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Drosophila melanogaster: A new model to study cisplatin-induced neurotoxicity

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ABSTRACT

Platinum-based compounds are widely used and effective chemotherapeutic agents; however, sensory peripheral neuropathy is a dose-limiting and long term side effect for 20–30% of patients. A critical question is whether the mechanisms of cell death underlying clinical efficacy can be separated from the effects on neurons in order to develop strategies that prevent platinum-induced neuropathy. In rodent dorsal root ganglion neurons (DRG), cisplatin has been shown to bind and damage neuronal DNA, inducing apoptosis; however genetic manipulation in order to study mechanisms of this phenomenon in the rodent model system is costly and time-consuming. *Drosophila melanogaster* are commonly used to study neurological disorders, have DNA damage-apoptosis mechanisms homologous to mammalian systems, and have readily-available, inexpensive tools for rapid genetic manipulation. We therefore sought to develop adult *Drosophila* as a new model to study cisplatin-induced neurotoxicity. Adult *Drosophila* were exposed to 10, 25, 50, 100, 200 and 400 μg/ml cisplatin for 3 days and observed for fly survival and geotactic climbing behavior, cisplatin-DNA binding and cellular apoptosis. On day 3, 50 μg/ml cisplatin reduced the number of flies able to climb above 2 cm to 43% while fly survival was maintained at 92%. 100% lethality was observed at 400 μg/ml cisplatin. Whole fly platinum-genomic DNA adducts were measured and found to be comparable to adduct levels previously measured in rat DRG neurons. Brain, ovaries, kidney and heart harvested from cisplatin treated flies were stained for active caspase 3. Apoptosis was found in ovaries and brain but not in heart and kidney. Brain apoptosis was confirmed by transmission electron microscopy. Expression of the anti-apoptotic baculoviral protein, p35, in neurons using the GAL4-UAS system prevented cisplatin-induced apoptosis in the brain and restored climbing behavior. In conclusion, cisplatin-induced behavioral and apoptotic changes in *Drosophila* resemble those seen in mammals. Furthermore, the use of lethality and climbing assays combined with powerful gene manipulation, make *Drosophila* a suitable model to study mechanisms of cisplatin neurotoxicity.

Introduction

Chemotherapy induced peripheral neuropathy is a serious, life long side effect that impairs quality of life for long-term cancer survivors. Cisplatin and oxaliplatin are used to treat ovarian, testicular and metastatic colorectal cancers. Unfortunately, platinum drugs induce neuropathy in 20–30% of patients, which is the dose-limiting side effect (Thompson et al., 1984; Cavaletti, 2008; Windebank and Grisold, 2008). Mammalian models have been developed to investigate the mechanisms of platinum-induced neurotoxicity (Cavaletti et al., 1998; Carozzi et al., 2010b; Carozzi et al., 2010a). Cisplatin and oxaliplatin induce sensory behavioral changes in mice, in vivo (Ta et al., 2009). Mice treated with cisplatin exhibit thermal hyperalgesia to heat stimuli and mechanical allodynia. The chemotherapeutic mechanism of cisplatin and oxaliplatin is through DNA binding, inducing DNA damage that leads to apoptosis. Likewise, data from mammalian *in vitro* and *in vivo* models have demonstrated that sensory neurons undergo cisplatin-induced apoptosis through a DNA damage mechanism that is similar to the mechanisms observed in cancer cells (Fischer et al., 2001; McDonald and Windebank, 2002; McDonald et al., 2005; Ta et al., 2006).

Drosophila melanogaster have been used to study the genetics of human neurodegenerative diseases (Feany, 2010). In Parkinson's disease (PD), mutations in *Drosophila* parkin and PINK1 have been linked to the mitochondrial pathology of PD. Expression of human α-synuclein in *Drosophila* using the GAL4-UAS system induces a PD phenotype and allows for the study of toxicity induced by α-synuclein aggregation (Botella et al., 2009). Alzheimer's disease can be modeled in transgenic *Drosophila* expressing genes which induce the production of amyloid-ß peptide. Aggregation of amyloid-ß is observed in these flies along with age related neurodegeneration and memory loss (Iijima and Iijima-Ando, 2008).

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The genotoxic and mutagenic effects of cisplatin have been studied in *Drosophila* (Woodruff et al., 1980; Katz, 1987). Genotoxic potency of cisplatin was measured using a wing spot assay (Katz, 1998). Cisplatin induced an increase in large spots indicating genotoxic alterations. Also, platinum-DNA adducts measured in *Drosophila* larva induced DNA damage in larval brain (Garcia Sar et al., 2008). Many critical biological systems are highly conserved between *Drosophila* and mammals with many genes involved in the mammalian apoptotic pathway having *Drosophila* homologues (Steller, 2008; Xu et al., 2009). It is therefore likely cisplatin induces cellular apoptosis in *Drosophila* similar to that seen in mammals (Ta et al., 2006; Wang and Youle, 2009).

We and others have used *in vitro* and *in vivo* rodent models to study cisplatin-induced neurotoxicity (Cavaletti et al., 1998; Meijer et al., 1999; Cavaletti et al., 2002; McDonald and Windebank, 2002; McDonald et al., 2005; Ta et al., 2006; Ta et al., 2009). Cell culture is a simple model system to study the direct effect of drugs on specific cell populations and rodent models are an effective way to study whole body drug effects. However, culture systems have limitations and molecular manipulation of death pathways in rodent models is costly and time consuming. *Drosophila* melanogaster are an established model in the study of disease genetics. Transgenic animals are easy to generate with a variety of tools to control the expression or inhibition of specific genes and can be done in a relatively short amount of time with little cost. Established neurobehavioral assays have also been developed to reliably quantitate phenotypes in *Drosophila*. Our experiments were designed to establish a model to study cisplatin neurotoxicity in *Drosophila* melanogaster.

Methods

Drosophila treatment

Oregon Red *Drosophila* melanogaster were raised in vials containing molasses, cornmeal, yeast, agar, TegoSept and proprionic acid at 25° C. For our experiments, flies were placed into empty vials with 50 flies per vial and 2–3 vials per condition. Flies were treated with 0, 10, 25, 50, 100, 200 and 400 μg/ml cisplatin (APP Pharma., Schaumburg, IL) diluted in 10% sucrose-Dulbecco's phosphate buffered saline (DPBS). After an 8–10 h starvation period, flies were given 200 μl of the appropriate drug concentration. Additional drug was given 24 and 48 h later. On day 3, flies were removed from the drug and placed into normal food vials to lay eggs. On day 6, the flies were removed from the vials and either harvested for immunostaining or placed into new food vials and kept until day 9.

Quantitation of platinum levels

To measure platinum levels in *Drosophila*, flies were treated with or without 100 μg/ml cisplatin for 3 days. *Drosophila* were anesthetized with CO_2, placed into 95% ethanol, dehydrated and weighed. Whole flies were homogenized in DPBS and hydrochloric acid was added to a concentration of 6 N for whole body platinum measurements. For platinum-total DNA measurements, genomic DNA was isolated from treated flies using a Genomic-tip, DNA isolation kit (Qiagen, Valencia, CA). Total DNA was measured using a Nano-Drop spectrophotometer (Bio-Rad, Hercules, CA) and the sample was diluted to 6 N hydrochloric acid in DPBS. Platinum levels were measured using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (McDonald et al, 2005) and expressed as ng platinum/mg fly weight or ng platinum/mg total DNA.

Lethality and climbing assays

On days 3, 6 and 9 of our experiments, live flies were counted and tested for geotactic climbing ability. Lethality was expressed as percent of surviving flies compared to the number of live flies at the beginning of the experiment. The climbing assay was modified from Orso and colleagues (Orso et al., 2005). From each test vial, 10 flies were placed into an empty vial with a mark placed 2 cm from the bottom. The flies were gently knocked to the bottom of the vial by tapping on the counter and the number of flies that climbed above a 2 cm line was counted after 20 s. This was repeated 5 times per vial with 2 vials per condition. Climbing ability was expressed as a percent of the number of flies above the 2 cm line as compared to the total number of flies.

Ovary apoptosis

Cisplatin treated flies were anesthetized on day 6 using CO_2. Ovaries were removed in PBS and fixed with 4% paraformaldehyde. The ovaries were stained with 1:500 anti-active caspase 3 (Cell Signaling Tech. Inc., Danvers, MA) polyclonal antibody and 1:200 anti rabbit Cy 2 (Jackson Immunoresearch, West Grove, PA) followed by 1 μg/ml bisbenzimide to stain nuclei. Ovaries were imaged using a Zeiss Axiovert fluorescent microscope (Carl Zeiss Microimaging Inc., Thornwood, NY). Oocytes contain multiple cells and not every cell within the oocyte is at the same stage of apoptosis making for an uneven morphology, we therefore counted the individual oocytes containing any caspase 3 activity. The caspase positive oocytes were counted and expressed as a percent of the total number of oocytes.

Heart, kidney and brain apoptosis

On day 6, flies treated with 50 μg/ml cisplatin were anesthetized with CO_2 and prefixed with a 1.25% formaldehyde-octane fixative for 30 min. The heads and bodies were separated and the thorax cut away from the abdomen. Fly brains were removed from heads and abdomens cut open down the midline followed by postfixation in 0.016 % formaldehyde for 90 min. Brain and abdomen were stained with 1:200 anti-active caspase 3 polyclonal antibody and 1:200 anti-rabbit FITC (Jackson Immunoresearch, West Grove, PA). Heart and kidney were removed from the abdomen and stained with 1 μg/ml bisbenzimide. The heart was additionally stained with 1:2 anti-pericardin (DSHB, U. of Iowa) monoclonal antibody and 1:100 anti-mouse Cy3 (Jackson Immunoresearch, West Grove, PA) to stain heart myocytes. Brains were also stained with 1:5 anti-elav monoclonal antibody (DSHB, U. of Iowa) and 1:100 anti-mouse Cy3 to stain all neurons. Images were acquired using a Zeiss LSM 500 laser scanning confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY). Brain apoptosis was quantitated by systematically acquiring images from all different brain regions. Four images were acquired per brain with 8–10 brains per condition. Percent apoptosis was calculated by counting the number of elav positive neurons compared to the number neurons co-labeled with active caspase 3.

Electron microscopy

Drosophila brains treated with 100 μg/ml cisplatin were dissected fresh, fixed in Trump's fixative consisting of 4% formaldehyde and 1% glutaraldehyde in PBS at pH 7.2, post fixed in 1% osmium tetroxide and stained *en bloc* with 2% uranyl acetate. The brains were embedded in mixture of Epon and araldite. All reagents were obtained from Electron Microscope Services (Ft. Washington, PA). Ultrathin, 100 nm sections were cut from the same blocks, mounted on 200 μm mesh copper grids, stained with lead citrate. Images were acquired using a Joel ExII Transmission Electron Microscope.

p35 experiments

Drosophila were bred to express p35 via the elav neuron-specific promoter. p35 is a baculoviral anti-apoptotic protein that inhibits caspase activity (Martin et al., 2009). Elav-GAL4 flies were obtained from Corey Goodman's Laboratory (University of California, Berkeley) and UAS-p35 flies were obtained from Bruce Hay's Laboratory (California Institute of Technology). Each fly strain was bred with w;Adv/SM5-TM6B balancer flies. Flies were selected from F1 with white eyes and curly wings. The elav-GAL4/balancers were bred to the

UAS-p35/balancers to create elav-GAL4/UAS-p35 expressing flies. These flies were treated with 100 μg/ml cisplatin for 3 days followed by survival and climbing assay. On day 6, flies were anesthetized with CO_2 and tissue fixed with a formaldehyde-octane fixative as previously described. Brains were stained with either 1:400 anti-active caspase 3 for apoptosis or 1:1000 anti-p35 (IMGENEX, San Diego, CA) polyclonal antibody for p35 expression and 1:800 anti-rabbit Cy 2 followed by 1:5 rat anti-elav antibody and 1:400 anti-rat Cy3 to stain all neurons. Ovaries were removed from the abdomen and stained with either 1:1000 anti-active caspase 3 or 1:1000 anti-p35 and 1:800 anti-rabbit Cy2 followed by 1 μg/ml bisbenzimide to stain nuclei. Images were acquired using laser scanning confocal microscopy.

Cyclophosphamide experiments

Drosophila were treated with 0, 200, 400, 800, 1400, 2000, 3000 and 4000 μg/ml cyclophosphamide using the same experimental parameters as cisplatin treatment. Cychophosphamide, like cisplatin, is a chemotherapeutic drug that binds the N7 position of guanine inducing inter- and intrastrand DNA crosslinks and DNA damage (Emadi et al., 2009), however, does not induce neurotoxicity. Survival and climbing behavior assays were performed on surviving flies followed by active caspase 3 immunostaining of brain, kidney and ovaries. On day 6, flies were anesthetized with CO_2 and tissue fixed with a formaldehyde-octane fixative as previously described. Brains were stained with 1:400 anti-active caspase 3 and 1:800 anti-rabbit Cy 2 to stain for apoptosis and with 1:5 rat anti-elav and 1:400 anti-rat Cy3 to stain all neurons. Ovaries and kidneys were removed from the abdomen and stained with 1:1000 anti-active caspase 3 and 1:800 anti-rabbit Cy2 and 1 μg/ml bisbenzimide to stain nuclei. Images were acquired using laser scanning confocal microscopy.

Statistical methods

Data was analyzed for means and SEM using one-way analysis of variance (ANOVA) of data with parametric distribution (Gaussian). Statistical significance was analyzed using Tukey-Kramer multiple comparison post-test. Brain apoptosis was analyzed by two-tailed paired t test.

Results

Fly platinum levels

Platinum levels were measured in whole fly and total DNA isolated from whole fly (Table 1). Whole body platinum levels were 18.9 ng elemental platinum/mg fly body weight in flies treated with 100 μg/ml cisplatin for 3 days. Platinum binding to genomic DNA was 118 ng platinum per mg total DNA which is equivalent to 1 platinum molecule per 2500 base pairs of DNA. Background controls measured 0.003 ng (whole body) and <0.002 ng (total DNA) elemental platinum/mg fly body weight.

Survival and behavior changes

Cisplatin killed *Drosophila* in a dose dependent manner (Fig. 1A). Flies were exposed to 0, 10, 25, 50,100, 200 and 400 μg/ml cisplatin and percent survival was determined at 3 days: 92%, 92%, 87%, 92%, 74%, 29%, 1%, 6 days: 90%, 89%, 82%, 89%, 60%, 16%, 0% and 9 days: 86%, 88%, 82%, 79%, 54%, 12%, 0%. There was no significant fly death induced by 10, 25 or 50 μg/ml cisplatin at 3, 6 and 9 days ($P>0.05$). Survival decreased with 100 and 200 μg/ml cisplatin ($P<0.01$ to $P<0.001$) and 100% lethality was observed with 400 μg/ml cisplatin.

Table 1
Platinum levels in whole fly and genomic DNA isolated from whole fly were measured by Inductively Coupled Plasma Mass Spectroscopy.

Cisplatin (μg/ml)	0	100
ng Pt/mg fly	0.003	18.9
ng Pt/mg tDNA	<0.002	118 (1Pt:2500 bp DNA)

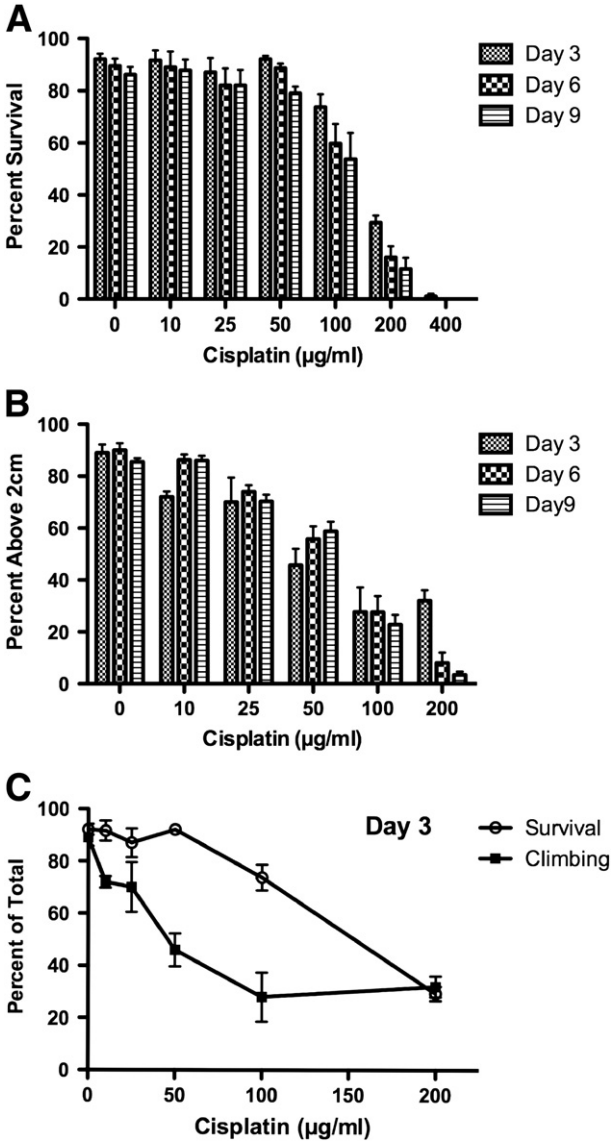

Fig. 1. Cisplatin decreased fly survival and inhibited geotaxis climbing behavior. Lethality and climbing behavior were measured in cisplatin treated flies on days 3, 6 and 9. Cisplatin induced a dose dependent decrease in fly (A) survival ($p<0.001$) and (B) geotaxis climbing ability ($p<0.0001$). (C) On day 3, climbing ability was impaired at non-lethal cisplatin concentrations. Survival in *Drosophila* treated with 50 μg/ml cisplatin was 92%, with only 46% of the flies able to climb above 2 cm.

Inhibition of geotactic climbing behavior by cisplatin was dose dependent (Fig. 1B). Flies above the 2 cm line at the end of the 20 s time period were counted. Percent of flies above the 2 cm line treated with 0, 10, 25, 50, 100 and 200 μg/ml cisplatin was 89%, 72%, 70%, 46%, 28%, 32% at 3 days, 90%, 86%, 74%, 56%, 28%, 8% at 6 days and 86%, 86%, 70%, 59%, 23%, 3% at 9 days. There were not enough flies to do the assay with 400 μg/ml cisplatin. No significant decrease in climbing was observed with 10 or 25 μg/ml cisplatin at 3 and 6 days ($p>0.05$). Climbing was significantly impaired with 50, 100 and 200 μg/ml cisplatin at 3, 6 and 9 days ($P<0.01$ to $P<0.001$). Inhibition of climbing behavior was observed at non-lethal doses of cisplatin (Fig. 1C). On day 3, 50 μg/ml cisplatin inhibited climbing behavior 46% while 92% of the flies survived.

Caspase activity

Caspase 3 activation was observed in ovaries and brain isolated from cisplatin treated flies. Oocytes are a rapidly dividing cell and were used as a positive control for cisplatin-induced apoptosis. Ovaries (Fig. 2A) were isolated from *Drosophila* treated with or without 50 μg/ml cisplatin and immunostained for active caspase 3 and bisbenzimide. Control ovaries (Fig. 2B) had normal nuclei as visualized with bisbenzimide and no active caspase 3 staining. Immunostaining of cisplatin treated ovaries (Fig. 2C) showed apoptosis with fragmented nuclei and positive caspase 3 staining in oocytes. Apoptosis was quantitated in ovaries isolated from flies treated with 0, 10, 25, 50, 100 and 200 μg/ml cisplatin (Fig. 2D) by counting the number of caspase 3 positive oocytes. Apoptosis was: 3%, 5%, 11%, 19%, 32% and 33%. When the concentration of cisplatin was increased to 100 μg/ml cisplatin reproduction was interrupted and the first generation of flies was lost (data not shown).

Drosophila brain (Fig. 3A) was harvested from cisplatin treated flies and stained for active caspase 3 and a neuron specific nuclear transcription protein, elav. Control brain stained for elav (Fig. 3B) and active caspase 3 (Fig. 3D) showed no caspase staining in neurons (Fig. 3F). Brain from cisplatin treated flies stained for elav (Fig. 3C) and active caspase 3 (Fig. 3E), showed positive caspase 3 staining co-localized with elav neuronal staining (Fig. 3G). Apoptosis was quantitated in brains treated with 50 μg/ml cisplatin (Fig. 3H). Control brain ($n=10$) had 1.11% apoptosis as compared to 16.23% apoptosis in cisplatin treated brain ($n=8$) ($P=0.0007$). Brain apoptosis was confirmed by electron microscopy. Images of control (Fig. 3I) brain show normal neurons with intact plasma and nuclear membranes while nuclear fragmentation in cells with intact plasma membranes were observed in brains treated with 100 μg/ml cisplatin (Fig. 3J).

There was no caspase 3 activation in heart or kidney harvested from cisplatin treated flies. *Drosophila* heart (Fig. 4A) was stained with antibodies for active caspase 3, pericardin and bisbenzimide. There was no nuclear fragmentation or active caspase 3 staining in control heart (Fig. 4B) or hearts treated with 50 μg/ml cisplatin (Fig. 4C). The same results were found in *Drosophila* kidney (Fig. 4D) with no active caspase 3 activity or nuclear fragmentation in control (Fig. 4E) or cisplatin treated kidney (Fig. 4F).

p35 inhibition of cisplatin neurotoxicity

Elav-GAL4 and UAS-p35 *Drosophila* were bred to express non-apoptotic p35 behind the elav promotor in neurons (Fig. 5A). Brains harvested from wild type and elav-p35 *Drosophila* were immunostained for p35 expression and caspase 3 activation. There was no expression of p35 in wild type brain (Fig. 5B). Expression of p35 was observed in elav-p35 brain neurons (Fig. 5C) and this expression prevented caspase activation in response to 100 μg/ml cisplatin treatment (Fig. 5E). To ensure p35 protection was neuron specific and drug treatment was sufficient to induce apoptosis, we stained wild type and elav-p35 ovaries for p35 and active caspase 3. No p35 expression was observed in wild type (Fig. 5F) and elav-p35 (Fig. 5G) ovaries and cisplatin induced caspase activation in elav-p35 ovaries (Fig. 5I). No caspase activation was observed in control elav-p35 brain (Fig. 5D) or non-treated elav-p35 ovaries (Fig. 5H). Elav-p35 *Drosophila* treated with or without 100 μg/ml cisplatin were observed on day 6 for survival (Fig. 5J) and climbing behavior (Fig. 5K). Survival of control flies was 96% in wild type and 100% in elav-p35 flies. Cisplatin treatment reduced fly survival to 66% and expression of p35 in brain neurons prevented fly death with 98% survival ($p=0.0347$). The percent of flies able to climb above 2 cm was 79% in wild type controls and 93% in elav-p35 controls. Climbing was reduced to 7% in wild type cisplatin treated flies and was significantly increased to 73% by p35 expression in neurons ($P<0.0001$).

Cyclophosphamide

Survival in cyclophosphamide treated flies decreases in a dose dependent manner while climbing behavior of surviving flies was

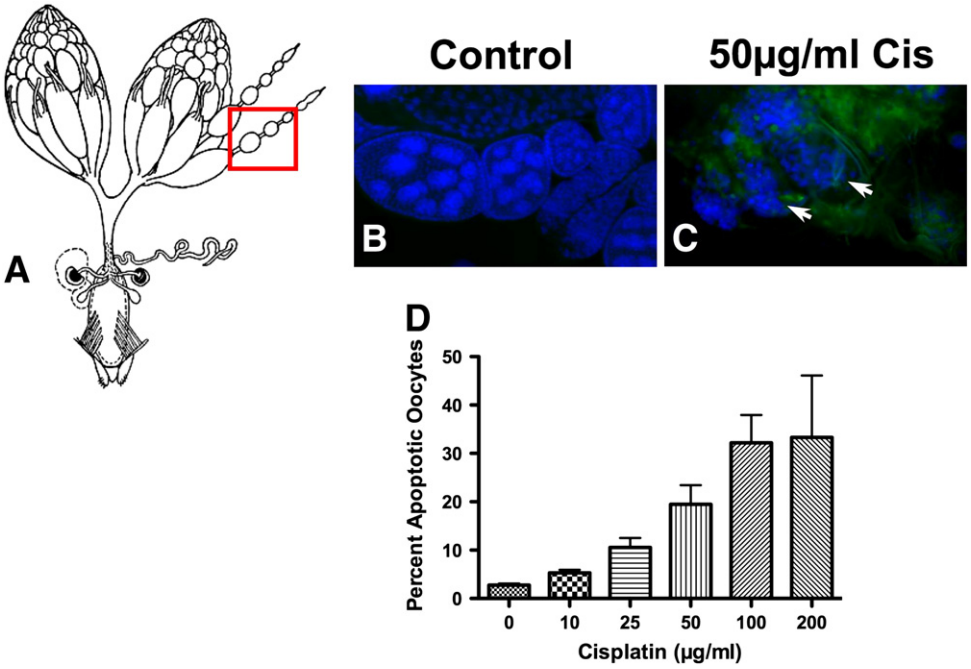

Fig. 2. Cisplatin-induced apoptosis in *Drosophila* oocytes. To determine if drug delivery was effective, ovaries harvested from flies treated with and without 50 μg/ml cisplatin were immunostained and oocytes were quantitated for apoptosis. (A) Ovaries (www.flybase.org/reports/FBim0000078 permissions requested) were stained for active caspase 3 and nuclei were visualized using bisbenzimide (blue). Normal oocytes with no caspase 3 activation and intact nuclei were observed in (B) controls. Fragmented nuclei (arrows) with positive caspase 3 staining (green) along with a disorganized morphology were observed in (C) cisplatin treated oocytes. (D) Caspase 3 positive oocytes were counted and expressed as a percent of the total number of oocytes ($p=0.098$). Despite the disorganized morphology, we were still able to count individual oocytes.

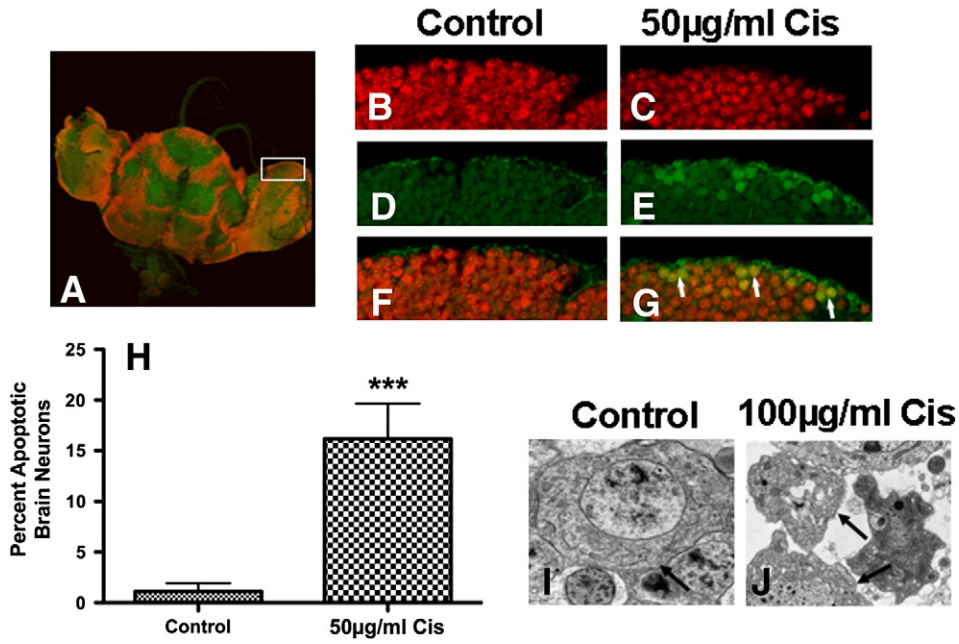

Fig. 3. Cisplatin-induced apoptosis in *Drosophila* brain neurons. (A) Brain harvested from cisplatin treated (50 μg/ml) flies were stained for active caspase 3 and elav, a pan neuronal marker. Control and cisplatin treated brain neurons stained for (B, C) elav (red) and (D, E) active caspase 3 (green), showed (F) no caspase 3 staining in control brain and (G) positive caspase 3 staining with co-localized elav staining in cisplaitn treated brain (arrows). (H) Caspase 3 staining was found in 1.11% of control brain neurons (n = 10) versus 16.23% in cisplatin treated brain neurons (n = 8) (p = 0.0007). Electron micrographs of (I) control brain and (J) brain treated with 100 μg/ml cisplatin show apoptosis with intact plasma membrane (arrows) and fragmented nuclei.

unaffected (Fig. 6A). Flies were treated with 0, 200, 400, 800, 1400, 2000, 3000, and 4000 μg/ml cyclophosphamide. On day 3 survival was 89%, 86%, 82%, 83%, 76%, 51%, 40% and 17% (p = 0.0001) while percentage of flies able to climb above 2 cm was 70%, 53% 78%, 76%, 84%, 65% and 88% (p = .0.0104). There were not enough flies to do the climbing assay with 4000 μg/ml cyclophosphamide. In *Drosophila* treated with 3000 μg/ml cyclophosphamide, no active caspase 3 was observed in brain neurons (Fig. 6B), however, active caspase 3 with fragmented nuclei was observed in both kidney (Fig. 6C) and ovaries (Fig. 6D).

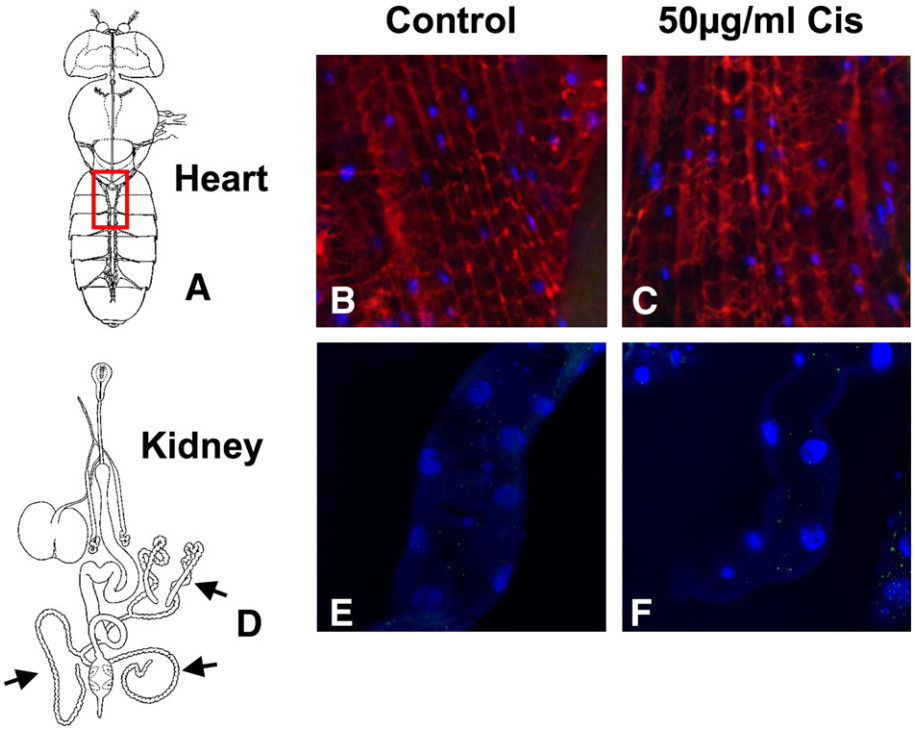

Fig. 4. No apoptosis was observed in cisplatin treated heart or kidney. Heart and kidney from *Drosophila* treated with and without 50 μg/ml cisplatin was observed for apoptosis. (A) Heart (www.flybase.org/reports/FBim0000047 permissions requested) was stained for active caspase 3 (green), pericardin (red) and nuclei visualized using bisbenzimide (blue). No caspase activity or nuclear fragmentation was observed in (B) control or (C) cisplatin treated heart. (D) Kidney (www.flybase.org/reports/FBim0000045 permissions requested) stained for active caspase 3 (green) and nuclei (blue) showed no positive caspase 3 staining or nuclear fragmentation in (E) controls or (F) cisplatin treated kidney.

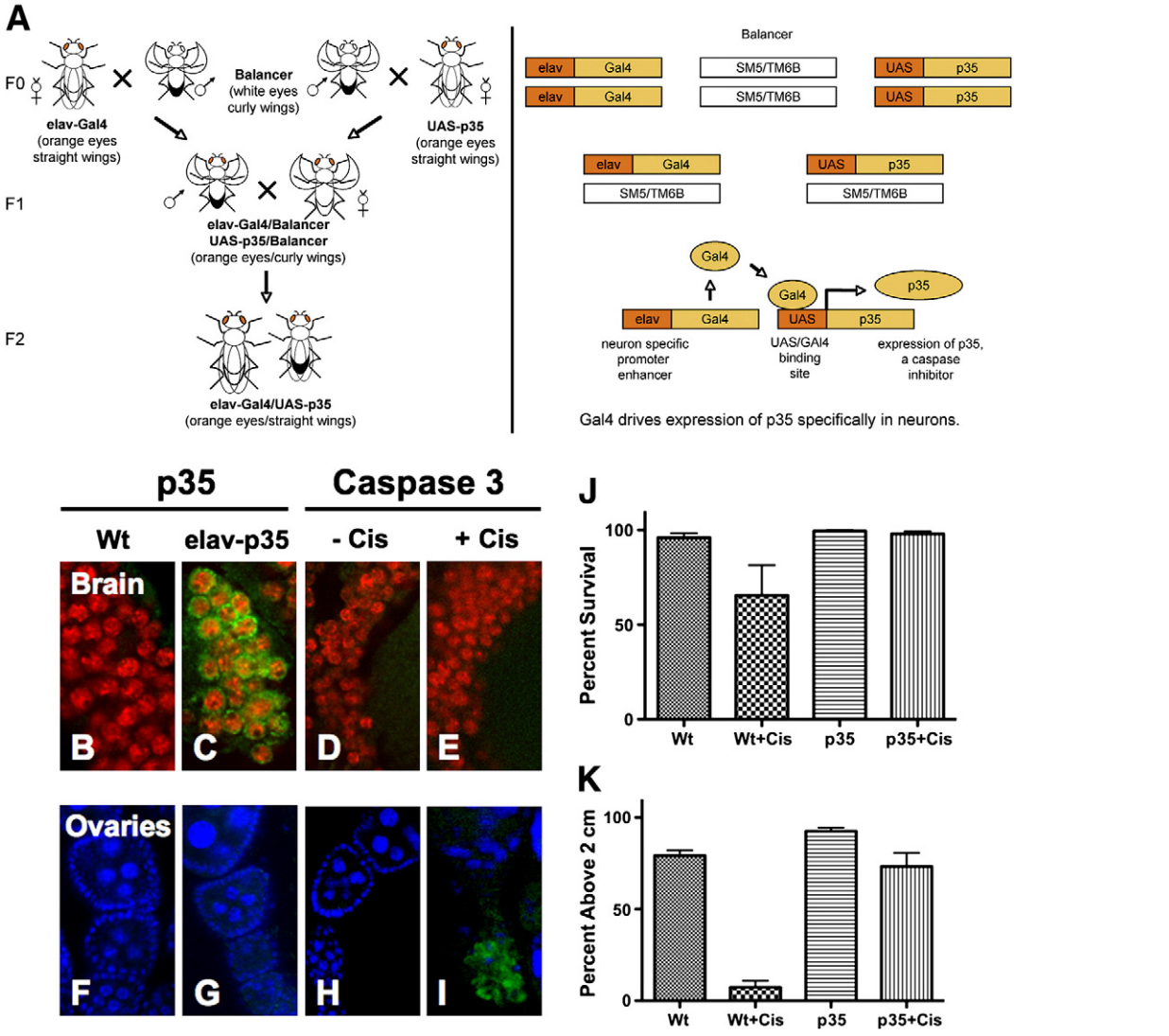

Fig. 5. Using the GAL4-UAS system, (A) elav-GAL4 flies and UAS-p35 *Drosophila* were bred with balancer flies to generate elav-p35 flies. Elav-p35 *Drosophila* targeted expression of p35, a baculovirus anti-apoptotic protein, specifically in neurons. Expression of the caspase-inhibitor p35 in *Drosophila* brain prevented cisplatin induced apoptosis, lethality and climbing inhibition. Elav-p35 *Drosophila* were treated with or without 100 μg/ml cisplatin and immunotstained for p35 expression and caspase 3 activation. p35 was not expressed in (B) wild type brain (Wt) but was expressed in (C) elav-p35 brain neurons as indicated by co-localization of p35 (green) with elav staining (red). Expression of p35 prevented caspase 3 activation in elav-p35 brains treated (D) without or (E) with cisplatin. In Ovaries, no expression of p35 was observed in either (F) Wt or (G) elav-p35 oocytes and no caspase 3 staining was observed in (H) control elav-p35 oocytes. Cisplatin treatment induced positive active caspase 3 staining in (I) elav-p35 oocytes. p35 expression in neurons improved (J) fly survival and prevented (K) climbing inhibition induced by cisplatin treatment. Wild type (Wt) and elav-p35 (p35) flies were treated with 100 μg/ml cisplatin. Cisplatin reduced fly survival and inhibited climbing ability in wild type flies (Wt + Cis). Survival was slightly improved ($p = 0.0347$) and climbing inhibition significant improved ($p < 0.0001$) by the expression of neuronal p35 in cisplatin treated elav-p35 flies (p35 + Cis).

Discussion

The goal of this study was to determine whether *Drosophila* has the potential to serve as a model system for studying cisplatin neurotoxicity. We determined that including cisplatin in the fly food resulted in tissue levels comparable to rodents and humans treated with the drug, that platinum DNA adducts were formed and that tissue damage was qualitatively similar to that seen in vertebrates. Cisplatin-induced neuronal apoptosis was associated with altered climbing behavior, which occurred at lower doses than what caused fly lethality. Importantly, the neuronal damage and behavioral changes were abrogated by targeting expression of an anti-apoptotic factor (p35) specifically in neurons.

Cisplatin binding to DNA in whole fly was similar to platinum levels found in cultured rat DRG. DNA isolated from embryonic rat DRG treated with 2 μg/ml cisplatin had an accumulation of 1 platinum adduct per 3700 base pairs genomic DNA (McDonald et al., 2005). *Drosophila* fed 100 μg/ml cisplatin had a whole fly DNA binding of 1 platinum molecule per 2500 base pairs genomic DNA. Although the delivery of the drug was administered orally to the *Drosophila* in our experiments, platinum levels bound to DNA are comparable to our rat DRG studies, *in vitro*. We had previously demonstrated (McDonald et al., 2005; Ta et al., 2006) that these tissue levels in rodents were comparable to those found in cisplatin treated patients and that the concentration of DNA platinum adducts in neurons was well in excess of levels associated with cell death in cancer cells. The data in our present study also establishes that while *Drosophila* has a rudimentary glial blood–brain barrier (Freeman and Doherty, 2006; Edwards and Meinertzhagen, 2010), there is entry of cisplatin into the *Drosophila* brain, which is associated with the observed effects on climbing behavior.

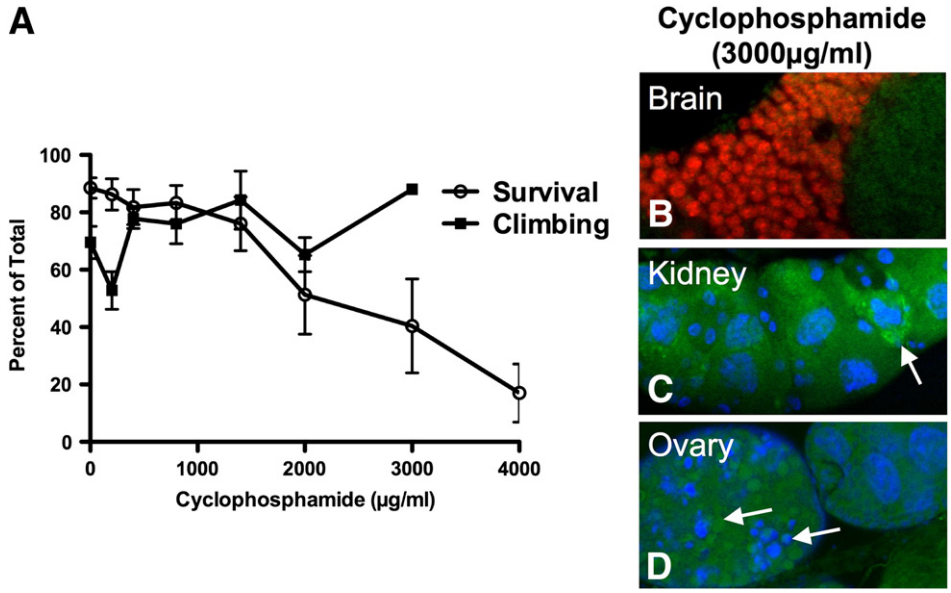

Fig. 6. Cyclophosphamide did not inhibit geotaxis, climbing behavior or induce apoptosis in brain neurons. (A) Climbing was not impaired in cyclophosphamide treated flies ($p = 0.0104$). At lethal concentrations, the surviving flies exhibited normal climbing ability. (B) No positive caspase 3 staining was observed in brain neurons, however, apoptosis was found in both (C) kidney and (D) ovaries.

Cisplatin induced cellular changes were observed in various organs and cell types within the same animal. Apoptosis occurred in both rapidly dividing oocytes and differentiated, post-mitotic brain neurons. Cardiomyocytes are also non-dividing cells under normal conditions in rodents and humans, and are unaffected by cisplatin. Similarly we have shown that cisplatin does not affect *Drosophila* cardiomyocytes. We also noted (data not shown) a dramatic increase in apoptosis in *Drosophila* gut cells which may mimic gastrointestinal changes in humans (Bearcroft et al., 1999). The cell damage in the gut may also reflect oral ingestion of the drug by flies. The model was less faithful in the kidney which is affected in humans and rats (Fisher et al., 2008) but was not affected in flies. It is possible that kidney tubule cells in *Drosophila* do not concentrate or handle the drug in the same way as vertebrates.

Cisplatin-induced lethality and behavioral changes were associated with brain neuronal apoptosis. Neuron specific expression of anti-apoptotic p35 in cisplatin treated flies improved fly survival, prevented climbing deficiencies and inhibited brain neuron caspase 3 activation while still inducing apoptosis in ovaries. To better understand the relationship of neuron apoptosis and climbing inhibition, *Drosophila* were treated with cyclophosphamide, a non-neurotoxic, chemotherapeutic drug with similar therapeutic DNA binding mechanisms to cisplatin. Cyclophosphamide is an alkylating agent that binds to N7 of Guanine inducing inter- and intrastand crosslinks and apoptosis. Cyclophosphamide did kill the flies at higher concentrations, however, there were no climbing deficiencies or brain neuron apoptosis in the surviving flies. Cyclophosphamide did induce apoptosis in kidney and ovaries and may play a role in lethality. Thus, there appears to be an association between altered climbing behavior and brain neuron apoptosis.

Multiple mechanisms are involved in cisplatin-induced neurotoxicity and have been difficult to separate. Furthermore, it is still not completely understood why neurons are the only post-mitotic cells that are preferentially affected by cisplatin. Rodent models have proven a difficult model in which to answer this question. *Drosophila* melanogaster has the potential to be an excellent model to study the mechanisms of cisplatin and other neurotoxic drugs. Using lethality and climbing behavior, we can determine drug neurotoxicity or neuroprotection and potentially develop an assay to rapidly screen several drugs at a time. Immunostaining can be used to characterize the effect of drugs on neurons as well as dividing cells within the same animal. The ability to target gene expression or gene knockdown in specific cell populations will be a powerful tool in dissecting out different mechanisms of cisplatin toxicity in different cells types. Also, use of genetically modified *Drosophila* can lead to a better understanding of the mechanisms involved in drug neurotoxicity.

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