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Serum or Target Deprivation Induced Neuronal Death Cause Oxidative Neuronal Accumulation of Zn^{2+} and Loss of NAD^+

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Abstract

Trophic deprivation mediated neuronal death is important during development, acute brain or nerve trauma, and neurodegeneration. Serum deprivation (SD) approximates trophic deprivation in vitro, and an in vivo model is neuronal death in the mouse dorsal lateral geniculate nucleus (LGNd) after ablation of the visual cortex (VCA). Oxidant-induced intracellular Zn^{2+} release, ($[Zn^{2+}]_i$), from metallothionein-3 (MT-III), mitochondria, or "protein Zn^{2+}" was implicated in trophic deprivation neurotoxicity. We previously showed that neurotoxicity of extracellular Zn^{2+} required entry, elevation in $[Zn^{2+}]_i$, reduction of NAD^+ and ATP levels causing inhibition of glycolysis and cellular metabolism. Exogenous NAD^+ and sirtuin inhibition attenuated Zn^{2+} neurotoxicity. Here we show that: 1) Zn^{2+} is released intracellularly after oxidant and SD injuries, and sensitivity to these injuries is proportional to neuronal Zn^{2+} content; 2) NAD^+ loss is involved; restoration of NAD^+ using exogenous NAD^+, pyruvate, or nicotinamide attenuated these injuries, and potentiation of NAD^+ loss potentiated injury; 3) Neurons from genetically modified mouse strains which reduce intracellular Zn^{2+} content (MT-III knockout), reduce NAD^+ catabolism (PARP-1 knockout), or increase expression of an NAD^+ synthetic enzyme (Wld^S) each had attenuated SD and oxidant neurotoxicities; 4) Sirtuin inhibitors attenuated, and sirtuin activators potentiated these neurotoxicities; 5) VCA induces Zn^{2+} staining and death only in ipsilateral LGNd neurons, and a 1ppm Zn^{2+} diet attenuated injury; 6) Finally, NAD^+ synthesis and levels are involved because LGNd neuronal death after VCA was dramatically reduced in Wld^S animals, and by intraperitoneal pyruvate or nicotinamide. Zn^{2+} toxicity is involved in serum and trophic deprivation induced neuronal death.

Keywords

visual cortex ablation; mouse; pyruvate; sirtuin; dorsal lateral geniculate nucleus

Target deprivation mediated neuronal death plays a large role during development, trauma, and neurodegeneration. In the developing nervous system, 20–80% of all neurons produced during embryogenesis die before reaching adulthood as a result of competition between neurons for innervation of their targets. This results in matching of the size of the target cell population with the number of innervating neurons (Oppenheim, 1991; Purves *et al*., 1988). Target deprivation mediated neuronal death is apoptotic and occurs by programmed cell death (PCD) (Deshmukh & Johnson, 1997; Martin *et al*., 1998). PCD is required for the

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elimination of excess cells during the sculpting of organs or tissues, and cells not required for a new developmental stage (Ellis *et al*., 1991). Target deprivation mediated neuronal death is also prevalent after trauma or neurodegeneration. Zn^{2+} staining was reported to have a one to one correspondence with those neurons undergoing developmental neuronal death (Lee *et al*., 2006). Furthermore, in vivo intracellular Zn^{2+} chelation using TPEN (N,N,N'N'-tetrakis(−)[2-pyridylmethyl]-ethylenediamine), was recently suggested to attenuate this developmental neuronal death (Cho *et al*.).

Serum Deprivation Models Target Deprivation, and Both Induce an Oxidative Injury

We and others have shown that serum deprivation (SD) induces substantial oxidative stress leading to partial ATP depletion, K^+ loss involving inhibition of the Na^+/K^+ ATPase, and the apoptotic cascade (Wang *et al*., 2003). We are using oxidative stress and serum deprivation as in vitro models approximating target deprivation to test therapeutics and implicate pathways. Reactive oxygen species (ROS), apoptosis, and target deprivation have also been suggested to play roles in progressive neurodegenerative diseases (reviewed in Martin *et al*., 1998). The ROS generated during serum or target deprivations was shown to be a required activating signal for immediate early genes causing an apoptotic cascade leading to caspase activation, mitochondrial dysfunction, and programmed cell death (reviewed in Chang *et al*., 2002; Deshmukh & Johnson, 1997; Martin *et al*., 1998; Martin *et al*., 2003). ROS mediated Zn^{2+} release plays a role in H_2O_2 mediated injuries perhaps through autophagy (Hwang *et al*., 2008). We propose that ROS generated by serum or target deprivation mediated injuries also causes ROS mediated Zn^{2+} release. Therefore, therapeutics that target Zn^{2+} neurotoxicity are effective against these ROS-mediated injuries (see model in Figure 1).

We chose the visual cortex ablation (VCA) induced death of LGNd neurons as our in vivo model of trophic deprivation because of its reproducibility and ease with the intent of defining mechanisms involved and therapeutic doses which would then be tested in more complex models of neurodegeneration. This model was shown to induce a Zn^{2+} mediated death of LGNd neurons after visual cortex (V1) ablation in adult mice that was associated with Zn^{2+} staining (Land & Aizenman, 2005). The cortical neurotrophic factors neurotrophin 4/5, nerve growth factor, and brain derived neurotrophic factor (BDNF) have been implicated as the target derived factors required for LGNd neuronal survival (Berardi & Maffei, 1999; Caleo *et al*., 2003). The hallmarks of trophic-factor deprivation induced apoptosis: ROS, DNA damage, and cytochrome C release have all been demonstrated in LGNd neurons after visual cortex ablation (Agarwala & Kalil, 1998; Al-Abdulla *et al*., 1998; Martin *et al*., 1998; Martin *et al*., 2003). We show here that $[Zn^{2+}]_i$, and $[NAD^+]_i$ concentrations are key to oxidant, and serum or target deprivation mediated neurotoxicities, and that the NAD^+ synthetic and sirtuin pathways are involved in vitro and in vivo (Figure 1).

MATERIALS AND METHODS

Cell culture and toxicity studies

Near-pure cortical neuronal cell cultures were prepared from E15 PARP-1 −/−, +/+, Wld^S, MT-III knockout, and Swiss Webster mice as previously described (Gottron *et al*., 1997; Sheline & Choi, 1998). Dissociated cortical neurons were plated in Eagles's minimum essential medium (MEM; Earle's salts, glutamine-free, Gibco/Invitrogen, Carlsbad, CA) containing 21 mM glucose, 5% fetal bovine serum, and 5% horse serum at a density of 5 hemispheres/plate onto poly-D-lysine/laminin coated 24-well plates. An additional 10 μM

$ZnCl_2$ was added to the plating medium for Zn^{2+} loaded cultures. At 2–3 days in vitro (DIV) cytosine arabinoside was added to 10 μM to inhibit glial growth. Slow toxicity was initiated by exposure to oxidants (ETH 20–60 μM, H_2O_2 40–100 μM, DeNO 200–400 μM) in minimal essential defined media for 24 hours (supplemented with 1 μM MK-801 and 50 ng/ml of BDNF, Cellsciences, Canton, MA) to keep near-pure neuronal cultures from undergoing excitotoxic cell death or spontaneous apoptosis in the absence of astrocytes or serum). Therapeutic compounds were present during the entire exposure period as indicated. Serum deprivation experiments were performed at DIV7 after washing 7 times to remove all serum, cultures were exposed in MEM + 1 μM MK-801 and cell death assayed 30 hrs later. Glucose deprivation experiments were performed after washing 7 times in MEM with amino acids and vitamins, but lacking glucose, cultures were exposed in MEM lacking glucose + 1 μM MK-801 and 50 ng/ml of BDNF for 5 hours followed by 2 times wash into MEM (21 mM glucose + 1 μM MK-801 and 50 ng/ml of BDNF) and cell death was assayed 24 hrs later. Cell death was assessed by lactate dehydrogenase efflux to the bathing medium, and staining with propidium iodide or trypan blue (Sheline & Choi, 1998).

Determination of levels of NAD^+, and NADH

Near-pure cortical neuronal cultures were used for these measurements. Nicotinamide adenine dinucleotide (NAD^+) measurements were made on cell lysates prepared by lysis in 0.2 N NaOH, 1 mM EDTA at 0C. This lysate was split and part of it directly hydrolyzed at 80C for 20 min, and the other part acidified followed by hydrolysis at 80C for 20 min. These lysates were neutralized and stored at −80C. Alkaline hydrolysis destroys NAD^+, whereas acid hydrolysis destroys NADH allowing for determinations of metabolites (FBP, ATP, ADP, NAD^+, and NADH) using the malate dehydrogenase/alcohol dehydrogenase cycling pair (Passonneau & Lowry, 1993). The dynamic range of this cycling assay can be varied (10^{-15} to 10^{-9} moles) by the amount of cycling enzymes used and the duration of the cycling time. 2 μl of acid extract (~1250 cells) was added directly to 100 μl of NAD^+ cycling reagent (100 mM Tris-HCl, pH 8.1, 2 mM β-mercaptoethanol, 2 mM oxaloacetate, 0.3 M ethanol, 0.02% BSA, and 25 μg/ml of yeast alcohol dehydrogenase and 2.5 μg/ml malic dehydrogenase) and incubated at 25°C for 1h to obtain 2000 cycles of malate amplification. Termination by heating at 100°C for 5 min was followed by addition of 1 ml of malate indicator reagent (50 mM amino-methylpropanol (pH 9.9), 5 mM L-glutamate, 0.2 mM NAD^+, 5 μg/ml malic dehydrogenase, and 2 μg/ml glutamate oxaloacetate transaminase). This reaction was incubated for 10 min at 25 °C. The NADH generated from malate was measured fluorimetrically (excitation at 365 nm, emission monitored at 460 nm) (Cai *et al*., 2006; Lin *et al*., 2001). The results obtained are compared to a calibration curve, and normalized to protein content; replicate and duplicate reactions are highly reproducible.

Visual cortex ablation

C57/Bl6, C57/Bl6/Wld^s, ZnT3, and MT-III knockout adult male mice (3–6 months of age) were used, and visual cortex ablation performed as described (Agarwala & Kalil, 1998; Land & Aizenman, 2005; Muessel *et al*., 2000). Briefly, anesthesia was induced by 5% isoflurane (Hospira, Inc., Lake Forest, IL), and maintained with 1.5% isoflurane through a mask. Mice were placed in a stereotax, and using aseptic precautions, a bone flap was made above the visual cortex and a unilateral lesion of visual cortex made by subpial suction (between 0.5 and 3.5 mm anterior to bregma, and 0.5 to 3.5 mm lateral to bregma, and the aspiration syringe inserted ~2 mm ventral to bregma, just touching the corpus callosum). The lesion was covered with a gelfoam sponge moistened in 0.9% saline, and the skin closed with 6-0 suture. The animals were temperature maintained at 37C for 3 hrs, and observed for 24 hrs post-surgery. Intraperitoneal injections of pyruvate, lactate, or nicotinamide were performed at 500 mg/kg 3×/week starting immediately following the surgery. Where indicated a zinc deficient diet (Harlan Teklad, Indianapolis, IN) supplemented with 1 ppm

zinc in the water was given to the mice for 4 weeks prior to and following the ablation (50 ppm is in normal rodent chow plus an indeterminate amount of zinc is present in the tap water normally given to mice). Animals were euthanized at 3 or 7 days post injury by an anesthetic overdose and cervical dislocation; the brains removed, fresh frozen, and processed for Zinpyr-1 (ZP1, TefLabs, Galveston, TX), Nissl, and FluoroJadeB stainings (FJB). These mouse strains were maintained at Washington University's transgenic animal facility. This was a double blind trial, where the animals were age matched, and efforts to minimize animal usage and suffering were taken. Animal usage was minimized by performing both ZP1 and FJB staining on the same animals. If excessive distress would have been noted, animals would have been given appropriate analgesics. Housing and anesthesia concurred with guidelines established by the institutional Animal Studies Committee, and were in accordance with the PHS Guide for the Care and Use of Laboratory Animals, USDA Regulations, and the AVMA Panel on Euthanasia guidelines.

Histology

Cryostat sections of the fresh frozen brain of 16 um thickness were post-fixed in 4% paraformaldehyde in PBS for 15 min, followed by alkaline dehydration and labeling using FluoroJadeB staining (Chemicon/Millipore, Billerica, MA, 0.0004% for 20 min). Sections were mounted and fluorescence microscopy used to detect staining of dead or dying neurons using FluoroJade B (ex. 460 and em. 530). Photomicrographs of the LGNd were taken of 20 sections separated by 5 sections spanning the entire injury. Quantification of neuronal death was determined after counting of these stained neurons by a rater blinded to the genotype, or treatment condition. The average of the total number of stained neurons in 20 sections is presented ± SEM.

Zinc staining

PNC cultures were loaded with 5 μM TSQ to determine basal zinc loading, or were preloaded with 2.5 μM FluoZin3-AM (Invitrogen/Life Technologies, Carlsbad, CA) for 30 minutes prior to oxidant or therapeutic exposures for the indicated times. FluoZin3 staining was quantitated using Metamorph software (Molecular Devices/Danaher, Washington, DC). Briefly, background fluorescence was determined in regions with no cellular structures, and fluorescence signals of greater than 250% of background from structures > 4 um and < 15 um were quantitated. Data is expressed as percent of the fluorescence of control cultures at 1 hour with an n>300.

For in vivo Zn^{2+} stainings, fresh frozen cryostat sections were air-dried and immersed in 5 μM zinpyr-1 (ZP1) in PBS for 2 min, and rinsed in normal saline before fluorescent microscopy (ex. 460 nm, em. 530nm). FluoroJadeB staining was performed on adjacent cryostat sections to correlate the changes in Zn^{2+} staining pattern with neuronal death. Quantification of Zn^{2+} staining was determined after counting of these stained neurons by a rater blinded to the genotype, or treatment condition. The average of the total number of stained neurons in 20 sections spanning the injury site is presented ± SEM.

Genetically manipulated mice

Experiments described utilized genetically manipulated mice. As is true for all knockout animals, there is the possibility of a compensatory change in expression of another family member, or an alternative pathway. To minimize strain differences we have backcrossed the zinc transporter 3, (ZnT3) and MT-III knockout mice (originally prepared in a hybrid 129/Sv background) into C57/Bl6/J for at least 13 generations. We have used the appropriate wildtype littermates, C57/Bl6/J or hybrid line parental controls for the MT-3 or PARP-1 knockouts, and C57/Bl6/Ola for the Wld^{s} lines. The ZnT3 and MT-III knockout mice were kindly supplied by Dr. Palmiter and were then backcrossed. The PARP-1 knockout and the

Wld^S mice are from Jackson Laboratories (Bar Harbor, ME) and Harlan Labs (Loughborough, UK) respectively.

Reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co (St. Louis, MO).

Data analysis and statistics

The changes in neuronal death, and $[NAD^+]_i$ were determined in cultures under the conditions and genotypes stated. The mean ± SEM are plotted and the n is given for each experiment in the figure legends. Results were compared to sham wash or saline injection controls, and toxin or injury exposure alone. One-way ANOVA was used to assess variance in each set of experiments, followed by a Bonferroni test. Significance was achieved by a *P* value of less than 0.05.

RESULTS

Prior Zn^{2+} loading of neuronal cultures potentiated SD and oxidant neurotoxicity, and Zn^{2+} chelation attenuated SD and oxidant neurotoxicity

Neuronal cultures derived from embryonic cortex which has not yet been loaded with Zn^{2+} (typically occurs at postnatal day 14–28) would be expected to have reduced Zn^{2+}. Near-pure cortical neuronal cultures derived from E15 embryos do not stain well with 6-Methoxy-(8-p-toluenesulfonamido)quinoline (TSQ) (DIV 7–9), but cultures grown in the presence of media supplemented with 10 μM Zn^{2+} in addition to the ~1–3 μM Zn^{2+} in the growth medium (MS + 10% FBS) stain better with TSQ, without affecting the number of neurons in the cultures (data not shown). The additional 10 μM Zn^{2+} makes the Zn^{2+} concentration in the growth medium approximately equal to that in cerebral spinal fluid or serum of mice or humans (13–20 μM) (Cesur *et al*., 2005; Davis *et al*., 1998). These Zn^{2+} loaded cultures were more susceptible to oxidant-mediated neurotoxicity (ethacrynic acid-ETH, H_2O_2, serum and glucose deprivation), and the intracellular Zn^{2+} chelator, TPEN, attenuated these neurotoxicities. In addition, the nitric oxide generator diethyl amine NONOate (DeNO), induced neuronal death that was equally attenuated with TPEN, and was potentiated by Zn^{2+} loading (Figure 2 and Supplementary Figure 1). H_2O_2 and serum deprivation were especially responsive to TPEN whereas glucose deprivation was not. Zn^{2+} loading potentiated NAD^+ loss, and Zn^{2+} chelation attenuated loss (Table 1).

Oxidative injuries induced an increase in intracellular free zinc $[Zn^{2+}]_i$

PNC cultures were preloaded with the zinc specific fluorescent dye, FluoZin3-AM at 2.5 μM for 30 min. Excess dye was washed out and cultures were exposed as indicated. All three oxidant exposures caused a significant increase in $[Zn^{2+}]_i$ at 1 and 5 hours post exposure. This increase in $[Zn^{2+}]_i$ was prevented by addition of the Zn^{2+} chelator, N,N,N'N'-tetrakis(−)[2-pyridylmethyl]-ethylenediamine (TPEN), and was attenuated by the ROS scavenger, trolox (500 μM), but not by pyruvate, nicotinamide, or NAD^+ (Figure 3). These compounds also do not affect $^{65}Zn^{2+}$ influx, and where determined, have a very low Kd for Zn^{2+} (Martell, 1995;Sheline *et al*., 2004). TPEN also reduced the levels of background and control fluorescence.

Compounds which restore $[NAD^+]_i$ attenuated SD and oxidant neurotoxicities, while 3-AP potentiated toxicities

We investigated the effects on ETH, H_2O_2, serum and glucose deprivation mediated neurotoxicities of decreasing or increasing intracellular NAD^+ levels ($[NAD^+]_i$) using pretreatment with 3-acetyl pyridine (3-AP, 50 μM) to generate inactive NAD^+, or addition

of exogenous pyruvate, nicotinamide or NAD^+ to increase $[NAD^+]_i$. Ethacrynic acid is being used as an inducer of intracellular ROS, as it is thought to both induce intracellular ROS, and reduce levels of glutathione (Rizzardini *et al*., 2003). H_2O_2 is being used as an inducer of extracellular ROS. As shown in figure 4, 3-AP potentiated most of these neurotoxicities, and exogenous pyruvate, nicotinamide, and NAD^+ (6–10 mM) reduced these neurotoxicities to varying extents. Lactate was ineffective or detrimental. Pyruvate and ZVAD (100 μM) attenuated the basal death of control cultures (normally occurring apoptosis of PNC cultures), whereas the other compounds did not have significant basal effects. The death induced by oxidants was only mildly attenuated by inhibitors of apoptosis or autophagy, whereas glucose and serum deprivation induced death were significantly attenuated (Supplementary Figure 2A–B).

Pyruvate, nicotinamide and NAD^+ restored $[NAD^+]_i$, and glycolytic flux that was inhibited by oxidant neurotoxicities

Pyruvate, nicotinamide, and NAD^+ have been previously shown to restore $[NAD^+]_i$ and glycolytic flux, and to attenuate both chronic and acute Zn^{2+} neurotoxicity, while 3-AP potentiated Zn^{2+} neurotoxicities (Cai *et al*., 2006; Sheline *et al*., 2000). A 30–70% loss of NAD^+ levels occurred before the onset of SD and oxidant neurotoxicities, and pyruvate, nicotinamide, and TPEN restored $[NAD^+]_i$. Furthermore, levels of FBP were elevated by these exposures suggesting glycolytic inhibition, and were restored by pyruvate, nicotinamide or TPEN (Table 1).

Near-pure neuronal cultures from PARP-1 and MT-III knockouts were susceptible to ethacrynic acid mediated neurotoxicity, but H_2O_2-, and SD-mediated toxicities were reduced; Wld^S cultures were somewhat resistant to ETH, and SD

NAD^+ levels were reduced prior to oxidant-induced neurotoxicity, and compounds which restored $[NAD^+]_i$ attenuated toxicity. This led us to study the most prevalent NAD^+-catabolic enzyme, PARP-1. PARP-1 induces poly-ADP ribosylation (PAR) of proteins using NAD^+ as substrate in response to DNA nicking caused by oxidative stress. Oxidative injuries which target DNA (peroxynitrite, superoxide, and MNNG) have been shown to be very responsive to poly-ADP ribose polymerase (PARP-1) inhibition or knockout (see above). We previously demonstrated by Western blots that exogenous Zn^{2+} exposure did not induce PAR in neurons, but did induce PAR in glia (Sheline *et al*., 2003). Wld^S animals overexpress the NMNAT-1 protein, and have been suggested to better maintain NAD^+ (Bedalov & Simon, 2004). MT-III knockouts have reduced brain Zn^{2+}, and therefore should have less oxidant releasable Zn^{2+} (Erickson *et al*., 1997). Near pure neuronal cultures from PARP-1 knockouts (and it's control 129) and MT-III knockouts or Wld^S (and their control B6), were exposed to ethacrynic acid, H_2O_2, or serum deprivation. Neuronal death was determined to be modestly reduced for Wld^S compared to controls for ETH, and was reduced for MT-III and PARP-1 knockouts and controls for H_2O_2. All 3 mouse lines showed significantly less death after serum deprivation (Figure 5). Larger effects for these genetic variants (Wld^S, MT-III KO, and PARP-1 KO) have been reported for in vivo mediated neuronal injuries such as Wallerian degeneration, seizures, and ischemias respectively. The lesser effects seen in vitro could be due to lower levels of expression of the target proteins in cultures from embryonic tissue with a limited number of days in vitro for development.

Neuronal death induced by ROS generators, and serum or glucose deprivations were attenuated by sirtuin inhibitors, and potentiated by sirtuin activators

The sirtuin pathway catalyzes the NAD^+-dependent deacetylation of proteins resulting in modulation of their function; the substrates for the reaction are acetylated protein (at lysines) and NAD^+ with the products being O-acetyl ADP-ribose, nicotinamide, and de-acetylated

protein. We utilized chronic administration of the sirtuin-pathway inhibitors sirtinol and its precursor, 2- hydroxynaphthaldehyde at 10 μM to attenuate SD and oxidant neurotoxicity. We also utilized a 3 hr pretreatment with the sirtuin-pathway activators resveratrol and fisetin at 10 μM to potentiate SD and oxidant neurotoxicity (Figure 6). Activators of the sirtuin pathway further reduced NAD^+ levels (Table 1). In a manner similar to that seen in exogenous Zn^{2+} toxicity (Cai *et al*., 2006), sirtinol did not significantly restore NAD^+ levels after ROS or SD neurotoxicities.

Visual cortex ablation induces Zn^{2+} staining and death in lateral geniculate nucleus neurons; LGNd neuronal death in pyruvate-, nicotinamide-, or reduced zinc diet-treated or Wld^S animals was attenuated

Recently, target deprivation of dorsal lateral geniculate nucleus (LGNd) neurons by ablation of the visual cortex was shown to induce intracellular Zn^{2+} staining, and LGNd neuronal death. LGNd neurons are normally devoid of synaptic/stainable Zn^{2+} suggesting intracellular Zn^{2+} release (Land & Aizenman, 2005). We have now reproduced the results of Land, and examined LGNd Zn^{2+} staining (ZP1) and death (FluoroJadeB) after cortical ablation in C57/Bl6 controls −/+ intraperitoneal injection of pyruvate or nicotinamide, and in Wld^S mice (Figures 7 & 8, and Table 2). Both ZP1 and FluoroJadeB staining of LGNd neurons increased only on the ipsilateral (injured) side 3 or 7 days after the visual cortex ablation in wildtype animals. Pyruvate or nicotinamide (500 mg/kg i.p. 3×/week) attenuated LGN neuronal death while the number of LGNd neurons staining for Zn^{2+} remained elevated. A similar lactate injection was ineffective. In addition, a reduced zinc diet (1ppm versus 50 ppm) attenuated LGN neuronal death and Zn^{2+} staining after VCA (Table 2). The number of dead LGNd neurons in Wld^S mice was reduced to 15% compared to wildtype mice, and Zn^{2+} staining was reduced as well. This dramatic attenuation of LGNd death and Zn^{2+} staining by the Wld^S protein perhaps suggests that axonal/synaptic protection may play a role, and that Wld^S may attenuate the mechanisms of Zn^{2+} accumulation. ZnT3, and MT3 knockout mice had a similar susceptibility to VCA compared to controls, whereas LGNd death in PARP1 knockouts was mildly attenuated (Figure 7 and Table 2).

DISCUSSION

In these studies we show that our neuronal cultures normally have reduced Zn^{2+}, and Zn^{2+} proficiency increased sensitivity to SD and oxidative injuries, and a Zn^{2+} chelator attenuated these injuries (Figure 2, Supplementary Figure 1). SD and oxidative injuries induced an increase in $[Zn^{2+}]_i$ (Figure 3). SD and oxidative injuries induced a loss of NAD^+ resulting in an increase in FBP; restoration of NAD^+ using exogenous NAD^+, pyruvate, or nicotinamide attenuated the increase in FBP and these injuries, and potentiation of NAD^+ loss potentiated injury (Figure 4, Table 1, Supplementary Figure 2). Genetically modified or inbred mouse strains which reduce intracellular Zn^{2+} content (MT-III knockout), reduce NAD^+ catabolism (PARP-1 knockout), or increase NAD^+ synthesis (Wld^S) attenuated SD and oxidative injuries to different extents (Figure 5). Inhibitors of the sirtuin pathway attenuated SD and oxidant neurotoxicity, and activators potentiated these injuries (Figure 6). Ipsilateral LGNd neuronal death after visual cortex ablation was particularly reduced in Wld^S mice, and substantially reduced in pyruvate, nicotinamide, or reduced Zn^{2+} diet treated mice, but lactate was ineffective (Figures 7, 8, and Table 2).

Injury Models and Intracellular Zn^{2+}

Some in vivo neuronal injuries may involve ROS-mediated intracellular Zn^{2+} release as opposed to synaptic Zn^{2+} release. These neuronal injuries may include kainate-induced seizure, target deprivation, and MCAO (Land & Aizenman, 2005; Lee *et al*., 2002; Lee *et al*., 2003). Kainate-induced seizure mediated CA1 and thalamic neuronal Zn^{2+} staining and

death were not responsive to knockout of ZnT3 (no synaptic Zn^{2+}), but were responsive to knockout of MT-III. This suggests that intracellular Zn^{2+} stores (especially MT-III) are more important than synaptic stores for seizure mediated CA1 and thalamic neuronal death. This result is in contrast to the equivalent sensitivity of MT-III knockout versus wildtype thalamic neurons to VCA reported here. One possible explanation is that MT3 is also an antioxidant, and its antioxidant function is more dominant in VCA in vivo than in seizure mediated injury or in vitro. It was suggested that Zn^{2+} was released from intracellular stores such as the mitochondrion or MT-III (Aravindakumar *et al*., 1999; Pal *et al*., 2004). Overexpression of metallothionein in glial cells provided for both an increased buffering capacity for $[Zn^{2+}]_i$ but also an increased pool of intracellular Zn^{2+} able to be released by oxidant exposure (Malaiyandi *et al*., 2004). Other pools of Zn^{2+} are present inside neurons including ribosomal, lysosomal, mitochondrial, and nuclear Zn^{2+}, and Zn^{2+} bound to proteins other than MT-III.

LGNd neurons lack synaptic Zn^{2+} suggesting that the increased $[Zn^{2+}]_i$ after VCA results from intracellular release (Mengual *et al*., 2001). The Zn^{2+} staining after VCA started at 2–3 days, preceding caspase-3 activation at 4–5 days and neuronal death at 5–7 days (Land & Aizenman, 2005). In contrast, other injuries, including global and retinal ischemias, and hypoglycemia, appear to result from synaptic release and reuptake of Zn^{2+} (Koh *et al*., 1996; Suh *et al*., 2005; Suh *et al*., 2007b; Suh *et al*., 2008; Yoo *et al*., 2004). Zn^{2+} neurotoxicity has also been implicated in neuronal oxidant-mediated injuries in vitro using a thiol oxidizing agent, peroxynitrite, or chemical glutathione depletion (Aizenman *et al*., 2000; Ho *et al*., 2008; Zhang *et al*., 2004). Alternate Zn^{2+} neurotoxicity mechanisms involving ROS, mitochondria, and activation of kinase cascades have also been proposed (Kim *et al*., 1999; Kim & Koh, 2002; Noh *et al*., 1999; Redman *et al*., 2009; Sensi *et al*., 2003; Sensi *et al*., 2000; Zhang *et al*., 2007).

NAD^+ Loss and Zn^{2+} neurotoxicity

Similar to what we show in this manuscript on intracellular Zn^{2+} release, we have previously demonstrated that nicotinamide adenine dinucleotide (NAD^+) levels are reduced upon Zn^{2+} exposures, and NAD^+ restoration attenuated exogenous Zn^{2+} neurotoxicity, whereas $[NAD^+]_i$ reduction potentiated Zn^{2+} neurotoxicity . The Zn^{2+}-induced loss of NAD^+ levels resulted in a block of glycolysis at GAPDH (increased levels of FBP/DHAP) in both neurons and glia, and under both chronic and acute Zn^{2+} exposure conditions; NAD^+ restoration unblocked GAPDH (Cai *et al*., 2006; Sheline *et al*., 2000). In addition, NAD^+ levels were shown to be reduced, and $[Zn^{2+}]_i$ increased in the rat permanent global ischemia model, after hypoglycemia, and other oxidative injuries (Alano *et al*., 2004; Plaschke *et al*., 2000; Suh *et al*., 2003; Virag *et al*., 2003; Ying *et al*., 2001).

We measure NAD^+ levels in total cellular lysates, and NAD^+ is small enough to permeate through the nuclear pores. Nuclear $[NAD^+]$ has been reported to be 70 μM suggesting this to be the cytoplasmic concentration as well (Fjeld *et al*., 2003). Since exogenous NAD^+ was neuroprotective and neurons can take up NAD^+, but mitochondria are not thought to take up NAD^+, then restoration of cytoplasmic/nuclear NAD^+ concentrations may be sufficient to attenuate Zn^{2+}, oxidative, and serum or target deprivation neurotoxicities (this manuscript, and Cai *et al*., 2006). Also, the efficacy of NAD^+ and nicotinamide against oxidative neurotoxicities were similar (Figure 3), but only nicotinamide is a catabolism inhibitor. This suggests that $[NAD^+]_i$ levels may be the critical determinant rather than inhibition of catabolism.

We previously examined the effects of Zn^{2+} on the predominant catabolizer of NAD^+, PARP, by measuring levels of poly-ADP ribosylation (PAR). In cortical cultures, only glia had significant PAR formation which was only induced by an acute 400 μM Zn^{2+} exposure,

not a chronic 40 μM Zn^{2+} exposure. Cortical neuronal cultures (neurons and glia) from PARP-1 −/− mice were found to be partially resistant to an acute 400 μM Zn^{2+} exposure, but appeared equally sensitive to a chronic 40 μM Zn^{2+} exposure (Sheline *et al*., 2003). PARP knockout or inhibition has been shown to attenuate oxidative injuries that induce DNA strand breaks (peroxynitrite, MNNG, and superoxide/hypoglycemia) (Alano *et al*., 2004; Suh *et al*., 2003; Virag *et al*., 2003; Ying *et al*., 2001). To the extent that PARP knockout spares $[NAD^+]_i$, then it would be expected to attenuate Zn^{2+} neurotoxicity, and does mildly attenuate VCA (Table 2, and Araki *et al*., 2004). In addition, Zn^{2+} induction of PARP-1 in astrocytes causes glycolytic inhibition and inhibition of glutamate uptake both of which would be detrimental to their role of supporting neurons (Sheline *et al*., 2004; Suh *et al*., 2007a). We recently demonstrated that neurons from slow Wallerian degeneration mice (Wld^s) were resistant to exogenous Zn^{2+} neurotoxicity (Cai *et al*., 2006).

Wld^s mice contain a spontaneous autosomal dominant recombination triplication mutation resulting in the overexpression of NMNAT-1 an important enzyme in the synthesis of NAD^+. The CA1, CA2 and caudate nucleus of Wld^s mice showed substantially less death after transient global ischemia and reperfusion, or oligodendrocyte death after spinal cord injury (Dong *et al*., 2003; Gillingwater *et al*., 2004). It has now been shown that the NMNAT-1 portion of the fusion protein is requisite for axonal protection (Araki *et al*., 2004), that NMNAT catalytic activity is required, and cytoplasmic overexpression of NMNAT1 is sufficient for Wld^s mediated axonal- and neuro- protection (Sasaki *et al*., 2009a; Sasaki *et al*., 2009b). The therapeutic effects in cultures derived from Wld^s embryonic tissue was less than that demonstrated for pharmacologic neuroprotectants, and much less than that demonstrated in adult Wld^s mice subjected to VCA (Figure 4, Table 2). This could be because the effects of the Wld^s phenotype require protein expression, or loss of protein expression which may not occur in embryonically derived neuronal cultures with a limited number of days in culture (Gillingwater *et al*., 2006). The large reduction in LGNd neuronal death after VCA in Wld^s mice, compared to the efficacy of pyruvate or nicotinamide, suggests that in addition to effects on NAD^+ levels, overexpression of NMNAT1 is especially beneficial in vivo perhaps through additional effects on axonal/synaptic integrity or Zn^{2+} accumulation. We recently demonstrated that exogenous Zn^{2+} neurotoxicity could also be attenuated by inhibition of another NAD^+ catabolic pathway, the sirtuin pathway (Cai *et al*., 2006).

The sirtuin family of proteins are NAD^+-dependent protein deacetylases which catalyze the removal of acetyl groups from lysine amino acids of histones and transcription factors resulting in their modulation. They have been implicated in genomic stability, transcriptional silencing, transcriptional regulation, mitochondrial regulation, and lifespan extension through effects on p53, forkhead transcription factors and histones (for review see Blander & Guarente, 2004). The inhibitors sirtinol and 2-hydroxy napthaldehyde, have been reported to be specific for sirtuin proteins and to not affect histone deacetylase or PARP activities (Grozinger *et al*., 2001). Previous neuroprotective effects of sirtuins were mediated through the deacetylation of target proteins, especially transcription factors (Blander & Guarente, 2004; Brunet *et al*., 2004; Lantz & Kaestner, 2005). These results are in contrast to our results where activation of the sirtuin pathway was detrimental. In Zn^{2+}, oxidant, or serum deprivation neurotoxicities, NAD^+, pyruvate or nicotinamide were not therapeutic if only a pretreatment was performed (data not shown, and Cai *et al*., 2006). Pretreatment with NAD^+ and SIRT-1 activation were required for the beneficial effects on Wallerian degeneration, which occurred through effects on the transcription/translation of target genes (Araki *et al*., 2004). This suggests that the mechanism involved in the negative effects of sirtuins may be NAD^+ loss. However, NAD^+ levels were not significantly restored by sirtuin inhibition, suggesting sirtuins may have another role, or that sirtinol inhibits deacetylation but not NAD^+ catabolism.

Pyruvate or nicotinamide also reduce death after many in vivo injuries including diabetes, global, focal, and retinal ischemias, and hypoglycemia (Chang *et al*., 2003; Hassan & Janjua, 2001; Lee *et al*., 2001; Suh *et al*., 2003; Suh *et al*., 2005; Visalli *et al*., 1986; Yamada *et al*., 1982; Yoo *et al*., 2004; Cai *et al*., 2006). These injuries all involve Zn^{2+} toxicity, and these compounds would be expected to ameliorate Zn^{2+} toxicity by restoring NAD^+ levels. There have been fewer reports of in vivo therapeutic efficacy of inhibitors of alternate Zn^{2+} neurotoxicity mechanisms such as kinase cascades, autophagy, or mitochondrial dysfunction. Pyruvate is converted to lactate by lactate dehydrogenase regenerating NAD^+ at the expense of NADH. Lactate is ineffective even harmful against exogenous Zn^{2+}, serum or glucose deprivation, or oxidant mediated neurotoxicities in vitro, or against global or focal ischemias, target deprivation, or hypoglycemia mediated neuronal death in vivo (Table 2, data not shown, and Cai *et al*., 2006; Koh *et al*., 1996; Sheline *et al*., 2000; Suh *et al*., 2005). This shows that NAD^+ restoration is required for efficacy, not availability of energy substrates. Beyond NAD^+ restoration, pyruvate can hyperpolarize and activate mitochondria, and is a crucial, permeable, endogenous α-keto acid which can inactivate H_2O_2 thereby acting as a sink for oxygen radicals (Holleman, 1904; Kauppinen & Nicholls, 1986). In addition to those injuries cited above, pyruvate has also been shown to be cytoprotective against: heart transplant (Kojima *et al*., 1993), quinolinic acid injection (Ryu *et al*., 2003), NMDA (Maus *et al*., 1999), H_2O_2 (Desagher *et al*., 1997), Zn^{2+}, ROS, SD, GD, and VCA (this manuscript and Sheline *et al*., 2000).

Nicotinamide can induce increased synthesis of NAD^+, or decrease its degradation by NAD^+-catabolizing enzymes. All NAD^+-catabolizing reactions that have nicotinamide as a leaving group would be inhibited by nicotinamide. Nicotinamide has also been shown to induce an increase in basal levels of NAD^+, and to restore NAD^+ and mitochondrial function after injury (Chong *et al*., 2004; Klaidman *et al*., 2003). It has been suggested to prevent PARP-activation and NAD^+ depletion, thereby reducing apoptosis of neurons induced by DNA damage (reviewed in Szabo & Dawson, 1998). Other “PARP inhibitors” were shown to be effective against global and focal ischemia (Endres *et al*., 1997; Plaschke *et al*., 2000). These “PARP inhibitors” are designed to look like nicotinamide, and therefore could be inhibiting any enzyme whose reaction involves nicotinamide as a leaving group including sirtuins.

These experiments have demonstrated that Zn^{2+}, NAD^+, and the sirtuin pathway are involved in target deprivation and oxidant-induced neurotoxicity. We believe the specific enzymes involved in the NAD^+ loss and neurotoxicity of exogenous Zn^{2+} and Zn^{2+} released intracellularly may be the same. Furthermore, these studies have validated and suggested mechanisms of action for pyruvate and nicotinamide as therapeutic compounds against target deprivation, hopefully spurring interest in clinical trials. Finally, since target deprivation occurs in many injury and neurodegenerative models, these compounds and pathways should be tested in these other models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SD	serum deprivation
LGNd	dorsal lateral geniculate nucleus
$[Zn^{2+}]_i$	intracellular Zn^{2+} levels
$[NAD^+]_i$	intracellular NAD^+ levels
VCA	visual cortex ablation
MT-III	metallothionein-3
PCD	programmed cell death
ROS	Reactive oxygen species
BDNF	brain derived neurotrophic factor
NAD^+	Nicotinamide adenine dinucleotide
TSQ	6-Methoxy-(8-p-toluenesulfonamido)quinoline
ZP1	zinpyr-1
ZnT3	zinc transporter 3
ETH	ethacrynic acid
DeNO	diethyl amine NONOate
Wld^s	Slow Wallerian degeneration mouse
PAR	poly-ADP ribosylation
PARP-1	poly-ADP ribose polymerase
TPEN	N,N,N'N'-tetrakis(−)[2-pyridylmethyl]-ethylenediamine
3-AP	3-acetyl pyridine
NMNAT	Nicotinamide mononucleotide adenyl transferase

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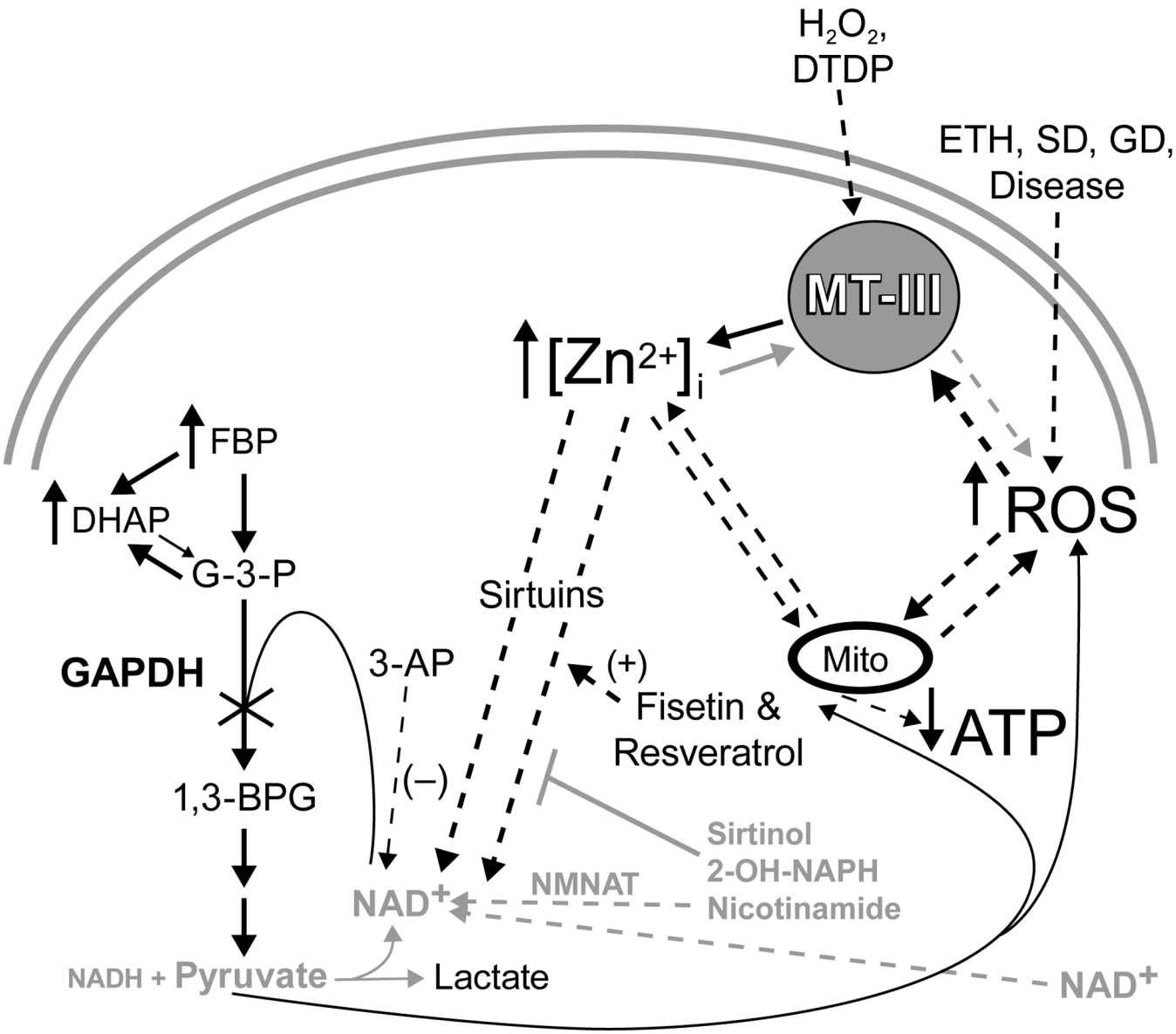

Figure 1. Model of Zinc Neurotoxicity

The resulting increased $[Zn^{2+}]_i$ may cause direct inhibition of mitochondria, or an indirect inhibition of GAPDH and mitochondria by a reduction in NAD^+ levels induced by an unknown NAD^+ catabolizing enzyme (sirtuins). Pyruvate and nicotinamide prevent NAD^+ loss and enzyme inhibition. Black = Toxic, Gray = Therapeutic.

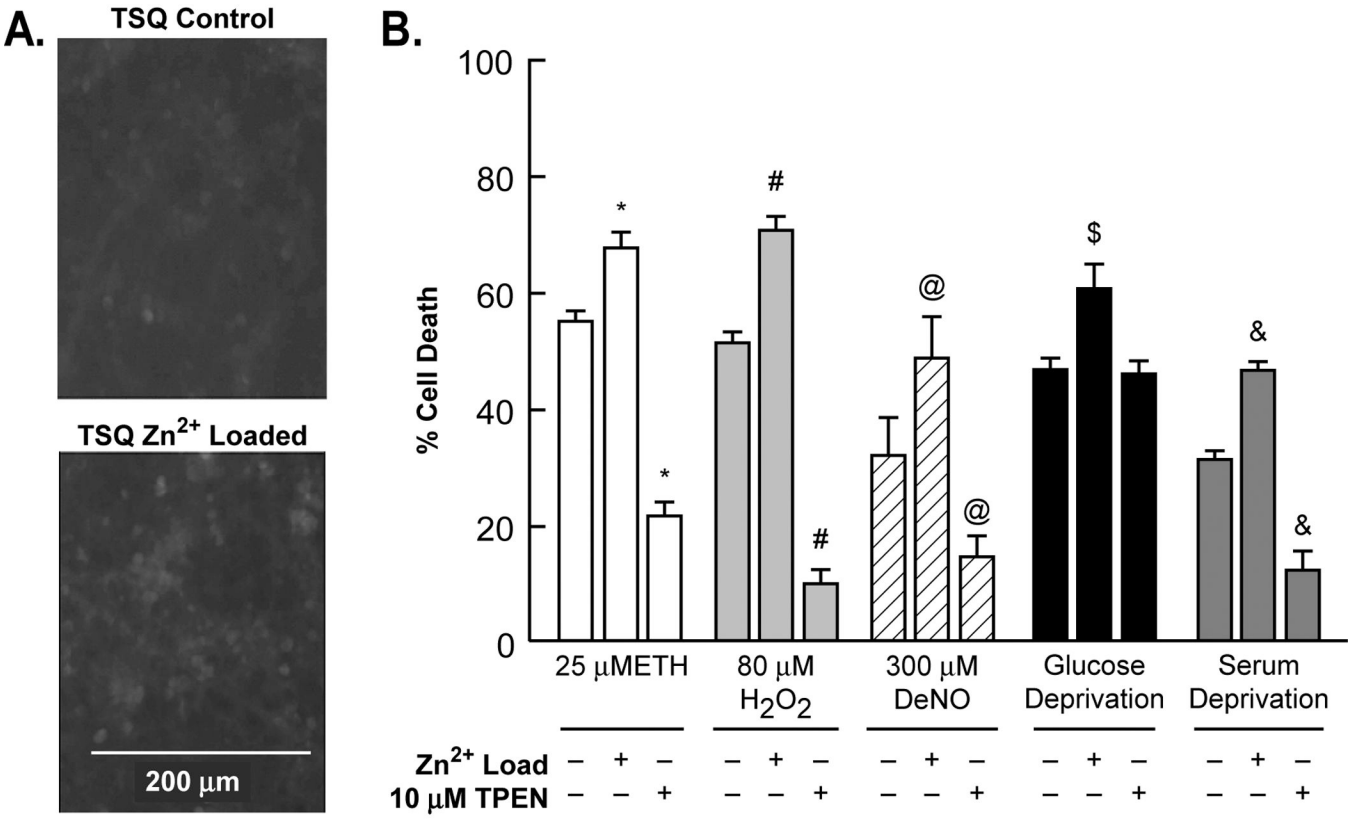

Figure 2. Zn^{2+} loading potentiated, and Zn^{2+} chelation attenuated SD, and oxidant mediated neurotoxicity

A. Near-pure neuronal cultures were cultured for 8 days - or + an additional 10 μM Zn^{2+} after which the neurons were stained for Zn^{2+} using TSQ and fluorescence photomicrographs taken. B. Near-pure cortical neuronal cultures were exposed chronically to the indicated conditions. Neuronal death was determined 24–36 hrs later by lactate dehydrogenase release to the medium scaled to the level associated with near complete death produced by exposure to 20 μM A23187 for 24 hrs, = 100 (mean ± SEM, n = 6–18 cultures per condition). * # $ & @ indicate difference from appropriate oxidant exposure in the absence of added Zn^{2+} at $P<0.05$

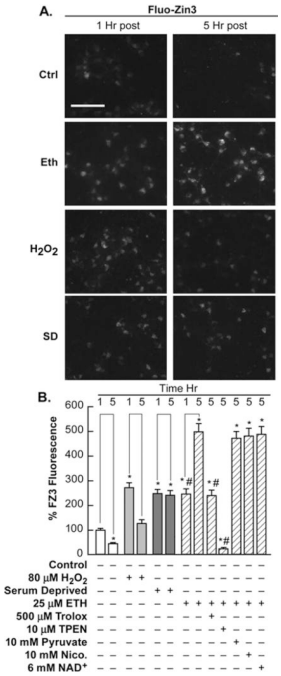

Figure 3. $[Zn^{2+}]_i$ was increased by SD and oxidant exposures

Near-pure neuronal cultures were cultured for 7–8 days after which the neurons were loaded for 30 minutes with 2.5 μM FluoZin3-AM (Zn^{2+} fluorescent indicator). A. These cultures were then washed and exposed to SD and oxidants as indicated and identical exposure fluorescence photo-micrographs were taken at 200x magnification, bar represents 50 microns. B. The fluorescence intensity of the cells under these conditions were quantitated using Metamorph and are presented as the percentage of baseline fluorescence. The effects of therapeutic concentrations of compounds against ethacrynic acid induced increases in $[Zn^{2+}]_i$ were also tested as indicated. * indicates difference from 1 hr control exposure at $P<0.05$; # indicates difference from 5 hr ethacrynic acid exposure at $P<0.05$

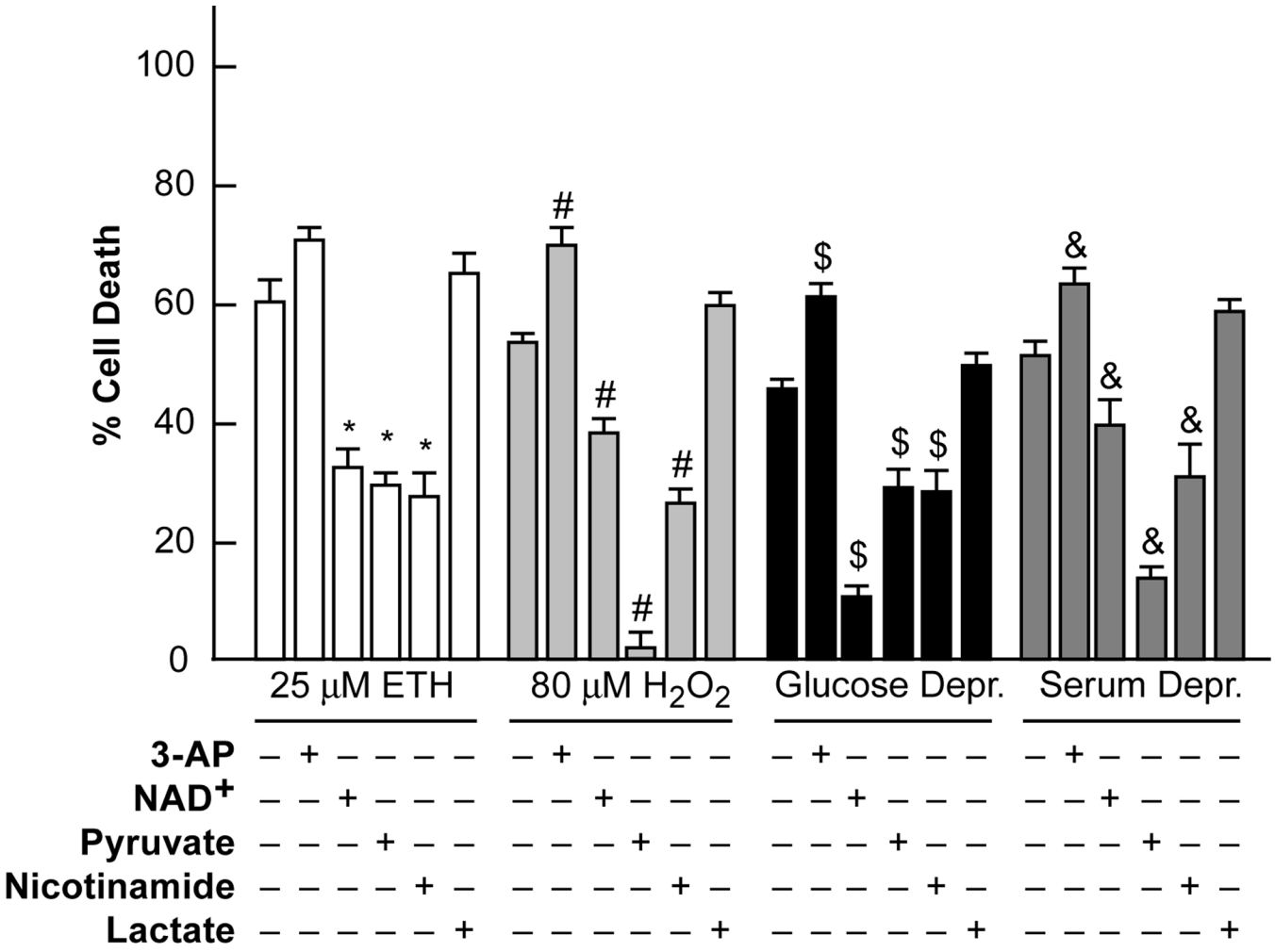

Figure 4. 3-AP potentiated SD and oxidant neurotoxicity, and increased $[NAD^+]_i$ attenuated them; lactate was ineffective

Near-pure cortical neuronal cultures were exposed to the indicated conditions. Neuronal death was determined 24 hrs later by propidium iodide staining scaled to the level associated with near complete death produced by exposure to 20 μM A23187 for 24 hrs, = 100 (mean ± SEM, n = 9–18 cultures per condition). 3-AP indicates the addition of 50 μM 3-acetyl pyridine, NAD^+ indicates the addition of 6 mM NAD^+. Pyruvate, nicotinamide and lactate were present as indicated at 10 mM. * indicates difference from ethacrynic acid (ETH) exposure, # indicates difference from H_2O_2 exposure, $ indicates difference from glucose deprivation, and & indicates difference from serum deprivation at $P < 0.05$ by one-way ANOVA followed by a Bonferroni test.

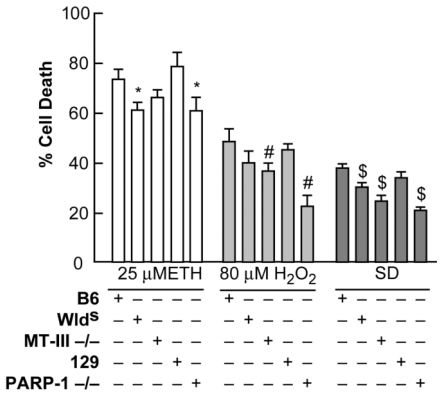

Figure 5. Neuronal cultures from MT-III and PARP-1 knockouts and Wld^S were differentially sensitive to SD and oxidant neurotoxicity

Near-pure cortical neuronal cultures from MT-III and PARP-1 knockouts, Wld^S and control cultures were exposed to the indicated conditions. Neuronal death was determined 24 hrs later by lactate dehydrogenase release to the medium. * indicates difference between B6 control cultures and Wld^S cultures after ETH exposure at $P < 0.05$; # indicates difference between control cultures and MT-III or PARP-1 knockouts after H_2O_2 exposure at $P< 0.05$. $ indicates difference between control cultures and MT-III or PARP-1 knockouts, or Wld^S cultures after serum deprivation at $P< 0.05$. B6 is the appropriate control for MT-III −/− and Wld^S, and 129 is the appropriate control for PARP-1 −/−.

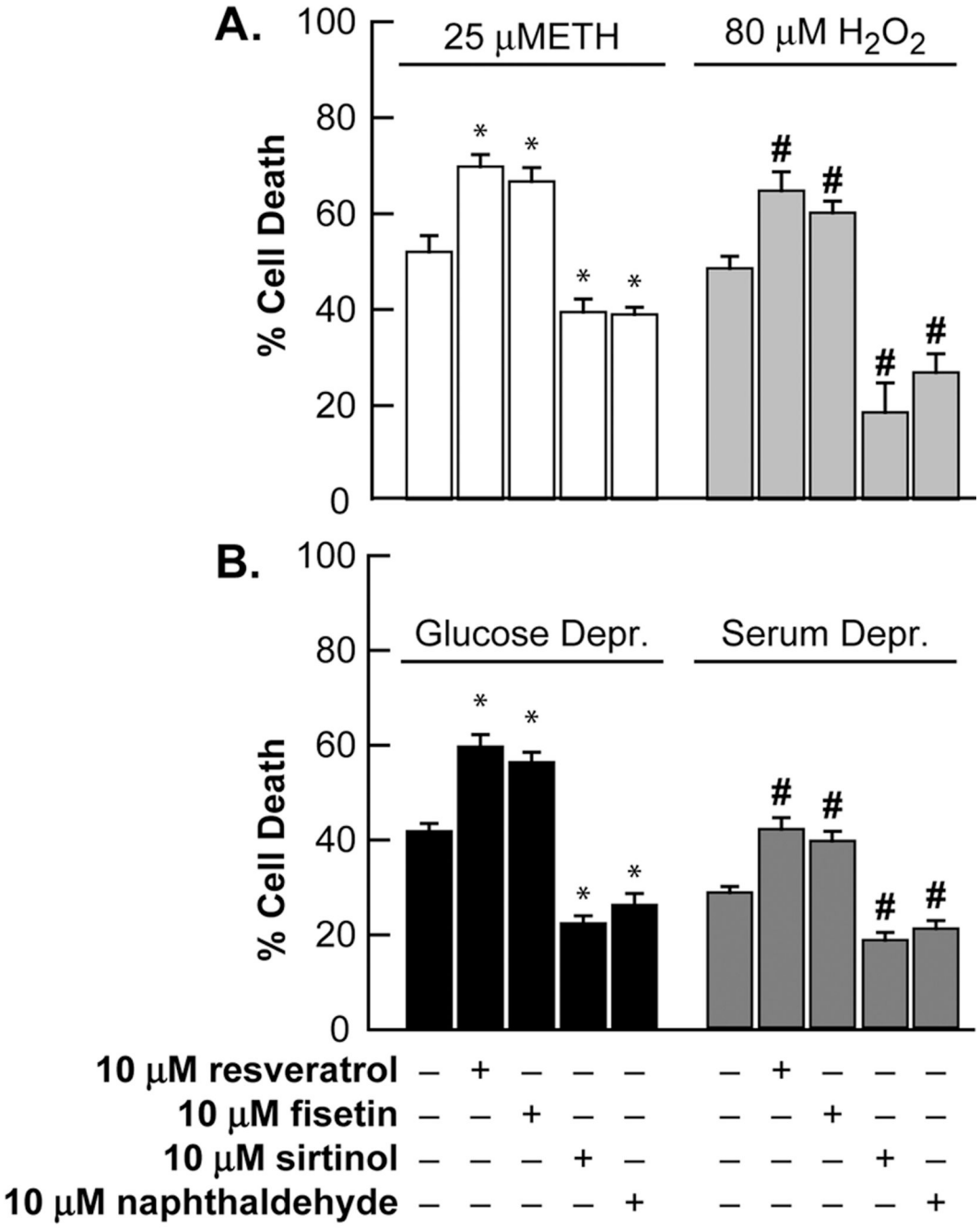

Figure 6. Ethacrynic acid, H_2O_2, serum or glucose deprivation mediated neuronal injuries were attenuated by sirtinol or 2-hydroxynaphthaldehyde, and these injuries were potentiated by resveratrol or fisetin

Near-pure neuronal cultures were exposed to A) 25 μM ethacrynic acid (ETH), or 80 μM H_2O_2 for 24 hrs, or B) to 5 hrs of glucose deprivation, or continuous serum deprivation (36 hrs) and neuronal death was assessed by LDH release to the bathing medium (mean ± SEM, n = 6–18 cultures per condition). Fisetin and resveratrol exposures were a 3 hr pretreatment at 10 μM. Sirtinol and 2-hydroxynaphthaldehyde were present chronically as indicated at 10 μM. * indicates difference from 25 μM ETH; # indicates difference from 80 μM H_2O_2; $ indicates difference from 5 hours of glucose deprivation exposure; and & indicates

difference from serum deprivation exposure at $P < 0.05$ by one-way ANOVA followed by a Bonferroni test.

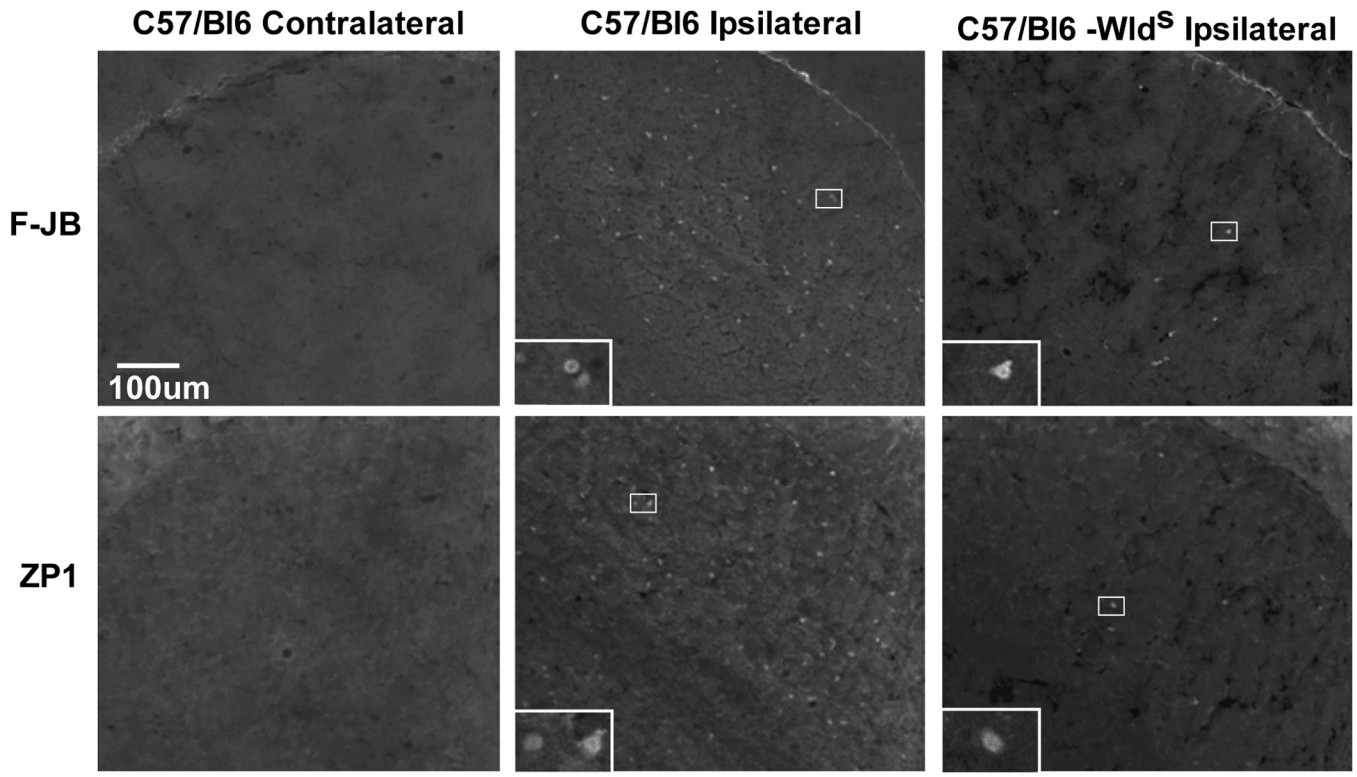

Figure 7. Ipsilateral LGNd neurons die after visual cortex ablation; Wld^S mice were resistant

Unilateral V1 ablation was performed and animals were sacrificed after 7–11 days, and the LGNd was stained for Zn^{2+} using ZP1, or death using FluoroJadeB. Identical exposure fluorescence photo-micrographs were taken at 100x magnification, bar represents 100 microns. Insets are the boxed area at 400x magnification, and the bar represents 25 microns.

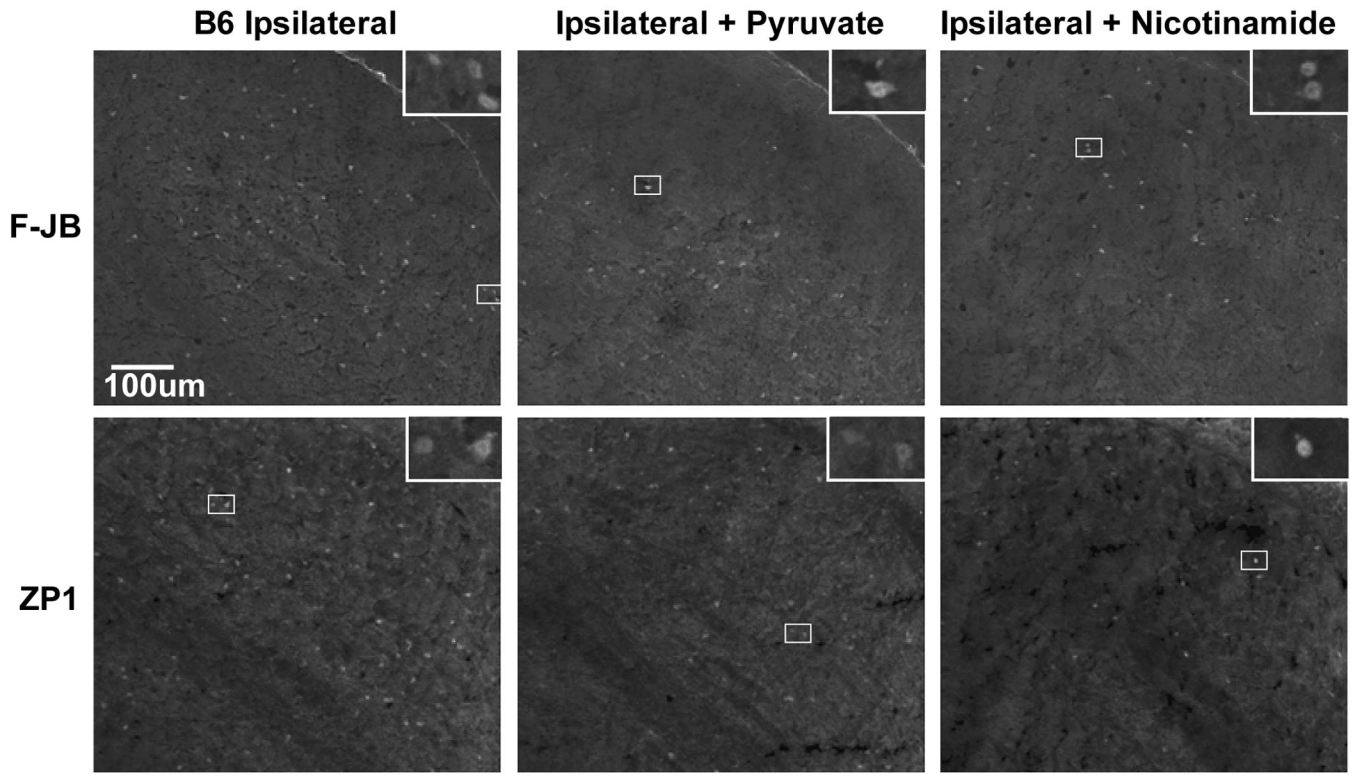

Figure 8. Only ipsilateral LGNd neurons stain for Zn^{2+} and die after visual cortex ablation; pyruvate, or nicotinamide treated mice were resistant

Unilateral V1 ablation was performed and animals were sacrificed after 7–11 days, and the LGNd was stained for Zn^{2+} using ZP1, or death using FluoroJadeB. Identical exposure fluorescence photo-micrographs were taken at 100x magnification, bar represents 100 microns. Insets are the boxed area at 400x magnification, and the bar represents 25 microns.

Table 1

NAD^+ levels are reduced by SD and oxidant exposures, and are restored by pyruvate, nicotinamide, and TPEN

Near-pure cortical neuronal cultures were exposed and total cellular NAD^+ was isolated at the times indicated which are prior to neuronal death (n >10).

Condition	NAD^+ (nmols/mg)	FBP (nmols/mg)
Control	2.9 ± 0.2	4.0 ± 0.3
+ Zn^{2+} preload	2.76 ± 0.2	N.P.
30 μM Ethacrynic acid (2 hr)	1.69 ± 0.15 *	11.3 ± 0.5 *
+ 10 mM pyruvate	2.27 ± 0.13 #	5.1 ± 0.3 #
+ 10 mM nicotinamide	2.35 ± 0.13 #	5.3 ± 0.4 #
+ Zn^{2+} preload	0.29 ± 0.03 #	N.P.
80 μM H_2O_2 (2 hr)	0.69 ± 0.03 *	14.3 ± 0.6 *
+ 10 mM nicotinamide	2.64 ± 0.18 #	6.4 ± 0.8 #
+ 10 μM TPEN	1.82 ± 0.07 #	7.2 ± 0.7 #
+ Zn^{2+} preload	0.29 ± 0.03 #	N.P.
Serum deprivation (6 hr)	2.01 ± 0.12 *	7.9 ± 0.3 *
+ 10 mM pyruvate	2.45 ± 0.04 #	5.0 ± 0.4 #
+ 10 mM nicotinamide	2.66 ± 0.05 #	5.2 ± 0.5 #
+ 10 μM TPEN	2.62 ± 0.2 #	6.2 ± 0.2 #
+ Zn^{2+} preload	1.39 ± 0.07 #	N.P.

* indicates a significant difference from control.

# indicates a significant difference from toxin alone at $P < 0.05$ by one-way ANOVA followed by a Bonferroni test.

Table 2

Quantitation of LGNd neuronal death after unilateral visual cortex ablation; Pyruvate and nicotinamide attenuated death, and Wld^S mice or mice pre-fed a zinc deficient diet were resistant to injury

C57/Bl6 with or without pyruvate, or nicotinamide, or lactate (500 mg/kg i.p. 3×/week) or C57/Bl6/Wld^S, or ZnT3, MT3, or PARP1 knockout animals (n=4–10) had a unilateral V1 ablation and 7–11 days later the animals were sacrificed. Brains were removed, fresh frozen, sectioned coronally at 16 um through the injury, and every fifth section was ZP1 stained for Zn^{2+}, or post-fixed and stained for FluoroJadeB (cell death). Photomicrographs of the LGNd were taken of 20 sections spanning the entire injury, and stained neurons were counted by a rater blinded to the genotype. The average of the total number of stained neurons in 20 sections is presented ± SEM. Genotype or treatment condition is as stated.

Animal	# Neurons Staining for Zn^{2+} (Injured Side)	# Neurons Staining for Zn^{2+} (Control Side)	# Dead Neurons (Injured Side)	# Dead Neurons (Control Side)
C57/Bl6	550 ± 47	2 ± 2	2512 ± 186	2 ± 2
C57/Bl6 + Pyruvate	450 ± 29	1 ± 1	1871 ± 136 *	1 ± 1
C57/Bl6 + Lactate	520 ± 37	NP	2399 ± 108	NP
C57/Bl6 + Nicotinamide	480 ± 32	NP	1819 ± 285 *	NP
C57/Bl6 + 1ppm Zn^{2+} Diet	300 ± 19 *	NP	1845 ± 142 *	NP
C57/Bl6/Ola	420 ± 31	2 ± 2	1957 ± 117	4 ± 2
C57/Bl6/Ola/Wld^S	285 ± 27 *	NP	480 ± 127 *	NP
ZnT3 −/−	461 ± 35	NP	2118 ± 270	NP
MT3 −/−	492 ± 32	NP	2646 ± 412	NP
PARP1 −/−	520 ± 39	NP	2032 ± 179 *	NP

* indicates difference between B6 control and treatment or genotype conditions at $P < 0.05$. ZnD diet denotes that animals were given 1 ppm total zinc in the diet (rather than 50 ppm) for 3 weeks before the surgery, and maintained on this diet after the injury. NP means not performed.