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Research Report

Neurotoxicity of reactive aldehydes: The concept of "aldehyde load" as demonstrated by neuroprotection with hydroxylamines

Paul L. Wood, M. Amin Khan, Sarah R. Kulow, Siddique A. Mahmood, Joseph R. Moskal*

The Falk Center for Molecular Therapeutics, Department of Biomedical Engineering, McCormick School of Engineering and Applied Sciences, Northwestern University, 1801 Maple Avenue, Suite 4306, Evanston, IL 60201, USA

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ABSTRACT

The concept of "oxidative stress" has become a mainstay in the field of neurodegeneration but has failed to differentiate critical events from epiphenomena and sequalae. Furthermore, the translation of current concepts of neurodegenerative mechanisms into effective therapeutics for neurodegenerative diseases has been meager and disappointing. A corollary of current concepts of "oxidative stress" is that of "aldehyde load". This relates to the production of reactive aldehydes that covalently modify proteins, nucleic acids, lipids and carbohydrates and activate apoptotic pathways. However, reactive aldehydes can also be generated by mechanisms other than "oxidative stress". We therefore hypothesized that agents that can chemically neutralize reactive aldehydes should demonstrate superior neuroprotective actions to those of free radical scavengers. To this end, we evaluated hydroxylamines as aldehyde-trapping agents in an in vitro model of neurodegeneration induced by the reactive aldehyde, 3-aminopropanal (3-AP), a product of polyamine oxidase metabolism of spermine and spermidine. In this model, the hydroxylamines *N*-benzylhydroxylamine, cyclohexylhydroxylamine and *t*-butylhydroxylamine were shown to protect, in a concentration-dependent manner, against 3-AP neurotoxicity. Additionally, a therapeutic window of 3 h was demonstrated for delayed administration of the hydroxylamines. In contrast, the free radical scavengers TEMPO and TEMPONE and the anti-oxidant ascorbic acid were ineffective in this model. Extending these tissue culture findings in vivo, we examined the actions of *N*-benzylhydroxylamine in the trimethyltin (TMT) rat model of hippocampal CA3 neurodegeneration. This model involves augmented polyamine metabolism resulting in the generation of reactive aldehydes that compromise mitochondrial integrity. In the rat TMT model, NBHA (50 mg/kg, sc, daily) provided 100% protection against neurodegeneration, as reflected by measurements of KCl-evoked glutamate release from hippocampal brain slices and septal high affinity glutamate uptake. In contrast, ascorbic acid (100 mg/kg, sc, daily) failed to protect CA3 neurons from TMT toxicity. In summary, our data support further evaluation of the concept of "aldehyde load" in neurodegeneration and the potential clinical investigation of agents that are effective traps for reactive aldehydes.

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* *Corresponding author*. Fax: +1 847 491 4810.
E-mail address: p-wood@northwestern.edu (P.L. Wood).

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1. Introduction

In spite of the heuristic value of current concepts of "oxidative stress" in apoptotic mechanisms that lead to neurodegeneration, the design of an anti-oxidant drug with significant clinical neuroprotective properties has remained elusive. Whereas it is clear that oxidative stress can initiate apoptotic pathways, oxidative stress does not appear to be the lone player in neurodegenerative paradigms or clinical conditions, based upon the limited success of a vast array of anti-oxidant drug candidates (Gilgun-Sherki et al., 2002). In the search for a common mediator of delayed neuronal cell death, studies of stroke and traumatic brain injury models have clearly defined the critical role of reactive aldehydes in the secondary cell death of the penumbra region surrounding an initial brain insult (Huang and Huang, 1990; Ivanova et al., 1998, 2002). The delayed cell death in the penumbra is preceded by large increases in reactive aminoaldehyde concentrations, generated by increased polyamine oxidase activity (Ivanova et al., 1998). It was our objective to determine if reactive aldehydes are also critical mediators of cell death in a non-ischemia model of delayed neuronal cell death.

Although oxidative stress is a well-established pathway leading to the generation of reactive aldehydes, as a result of lipid peroxidation (Bernoud-Hubac et al., 2001), aldehydes are also formed by oxidative pathways not involving oxidative stress. There are a number of metabolic pools and varied mediators that can generate reactive aldehydes in biological systems. An example of such a metabolic pool is polyamines. In many models of neurodegeneration, there are robust inductions of ornithine decarboxylase (ODC) and/or arginase resulting in the accumulation of polyamines and their subsequent metabolism via polyamine oxidase (PAO), diamine oxidase (DAO) and semicarbide-sensitive amine oxidase (SSAO) to generate reactive aldehydes (Ivanova et al., 1998, 2002; Seiler, 2000). Monoamine oxidases can also generate aldehyde byproducts via their metabolism of amines (Gubisne-Haberle et al., 2004; Toninello et al., 2004). In the glycolytic pathway, glyceraldehydes-3-phosphate dehydrogenase can regulate glyceraldehyde and methylglyoxal production (Kragten et al., 1998; Dukic-Stefanovic et al., 2001; Takeuchi and Yamagishi, 2004). The enzyme, myeloperoxidase (MPO), can also produce aldehyde products from tyrosine, serine and threonine (Hazen et al., 2000). In all cases, these reactive aldehydes are short lived as free aldehydes in that they rapidly form covalent bonds with proteins and nucleic acids. Studies, both in vitro and in vivo, have defined key roles for these reactive aldehydes in apoptotic mechanisms leading to both neuronal and glial cell death (Dogan et al., 1999; Ivanova et al., 1998; Kruman et al., 1997; McCracken et al., 2000; Ong et al., 2000).

Pharmacological approaches that have been shown to reduce intracellular aldehyde load include (i) inhibition of aldehyde production via inhibition of polyamine synthesis with the arginase inhibitor amino-6-boronohexanoic acid (Xu et al., 2003); (ii) inhibition of reactive aldehyde synthesis via inhibition of polyamine oxidase (Ivanova et al., 1998; Seiler, 2000); (iii) sequestration of aldehydes via formation of a thioacetal adduct with mercapto agents like *N*-(2-mercaptopropionyl)glycine (Ivanova et al., 2002; Oka et al., 2000); (iv) sequestration of aldehydes with amines, specifically aminoguanidine (Hipkiss, 2001), carnosine (Guiotto et al., 2005b; Hipkiss, 2002) and pyridoxamine (Nagaraj et al., 2002); (v) sequestration of aldehydes via production of hydrazone derivatives with hydralazine and dihydralazine (Burcham et al., 2002); (vi) sequestration of aldehydes with hydrazide and formation of cyclic acetals with 1,2-diol derivatives of carnosine (Guiotto et al., 2005a); and (vii) sequestration of aldehydes with hydroxylamines like *N*-*t*-butylhydroxylamine (Atamna et al., 2000, 2001; Hipkiss, 2001; Lee et al., 2004). Similarly, spin trap agents such as phenyl-*N*-*t*-butyl nitrone, which can be metabolized to *N*-*t*-butylhydroxylamine (Atamna et al., 2000), may also neutralize reactive aldehydes. In the case of *N*-*t*-butylhydroxylamine, this agent has been shown to be relatively non-toxic in tissue culture and to demonstrate mitochondrial protection against senescence (Atamna et al., 2000, 2001; Mir et al., 2003) and radiation damage (Lee et al., 2004). The mechanism proposed by these workers was the anti-oxidant action of *N*-*t*-butylhydroxylamine in its nitroxide form, but it was also speculated that removal of reactive aldehydes may play a role in mitochondrial protection (Atamna et al., 2001). However, in a direct comparison of nitroxides, and their corresponding hydroxylamines, for their ability to protect against radiation-induced cell damage, the nitroxides were superior (Xavier et al., 2002; Yan et al., 2005). In the case of aldehyde-mediated cell death, our study provides direct evidence for the ability hydroxylamines, but not anti-oxidants, to protect cells against reactive aldehydes.

As a result of the diverse biochemical sources of reactive aldehydes, we hypothesized that a drug candidate that could buffer reactive aldehydes from all sources would be superior to an anti-oxidant agent if reactive aldehydes are important mediators of neurodegeneration. In this study, we evaluated several hydroxylamines that can avidly react with aldehydes and metabolically inactive them. We chose to study 3-aminopropanal (3-AP) toxicity in vitro because this is a reactive aldehyde generated by increased polyamine metabolism in vivo (Ivanova et al., 1998, 2002) and previous workers have demonstrated its neuronal toxicity in vitro (Yu et al., 2004) and its pathologic relevance in vivo (Ivanova et al., 1998, 2002). Preliminary investigations evaluated *tert*-butylhydroxylamine (tBHA), *N*-benzylhydroxylamine (NBHA) and *N*-cyclohexylhydroxylamine (CHHA) in an in vitro neuronal cell death model induced by 3-AP. Subsequently, we evaluated the neuroprotective potential of NBHA in vivo in the rat trimethyltin (TMT) model of delayed glutamatergic pyramidal cell (CA3) neurodegeneration in the hippocampus (Harry and d'Hellenourt, 2003; Wood, 2003; Wood et al., 2006).

2. Results

2.1. *Strategy*

It was our objective to utilize a cellular assay to define an optimal aldehyde sequestering agent for investigation of the role(s) of reactive aldehydes in animal models of neurodegeneration. We chose 3-AP neurotoxicity in a retinal cell line as

our primary assay. Previous studies with HTB11 neurons (Ivanova et al., 1998, 2002), SH-SY5Y neuroblastoma (Yu et al., 2004), HTB14 glial cells (Ivanova et al., 1998, 2002), D384 glioma cells (Li et al., 2003) and J774 macrophages (Yu et al., 2003) have shown that 3-AP gains access to the intracellular space and is avidly accumulated by lysosomes over a 2- to 4-h period with subsequent mitochondrial damage and cell death. For our first animal model, we chose the TMT rat model of delayed cell death in the CA3 hippocampal region. We characterized this model and demonstrated that reactive aldehyde synthesis is dramatically (50-fold) increased prior to neuronal degeneration in the hippocampus. Additionally, we demonstrated that the aldehyde-sequestering agent NBHA can provide 100% neuroprotection against this toxic insult.

2.2. *3-AP neurotoxicity in vitro: effects of hydroxylamines*

3-AP demonstrated a concentration-dependent neurotoxicity to retinal precursor cells as reflected by increases in 24-h media LDH levels. The concentration–response curve was shifted to the left 6-fold in Opti-MEM® I, with reduced serum proteins as compared to DMEM with 10% FBS (Fig. 1). The TD_{50} in DMEM with FBS was 294 ± 30 μM whereas in Opti-MEM it was 47 ± 4.1 μM. These data clearly demonstrate the avidity with which 3-AP forms adducts with proteins. To evaluate the ability of compounds to sequester aldehydes in this cellular assay, we chose to maintain the 10% FBS so not as to introduce the variable of reduced serum levels on cellular viability.

The hydroxylamines *tert*-butylhydroxylamine (Fig. 2), NBHA (Fig. 3) and *N*-cyclohexylhydroxylamine (Fig. 4) all protected retinal cells from 3-AP toxicity as co-treatments. Studies of delayed hydroxylamine demonstrated that delayed treatment of 0.5–1.5 h was neuroprotective and that significant protection was still provided with delayed hydroxylamine administration of up to 3 h post-3-AP addition. These data demonstrate the ability of delayed administration of hydroxylamines to protect cells from 3-AP toxicity and support previous publications demonstrating that the entry of 3-AP into lysosomes is required to initiate cellular toxicity

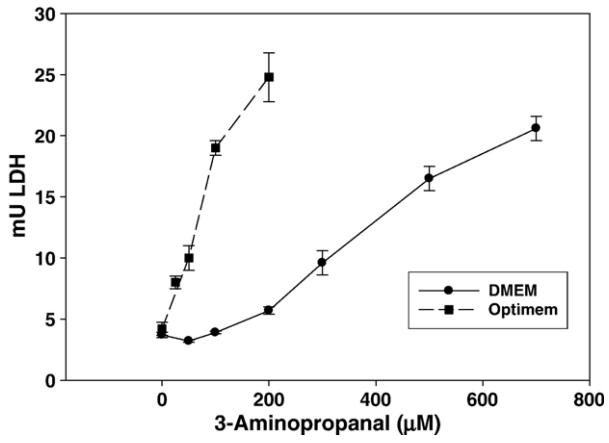

Fig. 1 – Concentration response for 3-AP neurotoxicity in DMEM with 10% FCS or OptiMem with reduced serum proteins. Twenty-four-hour media LDH levels. Mean ± SEM (*n* = 8 wells).

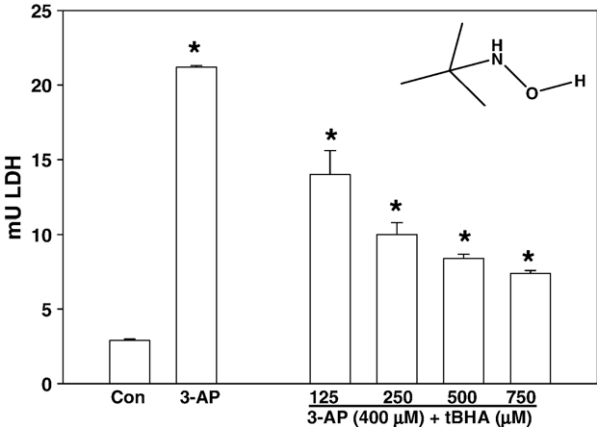

Fig. 2 – Prevention 3-AP neurotoxicity (400 μM), in DMEM with 10% FCS, by co-treatment with *t*-butylhydroxylamine (tBHA; 125–750 μM). Twenty-four-hour media LDH levels. Mean ± SEM (*n* = 8 wells). * *P* < 0.05 vs. controls (Con).

(Li et al., 2003; Yu et al., 2003). The hydroxylamines would act to form oximes with reactive aldehydes in the cytosol (Burcham et al., 2002; Hipkiss, 2001), thereby chemically sequestering the aldehydes. Additionally, the basic nitrogen of hydroxylamines makes them lysomotropic suggesting that

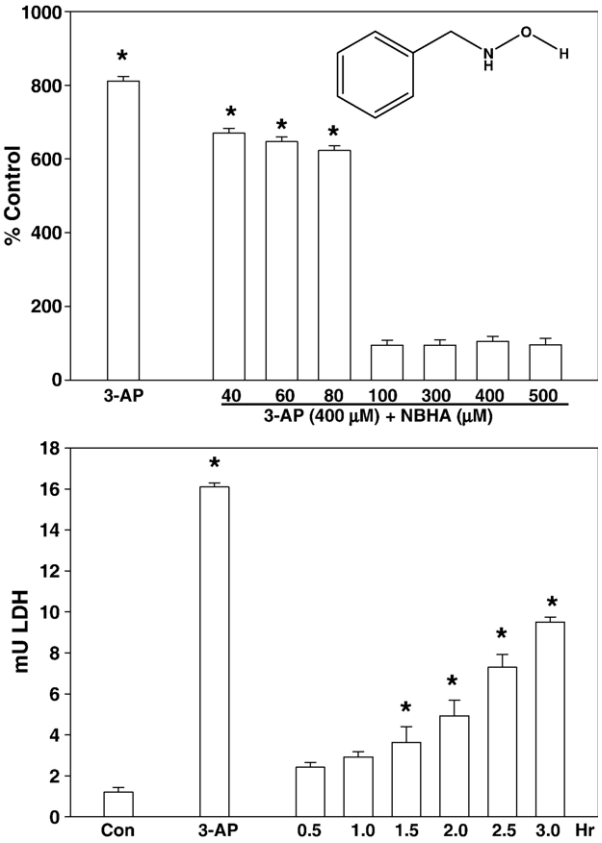

Fig. 3 – Prevention 3-AP neurotoxicity (400 μM), in DMEM with 10% FCS, by co-treatment with *N*-benzyl hydroxylamine (NBHA; 40–500 μM) and by delayed treatment (0.5–3 h) with 500 μM NBHA. Twenty-four-hour media LDH levels. Mean ± SEM (*n* = 8 wells). * *P* < 0.05 vs. controls (Con).

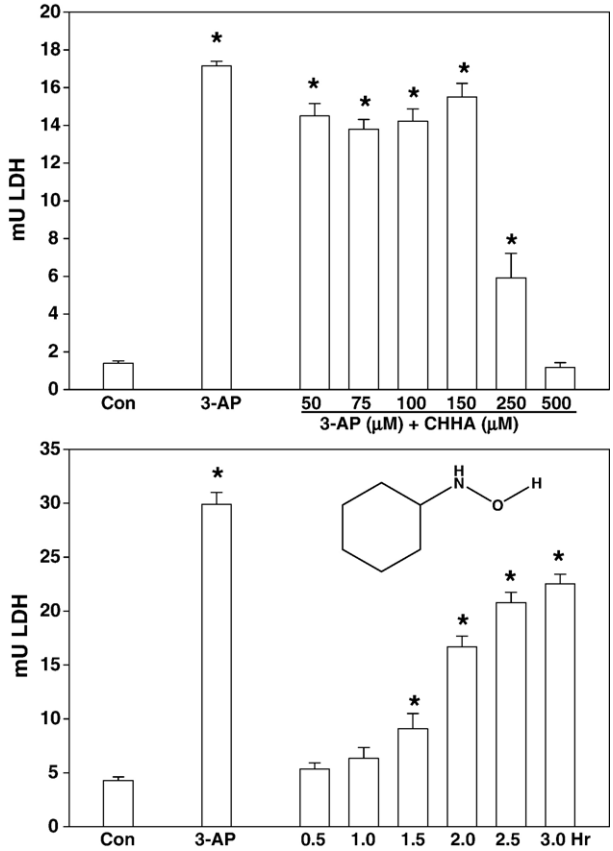

Fig. 4 – Prevention 3-AP neurotoxicity (400 μM), in DMEM with 10% FCS, by co-treatment with cyclohexylhydroxylamine (CHHA; 50–500 μM) and by delayed treatment (0.5–3 h) with 500 μM CHHA. Twenty-four-hour media LDH levels. Mean ± SEM (*n* = 8 wells). * *P* < 0.05 vs. controls (Con).

they may also neutralize aminoaldehydes that accumulate in lysosomes.

2.3. *3-AP neurotoxicity in vitro: effects of anti-oxidants, free radical scavengers, spin traps and anti-inflammatory compounds*

The nitroxide-free radical scavengers TEMPO and TEMPONE had no neuroprotective actions against 3-AP neurotoxicity (Fig. 5). Similarly, a number of anti-oxidants, free radical scavengers, spin traps, anti-inflammatory and neuroprotectant compounds were inactive in this assay of aldehyde-induced cell death (Table 1).

2.4. *Rat TMT model: activation of polyamine interconversion pathway*

TMT toxicity was associated with decreases in spermidine levels and increases in both putrescine and 3-AP (Fig. 6). These data demonstrate that the polyamine interconversion pathway (Seiler, 2000) is activated in this animal model of delayed cell death. This activation occurs prior to neuronal cell death which starts around day 7 (Harry and d'Hellenourt, 2003; Ishida et al., 1997). Both 3-AP (50-fold increase) and putrescine (3-fold increase) peak at day 6 but are maintained at high levels thereafter. The measured increases in 3-AP are presumably an underestimate based on the high reactivity of this aldehyde with proteins and the dilution effect of measuring 3-AP levels in the entire hippocampus rather than just the CA3 region. These are the first data to demonstrate the importance of the polyamine interconversion pathway in the neurotoxicity of TMT.

2.5. *Rat TMT model: effects of NBHA*

To avoid any potential influence of investigational compounds on the pharmacokinetics of TMT, drug dosing with NBHA was not initiated until 24 h post-TMT. Drug dosing was once daily (50 mg/kg). Using this paradigm, NBHA provided complete protection against the decrements in glutamate function in the terminal fields of CA3 hippocampal pyramidal neurons that degenerate within 7–14 days of TMT dosing. This was reflected by protection against decreases in synaptosomal glutamate uptake in the septum and in KCl-evoked glutamate release in the hippocampus (Fig. 7). Both high affinity glutamate uptake (Fonnum and Walaas, 1978; Naalsund et al., 1985; Wood et al., 1979) and KCl-evoked glutamate release (Gundersen et al., 1998) in CA3 terminal fields are validated indices of CA3 neuronal degeneration. The neuroprotection

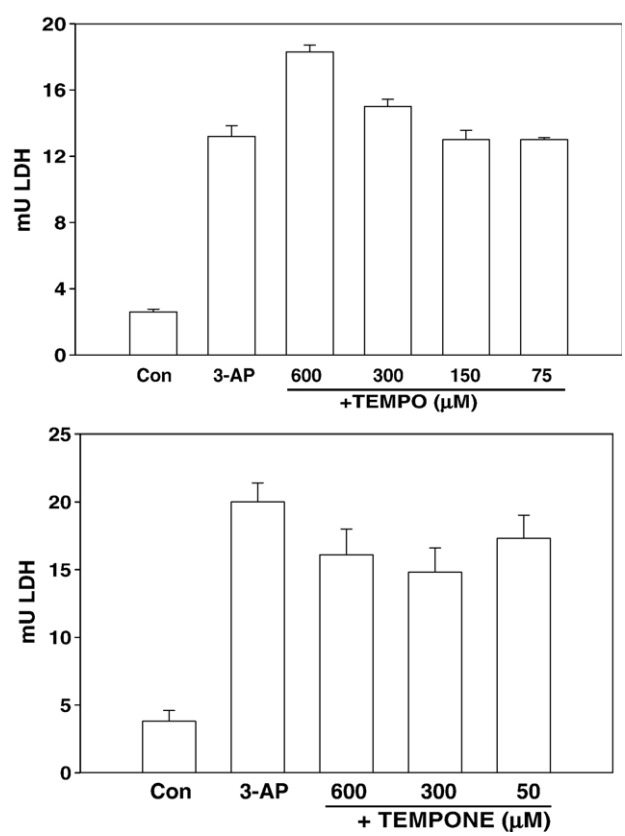

Fig. 5 – Lack of effect of co-treatment with TEMPO (75–600 μM) or TEMPONE (50–600 μM) on 3-AP neurotoxicity (400 μM), in DMEM with 10% FCS. Twenty-four-hour media LDH levels. Mean ± SEM (*n* = 8 wells). * *P* < 0.05 vs. controls (Con).

Table 1 – Inactive compounds in the 3-AP toxicity assay

Compound class	Compound	Concentration range (μM)
Amino acids	Leucine	300–600
	Leucine methyl ester	300–600
Anti-inflammatories	Oxaceprol	75–600
Anti-oxidants	Butylated hydroxytoluene	75–600
	3-Indolepropionic acid	50–500
	Lipoic acid	50–500
	Phenyl butylnitrone (PBN)	5–1000
	Cerovive (NXY-059)	100–1000
	Carnosine	75–600
	Ascorbic acid	300–600
	Thioproline	75–600
Hydroxamic acids	Acetohydroxamic acid	1–600
	2-Hydroxy-5-methyl benzohydroxamic acid	1–600
	Benzohydroxamic acid	1–600
NADPH oxidase inhibitor	Apocynin	100–5000
Neuroprotectants	Minocycline	12.5–100
	Dapsone	75–600

provided by NBHA at the neurochemical level was verified with H&E staining of frozen brain sections and also reflected behaviorally in that the dramatic increases in aggression in TMT-treated rats (aggression score = 4) were not observed in the rats receiving TMT plus NBHA (aggression score = 0).

In contrast, KCl-evoked GABA release from hippocampal slices was unaffected by TMT treatment or by treatment of TMT rats with NBHA. There were no differences in the tissue levels of glutamate or GABA between control and TMT-treated rats as reported previously (Wood et al., 2006).

2.6. *Rat TMT model: effects of ascorbic acid*

In the rat TMT model, ascorbic acid (100 mg/kg, sc, daily, starting 24 h post-TMT dosing) failed to provide neuroprotection against the decrements in glutamate function in the terminal fields of CA3 hippocampal neurons (Fig. 8). These data are consistent with the inactivity of ascorbic acid against 3-AP toxicity in the cellular assay (Table 1). Ascorbic acid in a pretreatment paradigm has been reported (Shin et al., 2005) to provide approximately 30% neuroprotection against TMT. However, as a delayed treatment, we found no neuroprotection in contrast to the efficacy of delayed treatment with NBHA.

3. Discussion

The production of reactive aldehydes and the associated accumulation of their adducts in neurological disorders is now well established (Zarkovic, 2003). The sources of reactive aldehydes are complex and varied and are not solely produced by oxidative stress. In a number of preclinical models of neurological disorders, there is induction of ODC and/or arginase activities, elevations of putrescine and decreases in spermine and spermidine. Additionally, whereas putrescine accumulation was found to correlate with neuronal lesion volume in preclinical stroke models, putrescine is not neurotoxic (Baskaya et al., 1997). These observations were once considered paradoxical until delineation of the role of polyamine and spermine oxidases in degrading spermine and spermidine to putrescine which also results in the production of the very reactive aldehydes 3-AP and acrolein (Seiler, 2000). These increases in reactive aldehyde levels are dramatic in stroke models (Ivanova et al., 1998, 2002).

The toxicity of reactive aldehydes can result from a number of actions. Cytotoxicity with 2-alkenals (e.g., acrolein), 4-hydroxy-2-alkenals (e.g., 4-hydroxy-nonenal) and ketoaldehydes (e.g., malondialdehyde) involves activation of the intrinsic apoptotic cascade, independent of lysosomes (Kruman et al., 1997; McCracken et al., 2000; Pocernich and Butterfield, 2003; Yu et al., 2004; Zarkovic, 2003). These aldehydes form covalent linkages with amino acids, proteins, nucleic acids and lipids; actions that can result in direct mitochondrial toxicity (Pocernich and Butterfield, 2003). Aminoaldehydes have the potential for these toxic actions also, but their lysomotropic actions appear to be more

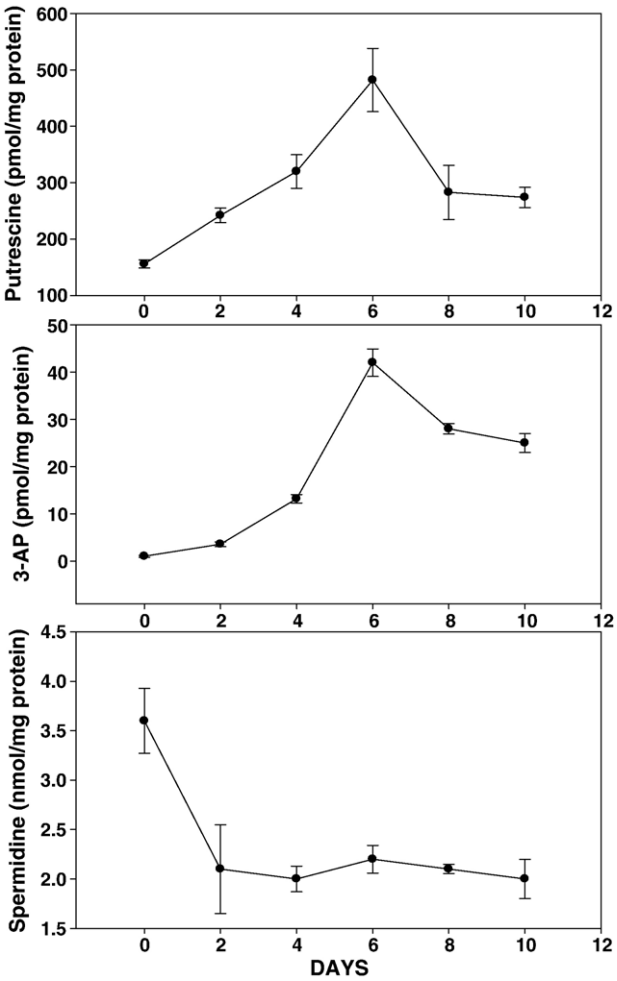

Fig. 6 – Accumulation of hippocampal putrescine and 3-aminopropanal (3-AP) and the associated decreases in spermidine induced by TMT. Mean ± SEM (n = 4 rats). All points are significantly different from day 0 values (P < 0.05) except for 3-AP at day 2.

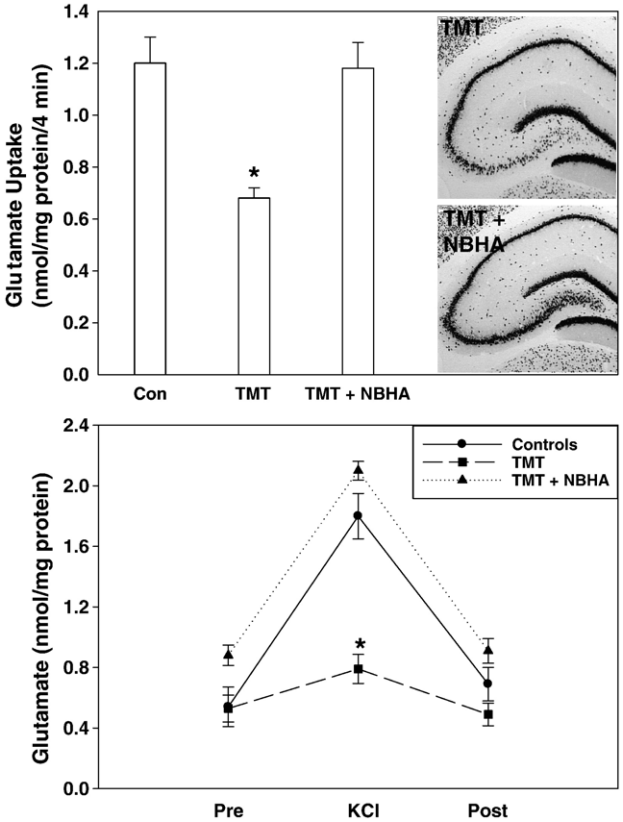

Fig. 7 – Prevention of TMT neurotoxicity by treatment with *N*-benzylhydroxylamine (NBHA), administered daily (50 mg/kg, sc) for 17 days, starting 24 h after TMT (8 mg/kg, sc) treatment. Measurements of septal high affinity glutamate uptake and KCl-evoked glutamate release from hippocampal slices were used as biochemical indices of CA3 neuronal loss. Mean ± SEM (n = 8–10 rats). * $P < 0.05$ vs. controls. Inserts demonstrate CA3 neuronal losses with TMT at day 19 and neuroprotection with NBHA.

important (Li et al., 2003; Yu et al., 2003). Robust insults with 3-AP can result in lysosomal rupture and cellular necrosis whereas lesser insults may result in lysosomal leakage of proteases that compromise mitochondrial integrity and thereby activate the intrinsic apoptotic cascade (Ivanova et al., 1998; Yu et al., 2003, 2004). The complex toxicities of reactive aldehydes combined with their multiple metabolic sources suggest that the concept of "aldehyde load" may be a useful corollary to current concepts of "oxidative stress" in neurodegenerative mechanisms.

The following is a brief overview of a number of neurological disorders in which elevated aldehydes (Zarkovic, 2003) have been demonstrated in the free and/or adduct forms (i.e., protein bound via covalent bonds). In Alzheimer's disease, 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), glyceraldeyhe, acrolein and methylglyoxal are all increased in the neocortex (Ahmed et al., 2005; Calingasan et al., 1999; Choei et al., 2004; Lovell et al., 2001; Montine and Morrow, 2005; Sayre et al., 1997). Additionally, increased polyamine metabolism as reflected by increased ODC and putrescine (Bernstein and Muller, 1995), increased neuronal MPO activity (Green et al., 2004) and induction of semi-carbazide-sensitive amine oxidase in cerebral blood vessels (Ferrer et al., 2002), are pathways that can augment reactive aldehyde production in Alzheimer's disease. Recent studies of CSF in Alzheimer's patients have demonstrated increased fructosyl-lysine and protein glycation by methylglyoxal, which correlates with cognitive decline (Ahmed et al., 2005). An extension of all these observations is the recent report of increased cortical 4-HNE and acrolein in patients with mild cognitive impairment suggesting that aldehyde accumulation occurs early in the pathological cascade that leads to Alzheimer's disease (Williams et al., in press). In stroke models of ischemia–reperfusion injury, there also are dramatic increases in both MPO (Maier et al., 2004) and in polyamine metabolism as indicated by increases in ODC and putrescine (Baskaya et al., 1997) and the associated increases in the reactive aldehyde, 3-AP (Ivanova et al., 1998, 2002; Li et al., 2003). Mechanistic studies with agents that remove 3-AP have demonstrated significant neuroprotection in the rat middle cerebral artery occlusion (MCAO) model. The reactive aldehydes 4-HNE and MDA are also elevated in stroke models (Imai et al., 2001). Similarly, in models of traumatic brain injury, elevations of 4-HNE and MDA have been reported (Ozsuer et al., 2005; Springer et al.,

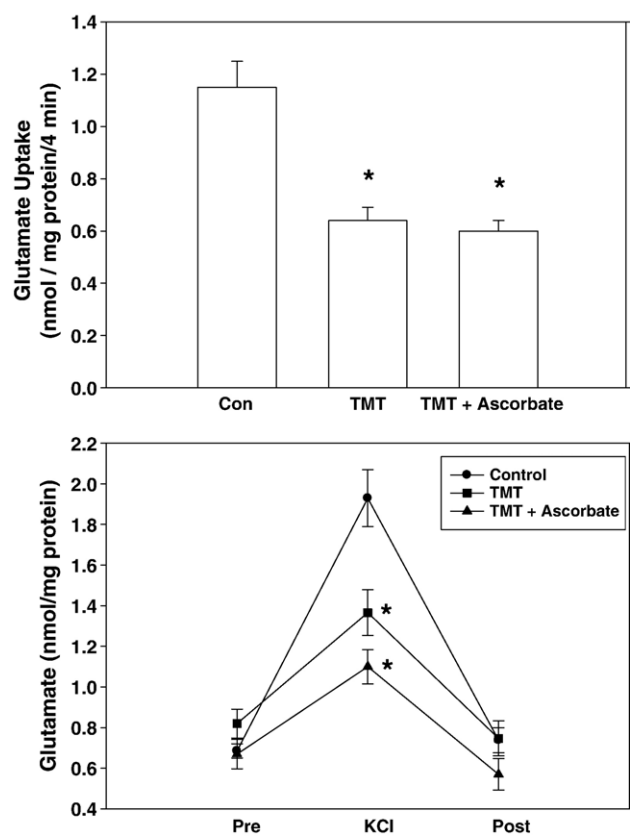

Fig. 8 – Lack of neuroprotection against TMT neurotoxicity by treatment with ascorbic acid (100 mg/kg, sc) administered daily for 17 days, starting 24 h after TMT (8 mg/kg, sc) treatment. Measurements of KCl-evoked glutamate release from hippocampal slices were used as an index of CA3 neuronal loss. Mean ± SEM (n = 8–10 rats). * $P < 0.05$ vs. controls.

1997) along with augmented polyamine metabolism, as reflected by ODC induction and increased putrescine levels (Dogan et al., 1999). Increased MPO activity also occurs in traumatic brain injury as a result of neutrophil infiltration. In murine EAE models of multiple sclerosis, there are increases in MDA and in MPO from invading blood cells. There also is a dramatic (287-fold) induction of arginase in the murine EAE model (Xu et al., 2003). Such dramatic increases in this enzyme would clearly augment polyamine metabolism and the generation of reactive aldehydes. Treatment of EAE mice with an arginase inhibitor provided clinical improvements in these mice (Xu et al., 2003). In preclinical seizure models, reactive aldehydes are potential mediators of neuronal cell death as reflected by increases in 4-HNE, MDA and glyceraldehyde (Jacobsson et al., 1999; Ong et al., 2000) and via increased polyamine metabolism with induction of ODC and increased putrescine levels (de Vera et al., 2002). Increases in brain levels of the reactive aldehydes 4-HNE, MDA and glyceraldehyde have also been demonstrated in autopsy studies of Parkinson's (Ilic et al., 1999; Selley, 1997) and ALS (Simpson et al., 2004) patients.

Our studies of 3-AP neurotoxicity in vitro clearly demonstrate neuroprotection by hydroxylamines against reactive aldehydes. Our data also support the proposed roles of reactive aldehydes in the pre-mitochondrial phase of apoptosis (Kruman et al., 1997; Li et al., 2003; Yu et al., 2003), as reflected by the ability of hydroxylamines to protect retinal cells against 3-AP toxicity after delayed addition to cultures. The mechanisms of aldehyde inactivation by hydroxylamines presumably include formation of oximes with aldehydes in the cytosol (Burcham et al., 2002; Hipkiss, 2001) and entry into lysosomes, as a result of the basic nitrogen, and inactivation of aldehydes in this cellular compartment (Yu et al., 2003, 2004). These combined actions of neutralizing the lysosomal toxicity of aminoaldehydes and blocking direct mitochondrial toxicity of aldehydes may contribute to the efficacy observed with hydroxylamines in our in vitro and in vivo studies. In this regard, the in vivo paradigm we chose to study was the TMT-treated rat model (Harry and d'Hellenourt, 2003; Wood, 2003; Wood et al., 2006). This is a model of delayed neuronal cell death, which is insensitive to treatment with glucocorticoids (O'Callaghan et al., 1991) and weakly responsive to pretreatments with anti-oxidants (Shin et al., 2005). We have previously reported that putrescine levels are dramatically elevated in the hippocampus of TMT-treated rats 14 days post-TMT (Wood et al., 2006) suggesting that polyamine oxidase activity is increased along with the generation of reactive aldehydes. This conclusion is now supported by our measurements of the time course of putrescine and 3-AP accumulation and by the total neuroprotection provided by NBHA in this animal model. These data demonstrating dramatic neuroprotection with aldehyde-trapping agents but not anti-oxidants in the rat TMT model of delayed hippocampal neurodegeneration, along with similarly robust effects of aldehyde-trapping agents in the rat MCAO stroke model (Ivanova et al., 1998, 2002) and in the rat model of global brain ischemia (Huang and Huang, 1990) strongly suggest that further evaluation of these agents in other models of neurodegeneration is warranted. This approach may lead to new drug candidates for evaluation in clinical neurodegenerative disorders.

4. Experimental procedures

4.1. Materials

Dulbecco's Minimal Essential Medium (DMEM) and Opti-MEM® I were purchased from GIBCO, Long Island, NY. Fetal bovine serum (FBS) was from Hyclone, Logan, UT. [$^2H_6$]GABA, [$^2H_5$] glutamate, [$^2H_6$]ornithine and [$^2H_4$]putrescine were purchased from CDN Isotopes, Pointe-Claire, Quebec, whereas L-[^3H] glutamate (52 Ci/mmol) was from Perkin Elmer, Boston, MA. Dowex AG 50W-X8 (200–400 mesh, hydrogen form) was from Biorad, Hercules, CA. The Cytotoxicity Detection Kit (LDH) was obtained from Roche Applied Science, Indianapolis, IN. The protein BCA kit was purchased from Pierce, Rockford, IL and 3-aminopropanal diethyl acetal was purchased from TCI America, Portland, OR. TEMPONE was purchased from Alexis Biochemicals (San Diego, CA). All other reagents were purchased from Sigma Chemicals, St. Louis, MO. The 1.5-ml screw top microfuge tubes were purchased from Sarstedt, Newton, SC.

4.2. 3-Aminopropanal (3-AP) synthesis

For the synthesis of 3-AP (Ivanova et al., 1998), 20 mmol of 3-aminopropanal diethyl acetal were mixed with 5 ml of 1.5 N HCl and stirred for 5 h at 25 °C. The reaction mix was applied to a column (3 × 6 cm) containing Dowex-50, in the H^+ form. 3-AP was eluted with 2 N HCl and concentrated in a Savant concentrator. The 3-AP was characterized by 1H NMR (500 MHz, D_2O): δ 7.61 (br, 1H), 3.22 (q, 2H), 3.15 (q, 2H), 2.15 (d, 1H), 1.98 (d, 1H) and quantitated with the Purpald reaction. Briefly, 100 μL of the synthesized 3-AP, diluted in PBS, was added to a 96-well microtiter plate and 100 μL of 34 mM Purpald in 2 N NaOH added. The plate was incubated for 20 min at room temperature with mixing before being read at 550 nm. Propionaldehyde (50–200 nmol per well) was used for the standard curve.

4.3. Retinal cell cultures

The rat retinal cell line, E1A-NR.3 (Seigel et al., 2004), was grown in DMEM, containing 10% FBS, in 75 cm^2 flasks. For neurotoxicity assays, cells were plated in 48-well tissue culture plates and exposed to 3-AP either in DMEM or in Opti-MEM® I, with reduced serum proteins, for 24 h. Media were collected and assayed for LDH using the Roche assay kit. Rabbit muscle LDH was used for the standard curve. Drug treatments with hydroxylamines were as co-treatments except in cases where delayed administration of the hydroxylamine was investigated. All drugs were dissolved in PBS.

4.4. Animals and tissue collection

Male Sprague–Dawley rats (200 g; Harlan) were administered trimethyltin (8 mg, sc) and housed individually as a result of the aggressive behavior induced by the neurotoxicant. The day following the TMT treatment, rats were started on a once daily dosing for 17 days with vehicle, NBHA (50 mg/kg, sc) or ascorbic acid (100 mg/kg, sc) in PBS. All drug solutions were

adjusted to pH 6–7. Rats were decapitated on day 19 and the hippocampus isolated and placed in chilled Hanks balanced salt solution containing 20 mM HEPES (HBSS–HEPES, 4 °C) for release experiments.

4.5. *Aggression scoring*

At the time of euthanasia and tissue harvest, rats were scored for aggressive behavior which is very marked in TMT-treated rats (Ishida et al., 1997): 0 = rat remains calm when approached and grasped; 1 = rat shies from hand when grasped; 2 = rat avoids hand by running and struggles when captured; 3 = rat leaps to avoid capture and struggles vigorously when captured; 4 = rat struggles and bites when captured. In our experience at day 18 post-TMT, rats consistently score 4 on this scale. All scoring was by a blinded investigator.

4.6. *Synaptosomal glutamate uptake*

The septum, a terminal projection field of CA3 glutamatergic pyramidal cells (Fonnum and Walaas, 1978; Wood et al., 1979), was homogenized in 0.32 M sucrose and the P2 synaptosomal fraction isolated to measure sodium-dependent high affinity glutamate (Fonnum and Walaas, 1978; Naalsund et al., 1985; Wood et al., 1979) using L-[^{3}H]glutamate (52 Ci/mmol). High affinity glutamate uptake has been validated as a reliable biochemical marker of CA3 neuronal cell losses (Fonnum and Walaas, 1978; Wood et al., 1979).

4.7. *KCl-evoked neurotransmitter release*

Chilled tissues were cut into 300 μM hippocampal slices. A single slice was incubated in 2 ml of HBSS–HEPES in 12-well culture plates at 37 °C for 30 min. The media was discarded and the slice incubated for 2 further 10-min periods, each with fresh media. Next, the slices were incubated for 5 min after which the media were isolated for amino acid analyses (pre-sample), followed by a 5-min incubation in which 50 mM KCl was added to evoke neurotransmitter release from the slices (KCl sample). Next, a final 5-min incubation in HBSS–HEPES was collected (post-sample).

4.8. *GC-MS analyses of GABA and glutamate release*

The amino acids released into the media were isolated by cation exchange chromatography and measured by GC-MS (Wood et al., 2006) with an Agilent bench-top GC-MSD (HP6890/MSD5973) under PCI conditions with ammonia as the reagent gas.

4.9. *GC-MS analyses of polyamines and 3-aminopropanal (3-AP)*

At various time points after TMT administration, rats were sacrificed by decapitation and the hippocampus rapidly frozen on dry ice. The tissues were sonicated in 1 N HCl and the 25,000 × *g* supernatants dried in a Savant concentrator. The dried samples were directly reacted as described above. Spermidine and putrescine were quantitated by GC-MS as described previously (Wood et al., 2006). In the case of 3-AP samples, 3-AP was isolated by cation exchange chromatography and the aldehyde function first derivatized with pentafluorobenzyl hydroxylamine (Wichard et al., 2005) and the amino terminal next derivatized with tBDMS (Wood et al., 2006). The internal standard was 3-aminocyclohexenone (3-ACHO). 3-AP and the internal standard were monitored in NCI with ammonia as the reagent gas. The ions monitored for 3-AP and 3-ACHO were 362 and 399, respectively. These were the $[M\text{-}HF]^-$ ions.

4.10. *Proteins*

The 25,000 × *g* protein pellets were solubilized in 0.5 N NaOH and the protein content assayed via the BCA procedure.

4.11. *Statistical analysis*

All data are presented as mean ± SEM for groups of 8 tissue culture wells. For the animal experiments, the *N* values are number of rats. Data were analyzed by one-way ANOVA followed by the Dunnett's *t* test for comparisons to control. TD_{50} values for 3-AP cytotoxicity were calculated via log concentration–probit analysis.

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