neuronal architecture. DRG sensory neurons were treated with either vehicle or cisplatin (5µg/ml) for 2 hours, and co-cultured with Thp-1 cells overnight. DRG neurons were fixed and stained with β3 tubulin and phalloidin. An increased neurite length was observed in cisplatin-treated DRG neurons following the uptake of monocyte-derived mitochondria.**** $p<0.0001$, one-way ANOVA and a Dunnett's post- hoc test for multiple comparisons. [A] Neurite length, [B] number and [C] total neurite growth was increased in cisplatin-treated DRG neurons following the uptake of monocyte-derived mitochondria. *** $P<0.001$, one-way ANOVA and a Dunnett's post-hoc test for multiple comparisons. Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. N = 27. Error bars are mean + SD.

Figure 5: Connexin 43 expression in DRG sensory neurons is increase as cisplatin treatment. [A] Representative image of DRG neurons treated with either vehicle or cisplatin (5µg/ml) for 2 hours, demonstrated expression of connexin 43. [B] Cisplatin induced sensory neurodegeneration demonstrated by reduced neurite growth. [C] Connexin 43 was increased following cisplatin treatment, [D] with increased expression identified on DRG sensory neurons that had less neurite growth. *** P<0.001, one-way ANOVA and a Dunnett's post-hoc test for multiple comparisons. Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. N = 27. Error bars are mean + SD.

Figure 6: Mitochondrial transfer from monocytes to SH-SY5Y is mediated by connexin 43. SH-SY5Y were treated with either vehicle or cisplatin (5µg/ml) and increasing concentrations of TATGap-19. Subsequently were [A] co-cultured with mitotrackcer labelled monocyte Thp-1 cells. [B] TATGap-19 treatment resulted in a decreased uptake of Mitotracker-labelled mitochondria by vehicle and cisplatin-treated SH-SY5Y. **** p<0.0001, one-way ANOVA and a Dunnett's post-hoc test compared to 0µM. Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. EC50; Veh = 3.582µM, Cis = 7.185µM. N = 9. Error bars are mean + SD.

Figure 7: Mitochondrial transfer from monocytes to DRG sensory neurons is mediated by Connexin 43 inhibition. DRG sensory neurons were treated with either vehicle or cisplatin (5µg/ml) and increasing concentrations of TATGap-19. Subsequently were [A] co-cultured with mitotrackcer labelled monocyte Thp-1 cells. [B] TATGap-19 treatment resulted in a decreased uptake of Mitotracker-labelled mitochondria by vehicle and cisplatin-treated DRG sensory neurons. **** p<0.0001, one-way ANOVA and a Dunnett's post-hoc test compared to 0µM. Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. Error bars are mean + SD.

Figure 8: Connexin 43 inhibition prevents mitochondrial transfer from monocytes to DRG neurons recovers neuronal architecture. [A] Representative images of DRG neurons treated with either vehicle or cisplatin (5µg/ml) and co-cultured with Thp-1 cells (neuron; β3 tubulin and F actin; phalloidin) in response to TATGap19. [B] An increased neurite length was observed in cisplatin-treated DRG neurons following the uptake of monocyte-derived mitochondria, which was prevented by connexin 43 inhibition (**** p<0.0001, one-way ANOVA and a Dunnett's post- hoc test for multiple comparisons). [C] Decreased cell death (Propidium Iodide) was observed in cisplatin-treated DRG neurons following the uptake of monocyte-derived mitochondria. This was prevented following connexin 43 inhibition (*** P<0.001, one-way ANOVA and a Dunnett's post-hoc test for multiple comparisons). Images were acquired using a Leica Thunder Microscope, Magnification X20. Scale bar; 100µm. Error bars are mean + SD.

Figure 1

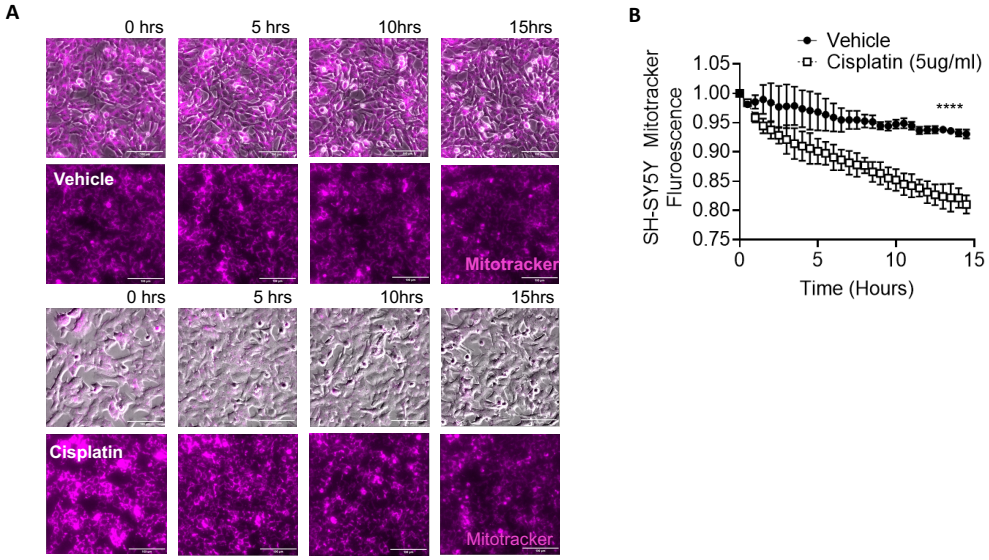

Figure 2

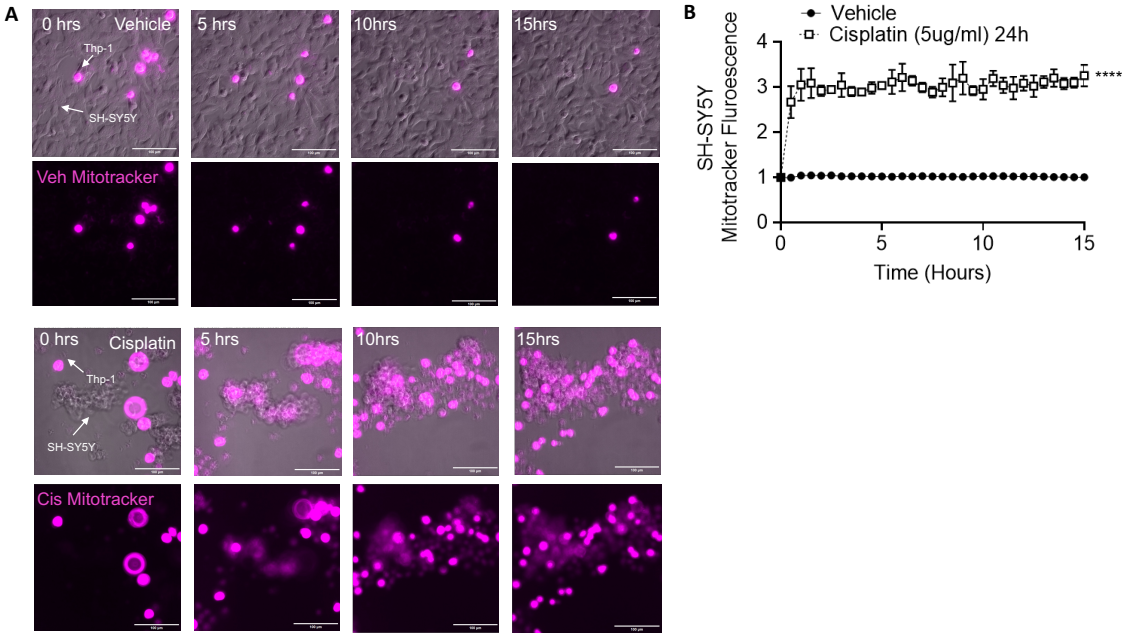

Figure 3

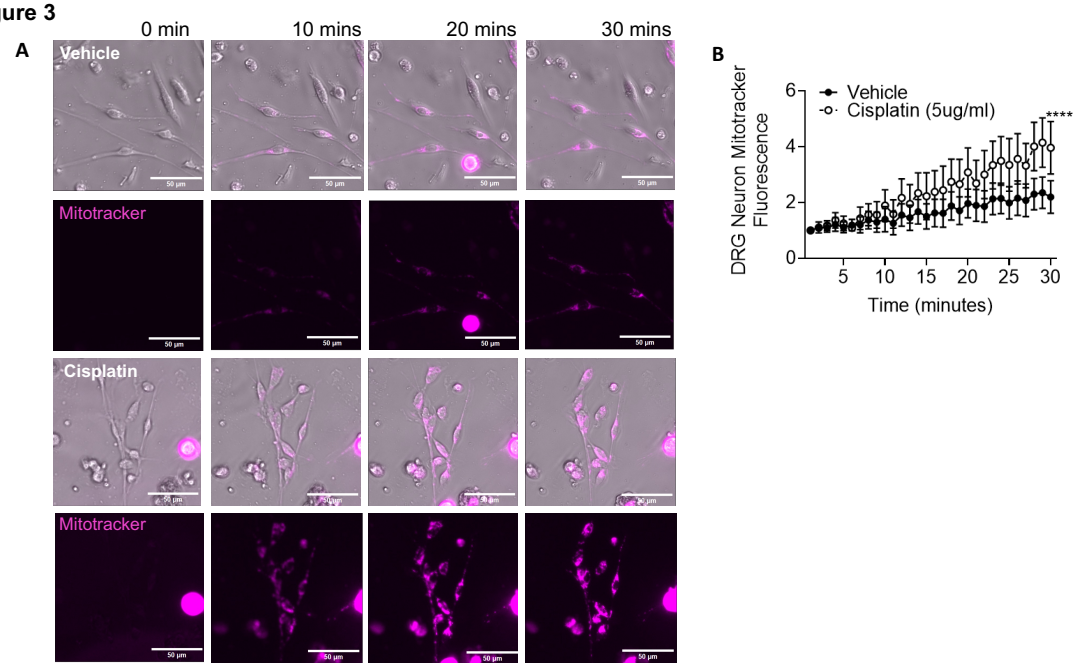

Figure 4

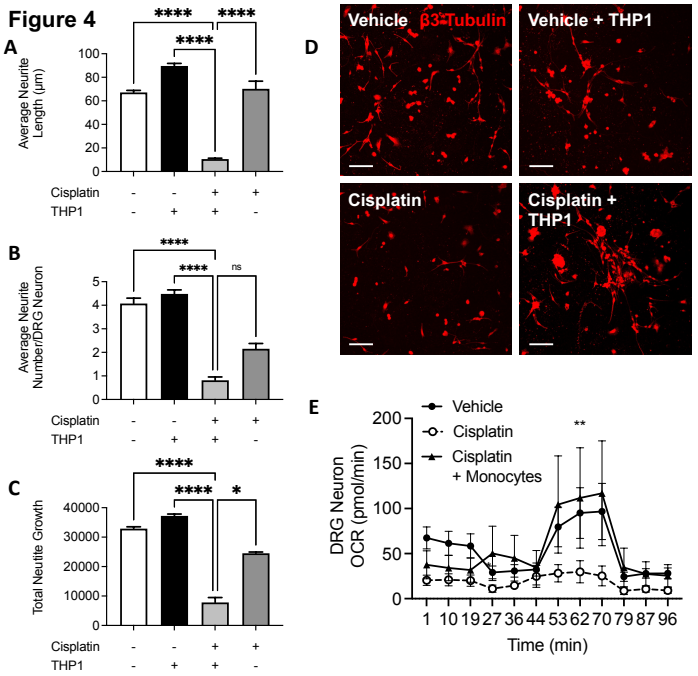

Figure 5

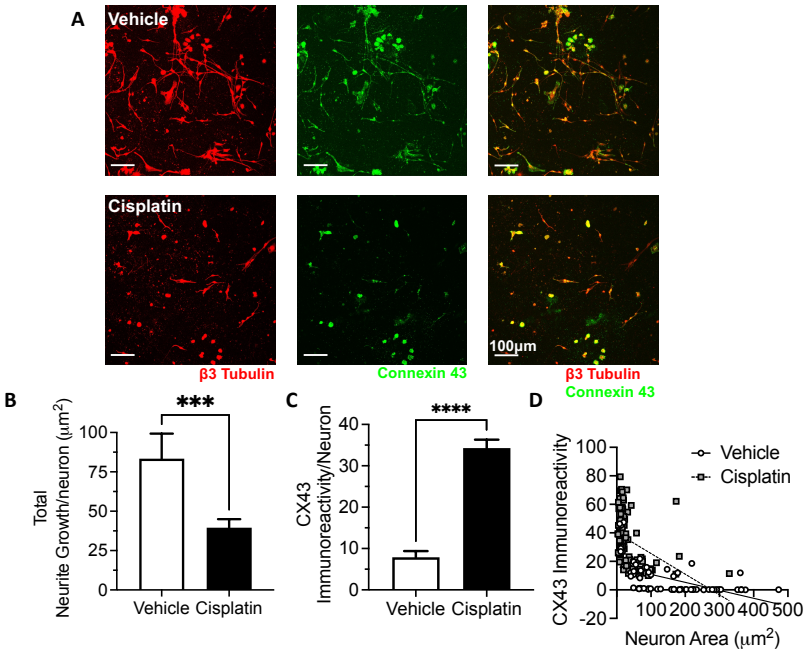

Figure 6

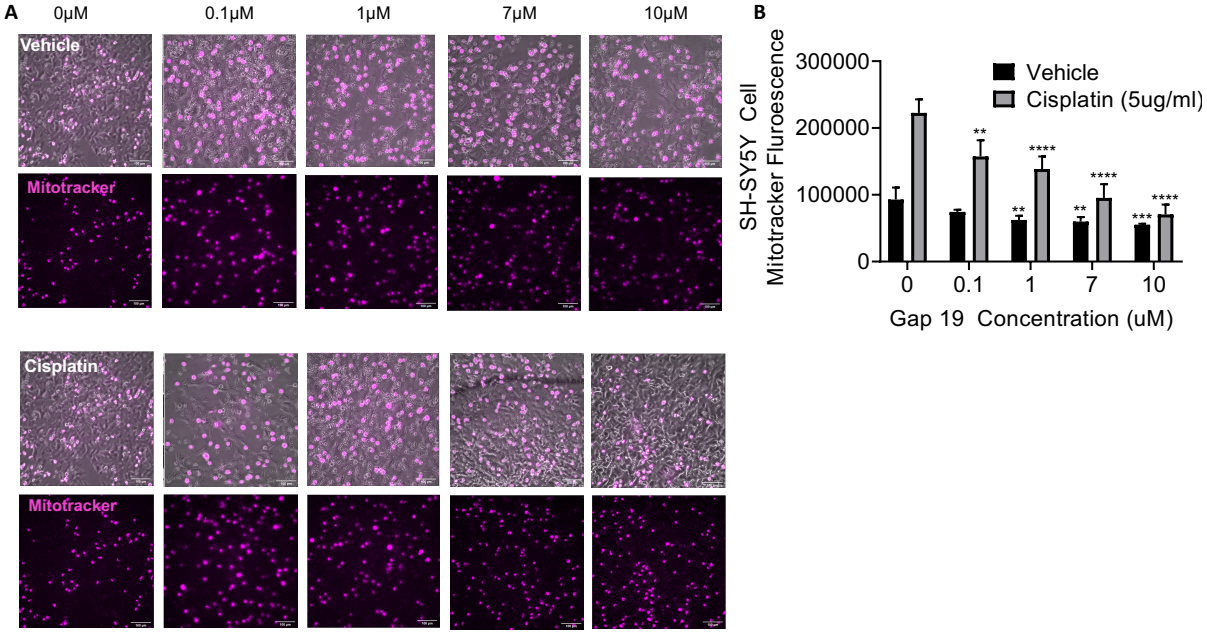

Figure 7

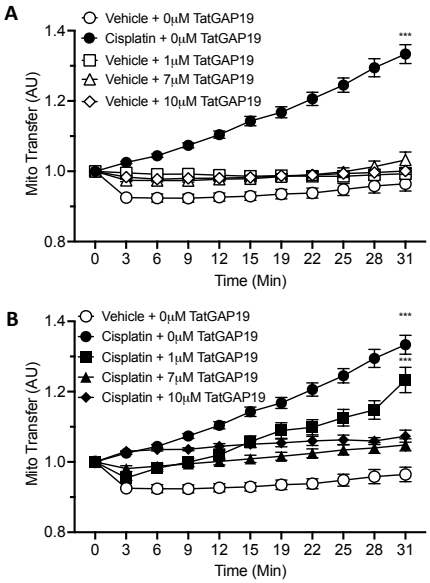
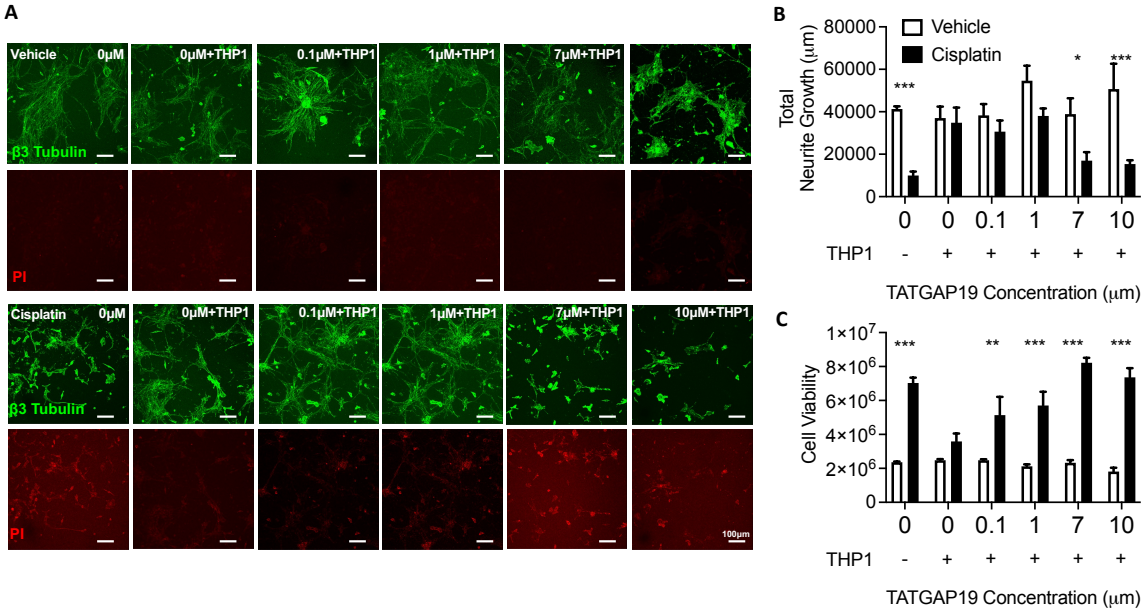
Figure 8