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Cisplatin Inhibits Hippocampal Cell Proliferation and Alters the Expression of Apoptotic Genes

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Abstract

The hippocampus, which is critical for memory and spatial navigation, contains a proliferating stem cell niche that is especially vulnerable to anti-neoplastic drugs such as cisplatin. Although the damaging effects of cisplatin have recently been recognized, the molecular mechanisms underlying its toxic effects on this vital region are largely unknown. Using a focused apoptosis gene array, we analyzed the early cisplatin-induced changes in gene expression in the hippocampus of adult Sprague-Dawley rats and compared the results to those from the inferior colliculus, a non-mitotic auditory region resistant to cisplatin-induced cell death. Two days after a 12 mg/kg dose of cisplatin, significant increases were observed in five proapoptotic genes Bik, Bid, Bok, Trp53p2 and Card6 and a significant decrease in one antiapoptotic gene Bcl2a1. In contrast, Nol3, an antiapoptotic gene showed a significant increase in expression. The cisplatin-induced increase in Bid mRNA and decrease in Bcl2a1 mRNA was accompanied by a corresponding increase and decrease of their respective proteins in the hippocampus. In contrast, the cisplatin-induced changes in Bcl2a1, Bid, Bik and Bok gene expression in the inferior colliculus were strikingly different from those in the hippocampus consistent with the greater susceptibility of the hippocampus to cisplatin toxicity. Cisplatin also significantly reduced immunolabeling of the cell proliferation marker Ki67 in the subgranular zone (SGZ) of the hippocampus two days post treatment. These results indicate that cisplatin-induced hippocampal cell death is mediated by increased expression of proapoptotic and antiapoptotic genes and proteins that likely inhibit hippocampal cell proliferation.

Keywords

Cisplatin; Apoptosis; Hippocampus; Bid; Bcl2a1

INTRODUCTION

Cancer patients undergoing chemotherapy often develop serious neurological and cognitive side effects, a condition sometimes referred as “chemo brain” (Weiss, 2008). Cisplatin, carboplatin and oxaliplatin, which are widely used to treat a variety of solid and disseminated neoplasms (Abe et al., 2009, Harter et al., 2011) are known to have serious side effects, but their effects on the brain, especially to vulnerable regions such as the hippocampus, are poorly understood (Amptoulach and Tsavaris, 2011, Kannarkat et al., 2007, Whitney et al., 2008). While platinum based chemotherapeutic drugs have dramatically increased the number of cancer survivors, it has also increased the frequency of neurological impairments. In patients that have undergone chemotherapy, estimates of mild cognitive impairment range from 10 to 40% (Matsuda et al., 2005, Soussain et al., 2009).

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Common neurological complaints of chemotherapy include acute encephalopathy, lukoencephalopathy, vasculopathy, stroke, headache, seizures and neuropathy (Fuse-Nagase et al., 1997, Highley et al., 1992, Soussain et al., 2009, Verstappen et al., 2003). Chemotherapy-induced cognitive dysfunction or "chemobrain" is generally manifested as anxiety, fatigue, pain, inability to concentrate, depression and memory decline (Hurria et al., 2006, Weiss, 2008). Cisplatin, widely used to treat ovarian (Morgan et al., 2012, Yang and Lee, 2012), cervical (Sehouli et al., 2012, Xu et al., 2012) and other solid tumors, is known to cause chemobrain (Whitney et al., 2008). Cisplatin exerts its therapeutic effects on cancers cells by binding with DNA hampering DNA replication and arresting cell division in cancer cells (Ortin et al., 2009); however, cell proliferation normally dividing adult stem cells in skin, bone marrow, muscle and adipose is also suppressed by cisplatin.

Stem cells are also present in two distinct niches in the adult mammalian brain, the subventricular zone and the subgranular zone (SGZ) of the hippocampus (Jin et al., 2003, Navarro-Quiroga et al., 2006, Tanaka et al., 2007, Valente et al., 2009). The significance of the hippocampal proliferative zone is that this region plays an important role in spatial navigation and the formation of new memories related to recently experienced events (Appleby et al., 2011, Kyd and Bilkey, 2005, Moita et al., 2003, Scoville and Milner, 2000). Roughly 250,000 new cells are born in the hippocampus each month (Cameron and McKay, 2001). Many of these newborn cells differentiate into neurons, integrate into the neural circuitry of the hippocampus and enhance learning and memory (van Praag et al., 1999, van Praag et al., 2002). The hippocampus is especially vulnerable to brain trauma, heavy metal toxicity, inflammation and Alzheimer's disease. Damage to the hippocampus is associated with memory loss, cognitive impairment, disorientation and mood disorders (Bekinschtein et al., 2007, Blank et al., 2002, Bolouri and Small, 2004, Dawe et al., 2011, Ekdahl et al., 2003, Femenia et al., 2012, Ishida et al., 1997, Ishikawa et al., 1997, Kesner and Williams, 1995, Kraus et al., 2010, Luft et al., 2008, Monje et al., 2003, Niedermeyer and Ghigo, 2011, Pietropaolo et al., 2007, Prestia et al., 2011, Vanderwolf, 2001). Stem cells in the hippocampus are especially vulnerable to the toxic effects of cisplatin such that treatments results in a significant depression in cell proliferation and neurogenesis (Dietrich et al., 2006, James et al., 2008, Piccolini et al., 2012, Rzeski et al., 2004). However, the biological mechanisms underlying cisplatin-induced hippocampal neurotoxicity and cell cycle arrest are largely unknown. Therefore, we used focused gene arrays and immunolabeling to investigate the mechanisms of cisplatin-induced cell cycle arrest and cell death in the hippocampus two days following cisplatin treatment, a period during which cell death is increasing and cell proliferation is decreasing in the hippocampus. For comparison, tissue was also evaluated from the inferior colliculus, a non-proliferative zone in the auditory pathway.

MATERIALS AND METHODS

Animals

Twenty nine male SASCO Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) were used for this study. The mean weights of the animals were 325 ± 50 gm. Nine rats were used for the qRT-PCR array studies, eight were used for the confirmatory q-RT-PCR studies, six were used for the Western blot studies and six were used for immunohistochemistry studies. The experimental protocol was reviewed and approved by University at Buffalo Institutional Animal Care and Use Committee.

Cisplatin Treatment

Cisplatin (*cis*-diamminedichloro-platinum (ll) (Sigma-Aldrich) was dissolved in saline at 1 mg/ml concentration. Experimental rats received a single dose of 12 mg/kg cisplatin which

was slowly administered by intraperitoneal injection using a perfusion pump at the rate of 150 μl per minute as described in detail in our earlier publication dealing cisplatin ototoxicity and hearing loss (Jamesdaniel et al., 2008). Control rats received an intraperitoneal injection of sterile saline following the same procedures. Cisplatin and saline injections were administered to the rats under isoflurane anesthesia (4% induction, 1.5% maintenance). Afterwards, rats were twice daily subcutaneously injected with 20 ml/kg of normal saline to minimize cisplatin-induced kidney damage. The weight loss ranged from 5–8% in the cisplatin treated rats. The cisplatin treated rats were slightly less active after the treatment than the control rats; however, none of the rats died from the treatments. At two days post treatment, the animals were euthanized by CO_2 and decapitated for tissue collection.

Total RNA Isolation and cDNA Construction

The hippocampus and inferior colliculus were dissected out in an RNase free environment. Since, inferior colliculus is a non-proliferating region; it was used as a negative control. Total RNA was isolated using a RNeasy lipid tissue extraction kit (Qiagen), following the manufacturers protocol. Tissue samples were homogenized in a RNA free 1.7 ml tube containing QIAzol lysis reagent and centrifuged at 12000 g at 4 °C for 15 minutes. The aqueous phase of the sample was transferred to a new tube. An equal volume of 70% ethanol was added and mixed with the sample and transferred to the RNeasy column. The column was incubated with DNase I for 15 minutes at room temperature to avoid genomic DNA contamination and afterwards total RNA was eluted using 30 μl RNase-free water. Total RNA concentration and purity were measured using a spectrophotometer (Beckman Coulter DU 640 or Thermo scientific, NanoDrop 2000). Equal concentrations of total RNA (1 μg) were used to construct cDNA using a RT^2 first strand cDNA kit (Cat #: C-03, SA Bioscience Corporation). SuperArray RT qPCR Master Mix (SA Bioscience Corporation) was used for the PCR reaction.

Apoptosis Gene Expression Changes

The Apoptosis RT^2 Profiler™ PCR Array – PARN-012A (SuperArray Bio-sciences Corp., MD, USA) was used to investigate the cisplatin-induced changes in apoptotic gene expression in five control and four cisplatin-treated rat hippocampus. Because the hippocampus undergoes apoptosis following cisplatin treatment whereas the inferior colliculus does not, we also used the gene arrays in five control rat inferior colliculus to compare the relative abundance of apoptosis and anti-apoptosis genes in hippocampus. Both brain regions were isolated from same animal for this comparison study. The RT^2 Profiler™ PCR array contained 84 apoptotic genes (table II) involved in the intrinsic and extrinsic apoptotic pathway, the calcium-induced cell death pathway and p53 signaling pathway. In addition, the array has primers for 5 housekeeping genes (Hprt, Ldha, Rpl13a, Rplp1 and Actb), a genomic DNA primer, 3 reverse transcription controls and 3 positive PCR controls to facilitate normalization, detect genomic DNA contamination and test the efficiency of cDNA conversion as well as PCR reaction. The PCR reaction was run with a two-step cycling program using Bio-Rad MyiQ Single Color Real Time PCR System The PCR program consisted of one cycle of a hot start (95 °C) for 10 minutes to activate the DNA polymerase and 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). Cycle threshold (C_T) values were measured for each gene on the array.

The initial results of the apoptosis gene array analysis form the hippocampus revealed large changes in the expression of four Bcl2 genes, Bcl2a1, Bid, Bik and Bok. Based on the initial hippocampal results, we carried out additional qRT-PCR experiments to determine if these four Bcl2 genes showed similar changes in expression in the inferior colliculus and hippocampus two days post-cisplatin. To accomplish this, we designed primers for these

four Bcl2 genes plus beta actin, a housekeeping gene (Invitrogen); the forward and reverse primers for these five genes are shown in table 1. Four cisplatin-treated rats (two days post cisplatin) and four control rats were used from this experiment. Isolation of total RNA from the hippocampus and cDNA construction is described above. The PCR reaction was run with same program which had been used to run apoptosis RT^2 Profiler™ PCR Array using SYBR Green fluorescence (SABiosciences) technology with 25 μl per reaction in Bio-Rad MyiQ Single Color Real Time PCR System.

Data Analysis

The C_T values of five housekeeping genes (Hprt, Ldha, Rpl13a, Rplp1 and Actb) were averaged and used to normalize the C_T values of apoptotic genes. The statistical analysis of cisplatin-induced changes in mRNA expression levels, in the hippocampus, were calculated using SA bioscience online data analysis resource (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The software automatically performs all ΔΔCt based fold-change calculations from uploaded raw threshold cycle data. The fold changes with p-value less than 0.05 were considered statistically significant. In addition, the expression levels of the 84 apoptotic genes relative to the housekeeping genes were compared in hippocampus and inferior colliculus. The relative abundance of the 84 apoptosis genes in the hippocampus and inferior colliculus were compared using linear regression analysis (GraphPad Prism software, version 5.01).

Western Blot

Expression of Bcl2a1 and Bid proteins were evaluated in the hippocampus of normal control rats (n=3) and cisplatin-treated rats (n=3; 12 mg/kg cisplatin, 2 days post-cisplatin). The hippocampi from control and cisplatin treated rats were dissected out and homogenized in RIPA buffer (Thermo scientific). The homogenates were centrifuged at 12000 rpm for 20 minutes and total protein concentration of the supernatant was measured using the Bradford method. The extracted protein samples (10 μg) were separated on 4–12% gradient NuPage gels (Invitrogen, Carlsbad, CA), transferred to polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA), membrane and blocked with 5% dry non-fat milk for 1 hr. After blocking, the membrane was incubated with mouse anti-Bid (Chemicon, 1:5000, overnight) or rabbit anit-Bcl2a1 (Santa Cruz Biotechnology, Inc.; 1:500) followed by two hours incubation with appropriate hydrogen peroxidase conjugated secondary antibody (Pierce Chemical Co., Rockford, IL). Protein bands were visualized using the chemiluminescence detection method (Pierce Chemical Co., Rockford, IL) and samples evaluated using a Fuji model LAS 1000 imaging system (Stamford, CT). Afterwards, the membrane was stripped and re-probed with an antibody against actin to facilitate normalization. National Institute of Health (NIH) ImageJ software was used for densitometric analysis of the gels.

Immunohistochemistry

Three control rats and three cisplatin-treated rats (12 mg/kg, two days post-treatment) were deeply anesthetized (86 mg/kg, i.p, Fatal Plus, Vortech, Pharmaceutical Ltd.) and then perfused intracardially with 0.1 M phosphate buffered saline (PBS) followed by 10% formalin in PBS. The brain was removed, post-fixed in 10% formalin for 24 hr, and then cryoprotected in 15% sucrose in PBS for 6 hr followed by 30% sucrose in PBS for 12 hr. Coronal sections, 40 μm thick, were cut on a cryostat and stored in a cryoprotection solution of 30% ethylene glycol and 30% glycerol in PBS at −20 °C. Free-floating sections (20–25 sections from each rat) were blocked with 1% bovine serum albumin (BSA), 1% normal horse serum and 0.1% TritonX-100 (TX) in PBS for 30 min as previously reported (Manohar et al., 2012). The rabbit anti-Ki67 (Novocastra Laboratories Ltd, UK; 1:3000) was added to the solution and the sections were incubated overnight at 4 °C. Sections were then rinsed and incubated in a secondary antibody (biotinylated goat anti-rabbit IgG, BA 1000,

Vector Laboratories) and processed with an avidin-biotin-peroxidase complex (ABC) kit (Vectastain, Vector Laboratories). Immunoreactivity was visualized using the glucose oxidase (Sigma-Aldrich)-diaminobenzidine (Sigma-Aldrich) method (Shu et al., 1988, Van Der Gucht et al., 2006). Sections were rinsed and mounted on Fisher "Superfrost" polarized slides (Fisher Scientific), allowed to dry, stained with cresyl-violet, dehydrated in ethanol, cleared in xylene and coverslipped with DePex mounting medium (Manohar et al., 2012). Microscopic images were photographed using a SPOT digital camera (SPOT Insight; Diagnostic Instruments, Inc). The images were assembled using Adobe Photoshop CS3 software. Quantification and statistical analysis were performed following a standard protocol (Kraus et al., 2010). Ki67 positive cells were counted in 25 sections from each normal and cisplatin-treated rat and Ki67 density was calculated by dividing the cell count by the length of sub granular zone. A two-tailed paired student's t-test was performed to determine the statistical significance.

RESULTS

Cisplatin Induced Hippocampal Apoptotic Genes

Cisplatin-induced changes in the expression profile of apoptotic genes in the hippocampus two days post treatment. Significant changes were detected in the mRNA expression levels of seven of 84 genes on the array; those classified proapoptotic and antiapoptotic are indicated in legend of figure 1. Bcl2a1 (B-Cell leukemia/lymphoma 2 A1), a member of B-Cell leukemia/lymphoma 2 (Bcl2) gene family, was downregulated (1.7 fold). Bcl2a1, a pro-survival gene, encodes a protein that prevents the release of the cytochrome C from the mitochondria into the cytoplasm (Vogler, 2012). Release of cytochrome c into the cytoplasm initiates apoptosis through APAF-1 (apoptotic protease activating factor-1) and downstream caspases (Henshall et al., 2001). In contrast, three members of the Bcl2 gene family were significantly upregulated; these were Bid (BH3 interacting domain death agonist, 1.55 fold increase), Bik (Bcl2-interacting killer, 1.6 fold increase) and Bok (Bcl-2-related ovarian killer protein, 1.8 fold increase). Bid encodes a proapoptotic protein that interacts with the Bax protein; together they promote the opening of voltage dependent anion channels and the release of cytochrome c from mitochondria (Devarajan et al., 2002, Wang et al., 2001). Bik and Bok encodes proteins promote p53 dependent apoptosis (Li et al., 2008) (Hur et al., 2006, Yakovlev et al., 2004). The expression of three others genes increased significantly two days post-cisplatin. Card 6 (caspase recruitment domain gene family member 6), which was classified as proapoptotic increased 1.7 fold. Nol3 (apoptosis repressor with card domain protein Nuclear protein 3), considered antiapoptotic, increased 1.5 fold. Finally, Trp53bp2, also known as apoptosis-stimulating of p53 protein 2 (ASPP2)) and classified as proapoptotic increased 1.6 fold. Thus, the cisplatin-induced changes in mRNA expression the in the hippocampus were predominantly reflected by an increase in five proapoptotic genes plus and a decrease in one antiapoptotic genes, with the exception of an increase one antiapoptotic gene. Many of the gene expression changes occurred in the Bcl2 family. To extend and confirm these observations, the protein expression levels of Bcl2a1 and Bid were evaluated in the hippocampus, two days post treatment. Immunoblots detected Bcl2a1 and Bid protein bands at apparent molecular weights of 37 and 23 kD respectively. Cisplatin induced a 1.6 fold increase in Bid protein expression and a 0.7 fold decrease in Bcl2a1 protein expression (fig. 2), consistent with the cisplatin-induced changes in their mRNA expression. Collectively, these results highlight the activation of Bcl2 regulated apoptotic pathway in mediating the cytotoxic effects of cisplatin in the hippocampus.

Cisplatin Induces Site Specific Modulation of Bcl2 Family Genes in Brain

To determine if the effects of cisplatin on the hippocampus might be due to a unique apoptotic gene expression profile, we compared the relative abundance of the 84 apoptosis

genes with those in the inferior colliculus, a region not known to be susceptible to apoptosis. Our results show that the baseline gene expression profiles of the hippocampus and the inferior colliculus were very similar to one another (Fig. 3). A linear regression analysis comparing the expression levels of the 84 apoptosis related genes in the hippocampus to those in the inferior colliculus revealed a high correlation ($r^2 = 0.9263$). Genes that were expressed at high or low levels in the hippocampus were also expressed at high or low levels in the inferior colliculus under basal conditions.

To validate the hippocampus gene array data and to determine if cisplatin would induce an apoptotic response in the inferior colliculus similar to that seen in the hippocampus, we measured the cisplatin induced changes in expression of Bcl2a1, Bid, Bik and Bok in these two brain regions two days post-cisplatin. The effects of cisplatin on the expression of Bcl2a1, Bid, Bik and Bok genes were strikingly different in the inferior colliculus than the hippocampus (Fig. 4). In the hippocampus, mRNA levels of the antiapoptotic gene, Bcl2a1, was down-regulated whereas it increased slightly in the inferior colliculus. In contrast, the three proapoptotic genes, Bid, Bik and Bok, were significantly up-regulated in the hippocampus whereas all three proapoptotic genes decreased in the inferior colliculus. Bid expression in the inferior colliculus decreased more than 70 fold while Bik and Bok decreased only slightly. These results indicate that the hippocampus responds in an apoptotic manner to cisplatin whereas the inferior colliculus mounts an antiapoptotic response.

Cisplatin Decreases Ki67 in the SGZ

In addition to inducing apoptosis, many anticancer drugs suppress cell proliferation and neurogenesis in the hippocampus (Dietrich et al., 2006). To determine if our cisplatin treatment affected cell proliferation, an antibody against Ki67 was used to quantify cell proliferation in the SGZ of the hippocampus of control rats and cisplatin treated rats sacrificed two days post-treatment (Bullwinkel et al., 2006, Kraus et al., 2010). Figure 5A–B show representative coronal sections from a control rat and a cisplatin treated rat respectively; sections were immunolabeled with Ki67 and counterstained with cresyl violet. Consistent with our previous results, Ki67 positive cells were present in the SGZ of the hippocampus of control rats (arrows, Fig. 5A) (Kraus et al., 2010). The high magnification inset located near the asterisk in figure 5A shows a cluster of Ki67 positive nuclei. In contrast, few Ki67 immunolabeled cells were seen in the hippocampus two days post-cisplatin. Ki67 positive cells were counted along the length of the SGZ of the hippocampus of three control and three cisplatin treated rats. The mean numbers of Ki67 cells per mm length of SGZ decreased from approximately 0.82 to 0.1, a decline of nearly 90% (fig. 5).

DISCUSSION

Although cisplatin does not readily cross the blood-brain barrier (Jacobs et al., 2010, Minami et al., 1998), it nevertheless has been found to exert neurotoxic effects. Clinically, the neurological side effects of cisplatin are manifested as psychological, memory, perceptual and cognitive deficits (Kaasa et al., 1988, Troy et al., 2000, Verstappen et al., 2003, Whitney et al., 2008). Cisplatin potently inhibits cell proliferation, differentiation and neurogenesis in the hippocampus, alters neurotransmitter concentrations and damages neurons and oligodendrocytes (Dietrich et al., 2006, James et al., 2008, Liu et al., 2003). Since the molecular mechanisms underlying the neurotoxic effects of cisplatin on the in hippocampus are poorly understood, we utilized an apoptosis array consisting of 84 genes to identify biologically and clinically relevant targets significantly upregulated or downregulated two days after cisplatin treatment. Our results suggest that cisplatin initially promotes apoptosis in the hippocampus by increasing the expression of five proapoptotic genes, Bid, Bik, Bok, Tp53bp2 and Card6 while reducing the expression of one ant-apoptotic gene Bcl2a1 (Fig. 1). In contrast, expression of one antiapoptotic gene, Nol3, was

significantly increased. Moreover, cisplatin almost completely inhibited cell proliferation in the SGZ of hippocampus consistent with previous results (Dietrich et al., 2006). These changes may provide insights into the biological mechanisms that give rise to the psychological, emotional and cognitive impairments that develop after cisplatin treatment (Shabani et al., 2012, Troy et al., 2000).

Hippocampal Cell Death Genes

The Bcl2 gene family, which codes for antiapoptotic, proapoptotic and BH3 (Bcl-2 homology 3) proteins, regulates essential cellular functions such as programmed cell death, immune responses and tissue turnover (Martinou and Youle, 2011, Youle and Strasser, 2008). Activation of the Bcl2 cell death pathway results in numerous changes such as loss of mitochondrial membrane integrity, release of cytochrome c and upregulation of executioner caspase 3 (Zhang et al., 2005). Cisplatin upregulated the proapoptotic genes Bid, Bik and Bok while down regulating the antiapoptotic gene Bcl2a1. These results are consistent with studies showing that upregulation of Bik significantly enhances cisplatin induced apoptosis (Li et al., 2008). Likewise, cisplatin promotes the calpain-mediated cleavage of Bid which enhances apoptosis (Mandic et al., 2002). Conversely, cells over expressing Bcl2a1 are resistant to cisplatin and other chemotherapeutic compounds (Cheng et al., 2000, Kim et al., 2004b, Vogler, 2012). Since, Bcl2a1 interacts with Bid (Chen et al., 2005b, Mandic et al., 2002, Werner et al., 2002), Bok (Hsu et al., 1997) and Bik (Chen et al., 2005b, Holmgreen et al., 1999, Li et al., 2008) to prevent the loss of mitochondrial outer membrane integrity (Zhang et al., 2005), the cisplatin-induced changes in these four genes strongly implicate Bcl2 genes as major contributors to cisplatin mediated cell death in the hippocampus.

Cisplatin also significantly increased the expression of Tp53bp2, which codes for the apoptosis stimulating protein of p53-2 (ASPP2). ASPP2 promotes apoptosis and regulates cell growth (Chen et al., 2005a). Card6 expression was also significantly elevated two days postcisplatin. The Card6 gene encodes a microtubule-associated protein containing a caspase recruitment domain that interacts with signaling pathways involved in inflammation and apoptosis (Dufner et al., 2006). In contrast to the preceding findings, cisplatin exerted antiapoptotic effects by significantly increasing the expression of Nol3 (also known as ARC, apoptosis repressor with CARD domain). Nol3 codes for an antiapoptotic protein that reduces enzyme activities linked to caspase 2, caspase 8 and tumor protein 53 (Medina-Ramirez et al., 2011, Mercier et al., 2005, Wang et al., 2012, Wei et al., 2010). Upregulation of Nol3 could contribute to cisplatin drug resistance.

In addition to direct effects, cisplatin also increases the expression of a number of genes coding for inflammatory proteins such us TNF , RANTES (CCL5), chemokine receptors CCR1/CCR5, IL-1, and IL-6 (Ju et al., 2008, Li et al., 2005, So et al., 2008). Inflammatory molecule IL-1 and IL-1 increase the expression of proapoptotic protein Bid and Bik (Mezosi et al., 2004, Wakahara et al., 2005) while cytokines INF-β and INF-γ upregulate Card6 (Dufner et al., 2008). Thus, the inflammation may promote the increase in Bid, Bik and Card6 mRNA and provide an indirect pathway by which cisplatin may promote apoptosis in the hippocampus.

Hippocampus and Inferior Colliculus

The hippocampus is vulnerable to many different forms of traumatic brain injury and neurotoxic compounds including many anticancer drugs (Chang, 1990, Geddes et al., 2003, Lowenstein et al., 1992). While high doses of cisplatin can lead to apoptosis in the hippocampus (Dietrich et al., 2006), we are unaware of any studies showing that cisplatin induces cell death in the inferior colliculus. Why the hippocampus, but not the inferior colliculus, is susceptible to cisplatin damage is unclear, but could be related to differences in

the genes or proteins expressed in these regions. To partially address this issue, we compared the apoptotic gene expression profiles of the hippocampus and inferior colliculus and found them to be remarkably similar in normal animals (Fig. 3). However, the effects of cisplatin on Bcl2a1, Bid, Bik and Box gene expression were distinctly different in these two regions. Cisplatin significantly increased the expression of the pro-apoptotic genes Bid, Bik and Bok in the hippocampus, but had the opposite or no effect on these genes in the inferior colliculus. Moreover, cisplatin decreased the expression of Bcl2a1 in the hippocampus (Fig. 1, 3), but significantly increased the expression this prosurvival gene in the inferior colliculus. Thus, in response to cisplatin, the hippocampus appears to mount a largely proapoptotic response whereas the response of the inferior colliculus is largely antiapoptotic, which could contribute to drug resistance.

Cisplatin Inhibits Hippocampal Cell Proliferation

The stem cell niche in the rat hippocampus gives rise to approximately 9000 newborn cells each day many of which differentiate into neurons or glial cells (Aimone et al., 2009, Cameron and McKay, 2001, Deng et al., 2010, Jessberger et al., 2009). Using Ki67 immunolabeling, we found that cell proliferation in the SGZ of the hippocampus was suppressed by nearly 90% 2-days postcisplatin. Many different factors can modulate cell proliferation in the hippocampus (Couillard-Despres et al., 2009, Hattiangady et al., 2004, Kim et al., 2004a, Mustafa et al., 2008, Veena et al., 2009). Since cisplatin forms intra- and interstrand crosslinks with DNA, this could be a factor that directly suppresses cell proliferation in the hippocampus (Dietrich et al., 2006). However, the uptake of cisplatin into the central nervous system is somewhat limited (Gregg et al., 1992, Jacobs et al., 2010); therefore other mechanisms need to be considered. Cisplatin is known to induce oxidative stress and strong inflammatory responses (Jung et al., 2009, Kang et al., 2009, Lee et al., 2006, Lim et al., 2005, Ramesh and Reeves, 2004, Tsuji et al., 2009) which may act to suppress neurogenesis (Bachstetter et al., 2010, Barha et al., 2011, Graciarena et al., 2010, Iosif et al., 2006, Shih et al., 2006). In addition, cisplatin increased the expression of Trp53bp2 mRNA is known to contribute cell cycle arrest (Chen et al., 2005a, Lane, 1992, Naumovski and Cleary, 1996).

CONCULSION

Although cisplatin does not readily enter the central nervous system, it nevertheless damages the hippocampus and blocks hippocampal cell proliferation. Cisplatin promotes cell death in the hippocampus by increasing the expression of several proapoptotic genes while reducing the expression of antiapoptotic gene; these gene expression changes were not seen in the inferior colliculus. Future studies are needed to determine the effects of long term treatment with cisplatin and its long term effects on the hippocampus

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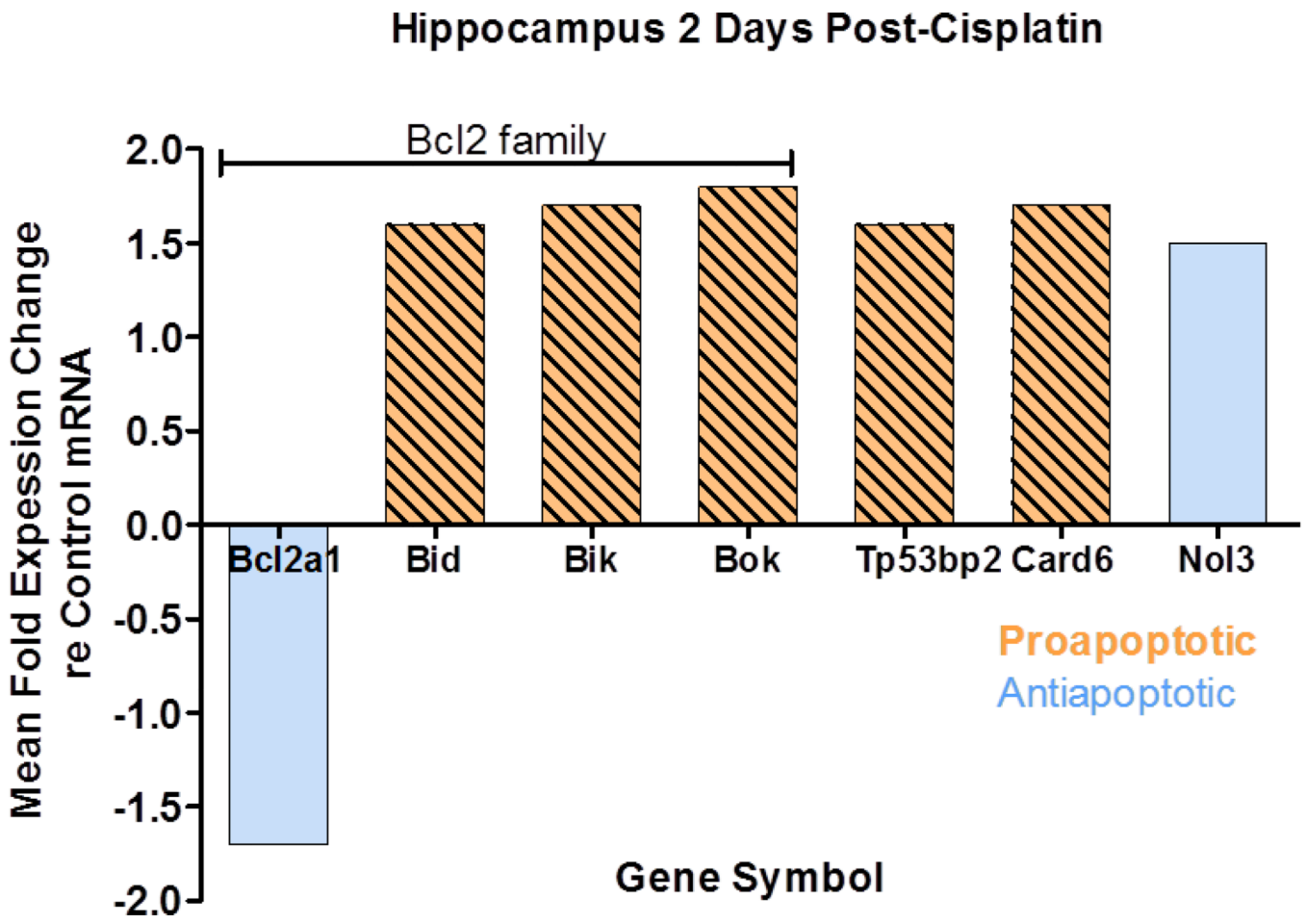

Fig 1.

Seven genes showed a significant change ($p<0.05$) in mRNA expression in the hippocampus at 2-days post-cisplatin (12 mg/kg); results show mean fold changes in expression. Bcl2a1, Bid, Bik and Bok are members of the Bcl2 gene family. All five genes classified as pro-apoptotic showed an increase in expression. One of two genes classified as an antiapoptotic showed a decrease in expression; the other showed an increase. Gene expression levels normalized to five housekeeping genes (Hprt, Ldha, Rpl13a, Rplp1 and Actb). Mean data shown for four cisplatin and five control rats.

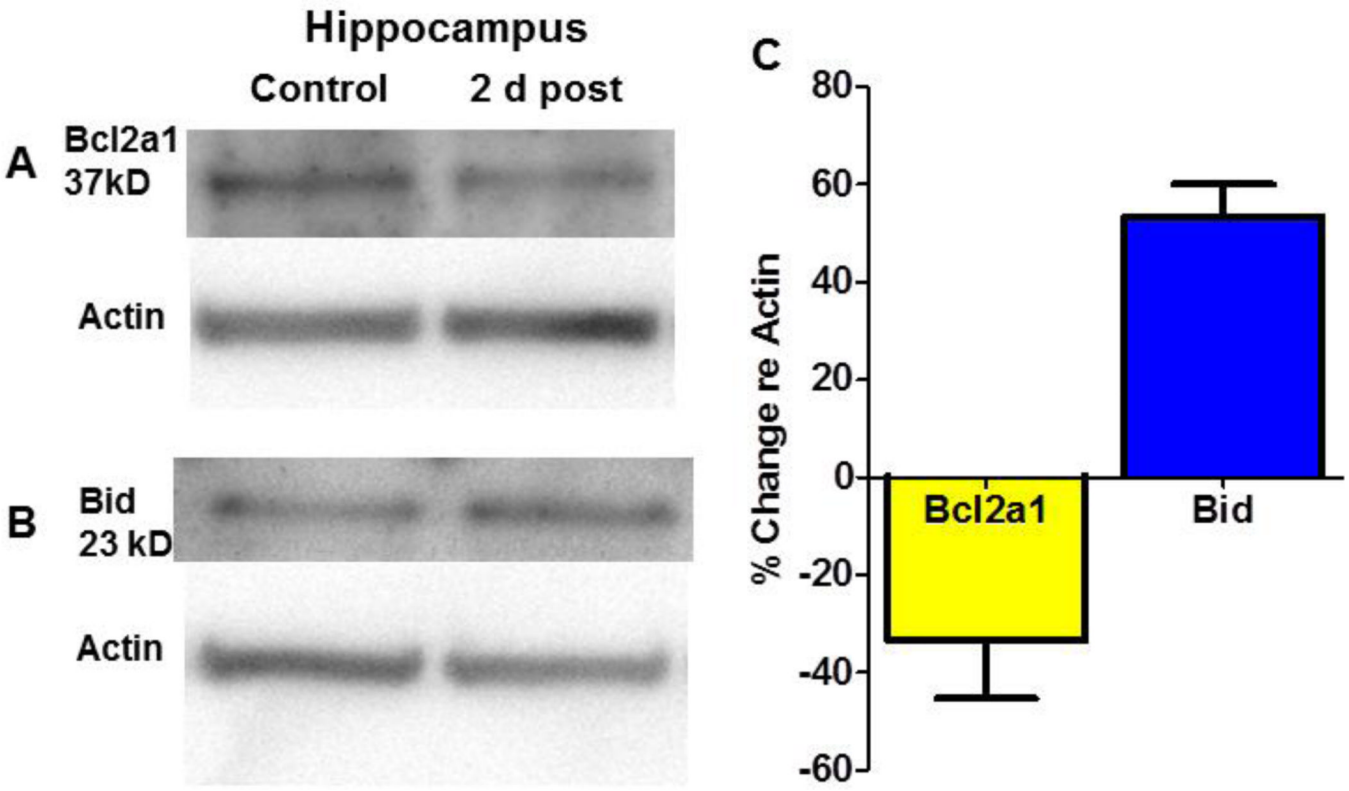

Fig 2.

Western blots showing changes in (A) Bcl2a1 (~37 kD) and (B) Bid protein (23 kD) expression in the hippocampus 2 days post-cisplatin and actin housekeeping protein shown below each protein. (C) Protein bands for Bcl2a1 and Bid proteins were normalized to actin housekeeping protein. Bcl2a1 was down-regulated approximately 30% while Bid was up-regulated nearly 60%.

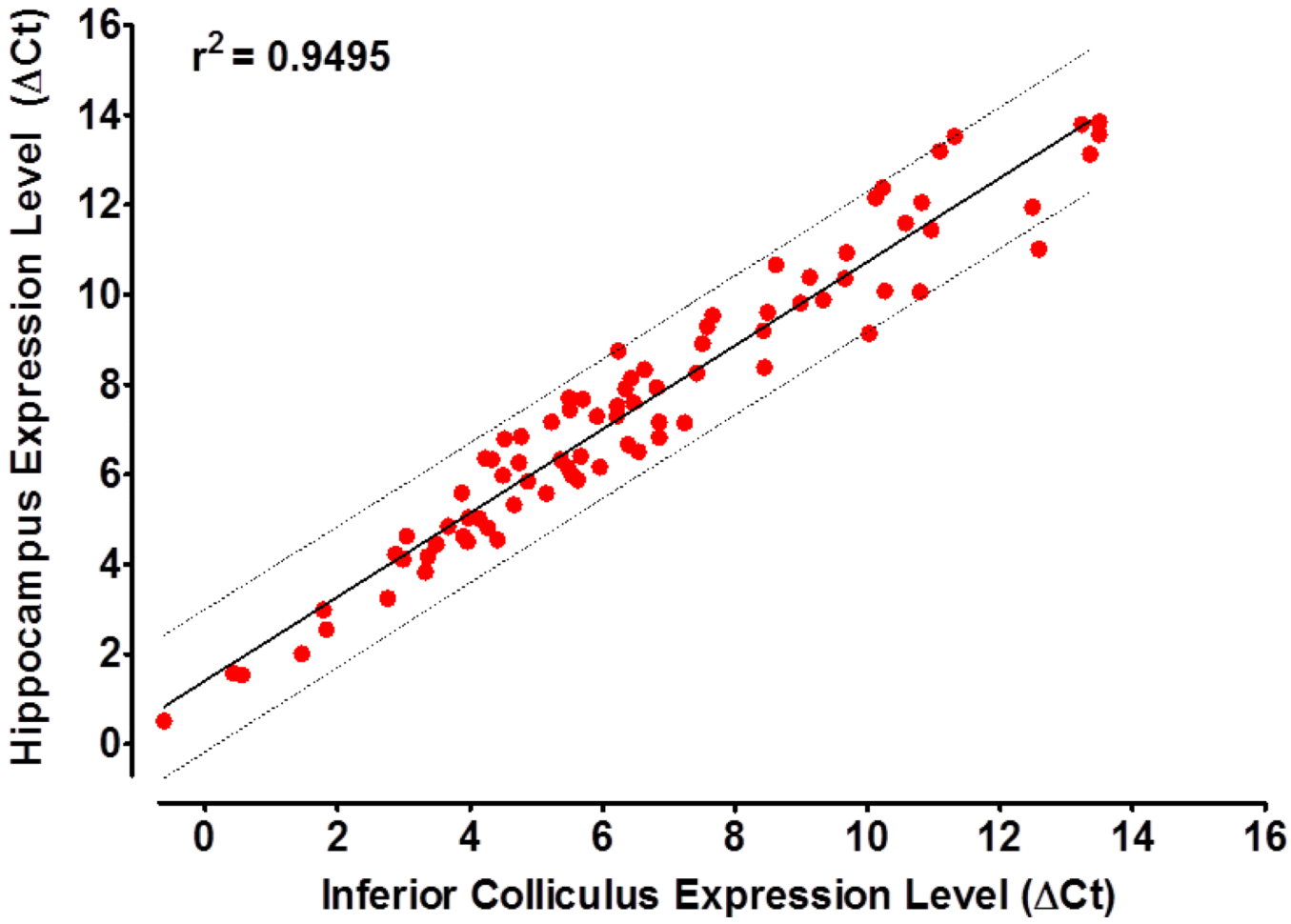

Fig 3.

Correlation of apoptotic gene expression levels (re housekeeping genes) in the hippocampus versus the inferior colliculus in normal controls. Linear regression showed a strong correlation (r^2=0.9495) in gene expression levels in these two regions. Thick solid line shows linear regression; dotted lines show the 95% confidence interval.

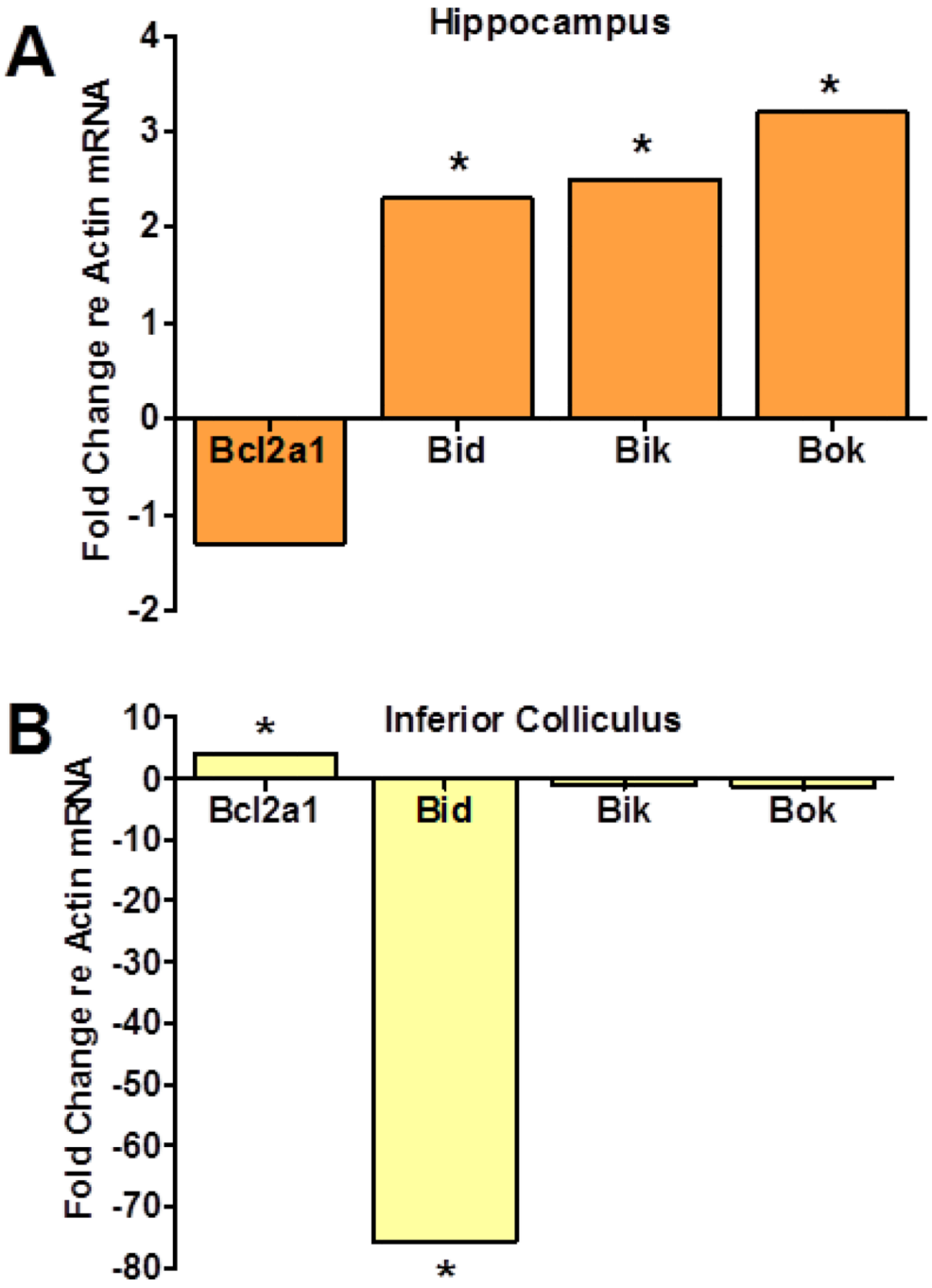

Fig 4.

Fold change in expression of four Bcl2 genes, Bcl2a1, Bid, Bik and Bok in hippocampus (A) and inferior colliculus (B) two days post-cisplatin. For each group, the mRNA expression was normalized to the beta-actin housekeeping gene. In the hippocampus (A), apoptotic genes Bid ($p = 0.001$), Bik ($p = 0.025$) and Bok ($p = 0.004$) were up-regulated significantly ($p < 0.05$) while antiapoptotic gene Bcl2a1 ($p = 0.22$) was down-regulated moderately. In contrast, in the inferior colliculus, apoptotic gene Bid was down-regulated significantly ($p = 0.0004$) while the antiapoptotic gene Bcl2a1 was significantly up-regulated ($p = 0.02$).

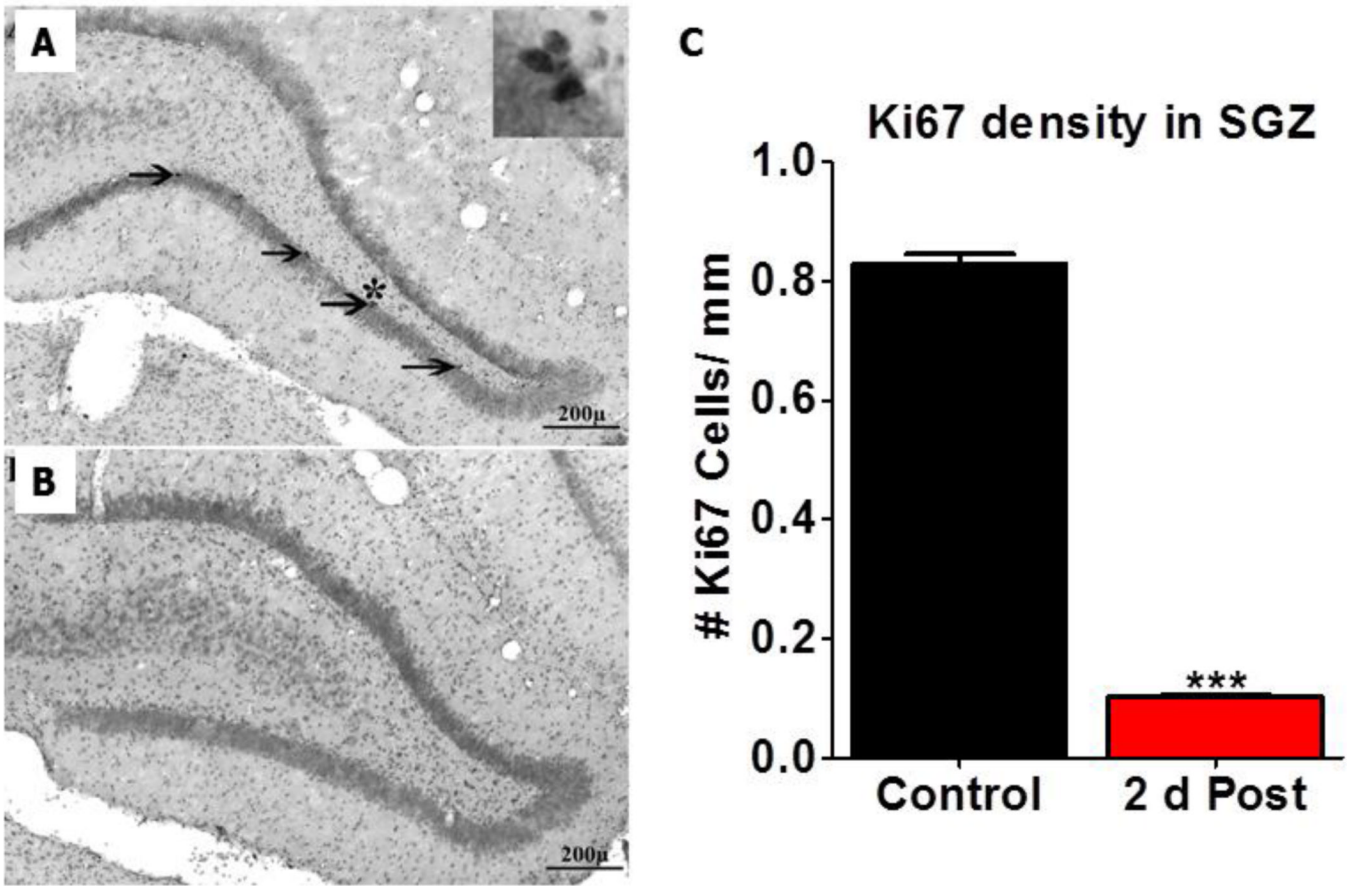

Fig 5.

Typical coronal sections from a control (A) and a cisplatin treated rat (B) immunolabeled with an antibody against Ki67 and counterstained with cresyl violet. In controls (A) Ki67 immunopositive nuclei (arrows) were present in the SGZ of the dentate gyrus. Insert in upper, right of panel A shows a cluster of Ki67 positive cells from the region with the asterisk ($p<0.001$, ***). Ki67 labeling was almost completely absent from the hippocampus 2 days after cisplatin treatment. (C) Histogram showing the mean (n=3/group, SEM) numbers of Ki67 positive cells per mm length of the SGZ. Ki67 immunolabeling was significantly ($t=35.67$ & $df=2$) reduced 2 days post-cisplatin relative to controls.

Table 1

Gene symbol, accession number and primer sequences for genes selected for validation by RT-PCR

Symbol	Accession No.	Forward	Reverse
Bok	NM_017312	ACGGACGTCCTCAAGTGTGTG	TCTCTCTGGCAACAGGAGGAAGA
BiK	NM_053704	AGGCGAGACTAATGGCCAGAGA	CCAGGCACCTCATGAAATCCAAG
Bid	NM_022684	AGGTCAGCAATGGCTCAGGC	CTGCAGCTCGTCTTCACGGT
Bcl2a1	NM_133416	ATGGAGGCTGGGAAGATGGC	TCTCAAGGGAGCCAGGGTTCT
Beta Actin	NM_031144	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG

Table II

Apoptosis related genes fold differences **(ΔΔCt)** relative to control hippocampus at 2 days post cisplatin-treated hippocampus (column 3). Control inferior colliculus (column 4) and hippocampus (column 5) apoptosis related genes mRNA expression **(ΔCt)** relative to average of five housekeeping (Hprt, Ldha, Rpl13a, Rplp1 and Actb) genes mRNA expression.

S.No	Gene Symbol	Fold different in hippocampus at 2 days post cisplatin treatment (ΔΔCt)	Inferior Colliculus (ΔCt)	Hippocampus (ΔCt)
1	Apaf1	1.06	6.39	6.67
2	Api5	1.03	1.83	2.55
3	Aven	1.16	4.74	6.26
4	Bad	1.52	3.05	4.63
5	Bag1	1.00	1.79	2.99
6	Bak1	1.26	6.22	7.31
7	Bax	1.25	3.67	4.85
8	Bcl10	−1.41	4.27	4.81
9	Bcl2	−1.10	5.96	6.17
10	Bcl2a1d	−1.70	8.44	8.38
11	Bcl2l1	1.40	2.87	4.23
12	Bcl2l11	−1.49	13.36	13.13
13	Bcl2l2	1.49	3.87	5.59
14	Bclaf1	1.13	2.76	3.24
15	Bid	1.56	6.22	7.52
16	Bik	1.67	11.09	13.19
17	Birc3	1.50	10.12	12.16
18	Birc5	−1.20	7.24	7.15
19	Bnip1	1.21	4.66	5.33
20	Bnip2	−1.01	5.54	5.98
21	Bnip3	1.14	0.56	1.54
22	Bok	1.81	3.37	4.18
23	Card10	1.98	8.62	10.67
24	Card6	1.70	7.66	9.54
25	Casp1	−1.07	5.70	7.67
26	Casp12	−1.22	8.50	9.61
27	Casp14	1.01	13.50	13.85
28	Casp2	1.30	6.82	7.94
29	Casp3	−1.01	4.13	5.02
30	Casp4	−1.19	5.37	6.33
31	Casp6	−1.02	5.50	7.44
32	Casp7	1.11	8.43	9.20
33	Casp8	−1.29	10.26	10.08

S.No	Gene Symbol	Fold different in hippocampus at 2 days post cisplatin treatment (ΔΔCt)	Inferior Colliculus (ΔCt)	Hippocampus (ΔCt)
34	Casp8ap2	1.30	4.52	6.79
35	Casp9	1.39	5.63	5.88
36	Cd40	−1.11	8.99	9.81
37	Cd40lg	1.09	13.50	13.83
38	Cflar	1.14	3.49	4.45
39	Cidea	1.07	7.42	8.26
40	Cideb	1.68	10.82	12.06
41	Cradd	1.10	5.67	6.40
42	Dad1	1.08	1.46	2.01
43	Dapk1	2.00	6.54	6.51
44	Dffa	1.52	6.43	8.14
45	Dffb	1.07	12.49	11.95
46	Fadd	1.80	6.47	7.61
47	Faim	−1.12	3.90	4.63
48	Fas	−1.09	10.58	11.59
49	Faslg	−1.38	7.51	8.91
50	Gadd45a	−1.25	4.87	5.85
51	Hrk	1.49	6.85	6.83
52	Il10	−1.12	11.32	13.52
53	Lhx4	4.00	9.68	10.93
54	Lta	1.82	13.23	13.79
55	Ltbr	2.77	6.24	8.75
56	Mapk8ip1	1.36	0.43	1.58
57	Mcl1	1.32	3.33	3.83
58	Naip2	1.29	10.96	11.44
59	Nfkb1	1.58	5.23	7.17
60	Nol3	1.47	4.23	6.36
61	Polb	1.49	4.34	6.34
62	Prdx2	1.12	−0.62	0.52
63	Prlr	4.02	12.58	11.01
64	Prok2	1.05	10.03	9.14
65	Pycard	−1.30	5.15	5.58
66	Ripk2	1.04	3.98	5.04
67	Sphk2	1.11	3.96	4.51
68	Tnf	−1.37	9.33	9.89
69	Tnfrsf10b	1.41	10.23	12.38
70	Tnfrsf11b	1.04	4.77	6.85
71	Tnfrsf1a	1.55	6.63	8.34

S.No	Gene Symbol	Fold different in hippocampus at 2 days post cisplatin treatment (ΔΔCt)	Inferior Colliculus (ΔCt)	Hippocampus (ΔCt)
72	Tnfrsf1b	1.46	9.13	10.39
73	Tnfsf10	−1.30	7.58	9.29
74	Tnfsf12	1.24	6.35	7.91
75	Tp53	1.02	6.85	7.17
76	Tp53bp2	1.59	4.42	4.55
77	Tp73	1.63	10.79	10.07
78	Tp73l	−1.21	13.50	13.56
79	Tradd	1.20	5.49	7.70
80	Traf1	1.22	9.66	10.36
81	Traf2	1.53	5.92	7.30
82	Traf3	1.29	4.50	5.98
83	Traf4	1.38	5.48	6.16
84	Xiap	1.09	2.99	4.12