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ORIGINAL ARTICLE

Neuronal Glutathione Content and Antioxidant Capacity can be Normalized *In Situ* by *N*-acetyl Cysteine Concentrations Attained in Human Cerebrospinal Fluid

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Abstract *N*-acetyl cysteine (NAC) supports the synthesis of glutathione (GSH), an essential substrate for fast, enzymatically catalyzed oxidant scavenging and protein repair processes. NAC is entering clinical trials for adrenoleukodystrophy, Parkinson's disease, schizophrenia, and other disorders in which oxidative stress may contribute to disease progression. However, these trials are hampered by uncertainty about the dose of NAC required to achieve biological effects in human brain. Here we describe an approach to this issue in which mice are used to establish the levels of NAC in cerebrospinal fluid (CSF) required to affect brain neurons. NAC dosing in humans can then be calibrated to achieve these NAC levels in human CSF. The mice were treated with NAC over a range of doses, followed by assessments of neuronal GSH levels and neuronal antioxidant capacity in *ex vivo* brain slices. Neuronal GSH levels and antioxidant capacity were augmented at NAC doses that produced peak CSF NAC concentrations of ≥50 nM. Oral NAC administration to humans produced CSF concentrations of up to 10 μM, thus demonstrating that oral NAC administration can surpass the levels required for biological activity in brain. Variations of this approach may similarly facilitate and rationalize drug dosing for other agents targeting central nervous system disorders.

Key Words Cysteine · Parkinson's disease · oxidative stress · human · cerebrospinal fluid · target engagement

Introduction

Oxidative stress contributes the pathogenesis of Parkinson's disease (PD) and other neurodegenerative disorders. The neuronal populations that are most affected in PD accumulate oxidized lipids, proteins, and DNA [1]. These markers of oxidative damage are accompanied by reduced levels of the major cellular antioxidant, glutathione (GSH), which is used by cells to both scavenge reactive oxygen species (ROS) and repair oxidized protein residues [2]. A causal role for oxidative stress and GSH depletion in PD is supported by both clinical and animal studies [1, 3–5]. In particular, factors that cause global impairments in neuronal GSH metabolism cause cytotoxicity preferentially in the neuronal populations most affected in PD [6–8].

Clinical trials have evaluated several agents targeting oxidative damage in PD, including coenzyme Q10, α-tocopherol, selegiline, and rasagaline; but disappointingly none of these trials have demonstrated an unequivocal slowing of disease progression [9, 10]. These negative results are difficult to interpret, however, because it is unknown whether the agents succeeded in having a significant effect on neuronal oxidative stress at the doses employed. This critical knowledge gap

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stems both from difficulties in measuring antioxidant effects *in vivo* and uncertainties about drug penetration across the human blood–brain barrier.

N-Acetyl cysteine (NAC) is a synthetic compound that enters cells by a presumably passive mechanism, where it is cleaved to generate intracellular cysteine [11]. Cysteine availability is normally the rate-liming factor in GSH synthesis [12], and consequently NAC can be used to promote GSH synthesis under conditions in which GSH consumption exceeds supply. NAC is currently approved by the US Food and Drug Administration for the treatment of acetaminophen toxicity and contrast nephropathy, and it has been identified as promising agent for treatment of PD [3, 13–15]. There is also a growing interest in the use of NAC to treat other neurological and psychiatric disorders in which oxidative stress has been implicated, including adrenoleukodystrophy, Alzheimer disease, schizophrenia, and compulsive disorders [15, 16]. However, there has been uncertainty as to the extent that NAC can cross the human blood–brain barrier [17], with resulting uncertainty about doses required for a rational clinical trial.

Here we present an approach for addressing these issues. The approach uses *ex vivo* assessments of neuronal GSH levels and antioxidant capacity in mice to identify the cerebrospinal fluid (CSF) concentrations of NAC required to have biological activity in neurons. Dosing in humans can then be tailored to reach or exceed these levels in human CSF.

Materials and Methods

Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) except where otherwise noted. Animal studies were approved by the San Francisco Veterans Affairs Medical Center animal studies committee. Sprague–Dawley rats, aged 3–6 months, were obtained from Simonsen Laboratories (Gilroy, CA, USA). Mice were either wild-type 3–6-month-old C57BL/6 strain (Jackson Laboratories, West Grove, PA, USA) or SLC1A1$^{-/-}$ mice that had been back-crossed to the C57BL/6 strain. The SLCA1$^{-/-}$ colony was maintained as homozygous with breeders back-crossed with C57BL/6 mice every 6 generations, to avoid genetic drift [6, 18]. SLC1A1 is more commonly known as excitatory amino acid transporter 3 or excitatory amino acid carrier 1 (EAAC1), and will be termed EAAC1 throughout this report.

Drug Administration

L-Buthionine-(S,R)-sulfoximine (BSO) was administered 5 h prior to brain harvest at a dose of 1.3 g/kg body weight by intraperitoneal (i.p.) injection. NAC was administered either i.p. or by oral gavage 5 h prior to brain harvest, at doses ranging from 0.1 to 75.0 mg/kg. The drugs were dissolved in 0.9 % sodium chloride solution and administered in approximately 3.0-ml volumes in rats or 0.3-ml volumes in mice. Vehicle-treated animals received equal volumes of 0.9 % sodium chloride only.

Mouse Brain Harvest

Mice were decapitated under isoflurane/nitrous oxide anesthesia and brains were quickly removed. The rostral third of each brain was immediately frozen in dry ice and stored at –70 °C for biochemical measurement of brain GSH content; the caudal third of each brain was fixed in phosphate buffered 4 % formaldehyde for GSH immunohistochemistry; and the middle third of each brain was sectioned in a vibratome for *ex vivo* assessment of functional antioxidant capacity (Fig. 1). In some studies slices were also taken through the upper midbrain to include neurons of the substantia nigra.

Ex Vivo Brain Slice Studies

The middle third of each brain was sectioned using a vibratome while maintained at approximately 0 °C by immersion in a solution containing 250 mM sucrose, 11 mM glucose, 2.5 mM KCl, 1.0 mM NaH_2PO_4, 2.0 mM $MgSO_4$, and 2.0 mM $CaCl_2$ [19]. Three or 4 slices of 275-μm thickness were obtained. Each slice

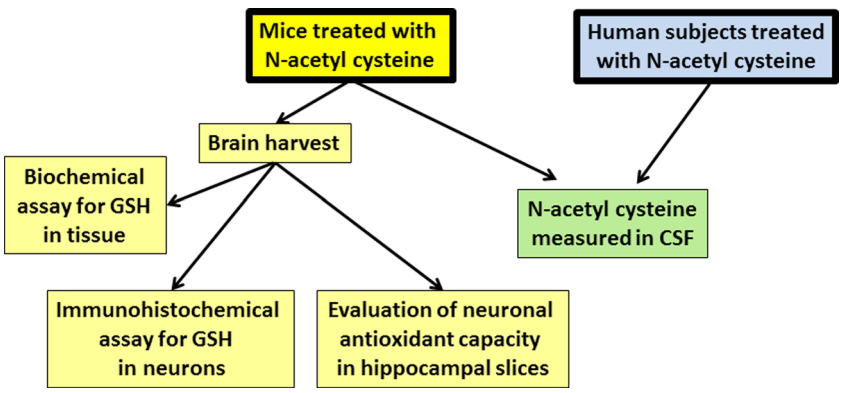

Fig. 1 Outline of experimental procedures. GSH = glutathione; CSF = cerebrospinal fluid

was divided in the midline and one half was placed in artificial CSF (ACSF) and the other half placed in ACSF containing 0–2.0 mM 3-morpholinosydonimide (SIN-1; Sigma-Aldrich, St. Louis, MO, USA), which forms superoxide, nitric oxide, and peroxynitrite in oxygenated solutions [20]. The ACSF contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 21.4 mM $NaHCO_3$, and 25 mM glucose, and was continuously bubbled with 95 % O_2/5 % CO_2. The sections were incubated in these solutions at room temperature (22 °C) for 30 min, then fixed overnight in phosphate buffered 4 % formaldehyde.

Immunostaining

The fixed, 275-μm-thick slices were incubated with antibodies to either nitrotyrosine (06-284; Millipore, Darmstadt, Germany) or 4-hydroxynonenal (HNE 11-S; Alpha Diagnostics, San Antonio, TX, USA), as described [21]. In each case the sections were also incubated with antibody to microtubule-associated protein 2 (MAB3418; Millipore) for identification of neuronal cell bodies. Using a confocal microscope, photomicrographs were taken of 4 randomly selected regions of the pyramidal cell layer in each hippocampal section. The images were taken at a tissue depth of 20 μm and with an optical thickness of 1 μm. Image acquisition parameters were the same in each experiment and set so that signal saturation did not occur. Control sections in which primary or secondary antibodies were omitted showed no signal under these acquisition conditions. The fluorescence signal intensity was measured in neuronal soma identified by the microtubule-associated protein 2 signal. All measurements were normalized to the mean values obtained in $EAAC1^{-/-}$ sections that had been incubated with SIN-1, immunostained, and photographed in parallel.

Immunostaining for GSH was performed on 40-μm cryostat sections prepared from formaldehyde-fixed brain tissue blocks. The sections were treated with 10 mM *N*-ethylmaleimide (NEM) for 4 h at 4 °C prior to incubation with antibody to GSH–NEM (clone 8.1GSH; Millipore), as described previously [21, 22]. Antibody binding was visualized using Alexa Fluor 488-conjugated goat antimouse IgG. Quantification of fluorescent labeling was performed in 4 evenly spaced sections collected through the hippocampus of each mouse and photographed under uniform conditions. Fluorescence intensity was measured in the neuronal soma of hippocampal of the CA1 pyramidal layer bilaterally in each section, and the values obtained in the 4 sections were averaged for each brain. All measurements were normalized to the mean values obtained in $EAAC1^{-/-}$ brains that were immunostained and photographed in parallel.

GSH Biochemical Assay

Brain tissue was sonicated with 5 % sulfosalicylic acid (200 mg/ml) and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was mixed with 1 mM dithiobis-2-nitrobenzoic acid and 1 mM ethylenediaminetetraacetic acid in 100 mM sodium phosphate buffer (pH 7.5), and 1 mM nicotinamide adenine dinucleotide phosphate and 200 U/ml GSH reductase were added [23]. Optical absorbance of samples and standards was measured at 405 nm and subtracted by values measured in the absence of GSH reductase.

Mouse and Rat CSF Collection

Animals were anesthetized with isoflurane/nitrous oxide and placed in a stereotaxic frame with the neck flexed forward. The skin and muscles were retracted to expose the cisterna magna. For rats, 100–150 μl CSF was removed from the cisterna magna by syringe puncture. For mice, the dura of the cisterna magna was carefully punctured with a capillary tube to draw up 6–7 μl of fluid per mouse. The animals were euthanized while still under anesthesia.

Human CSF Collection

These studies were approved by the institutional review board at the University of California, San Francisco, and the San Francisco Veterans Affairs Center. As detailed elsewhere [24], patients with PD were enrolled in the study between January 2012 and December 2013. Study inclusion criteria included age >40 years, a diagnosis of PD within 10 years, and adherence to a stable dopaminergic medication regimen for at least 2 weeks. Exclusion criteria included significant cognitive impairment, inability to sign informed consent, increased bleeding risk, or mass lesion on brain imaging. A Food and Drug Administration-approved solution form of NAC was obtained from McKesson (San Francisco, CA, USA). The capsule form was obtained from Professional Compounding Centers of America (Houston, TX, USA), a United States Pharmocopeia-certified pharmaceutical distributor, and was compounded by the University of California San Francisco Medical Center pharmacy. Patients took 4 oral doses of NAC, administered every 12 h over 48 h. Four dosing strategies were compared: 1) NAC solution at 7 mg/kg; 2) NAC capsules at 35 mg/kg; 2) NAC solution at 70 mg/kg; and 4) NAC capsules at 70 mg/kg. CSF was obtained by lumbar puncture in each participant prior to the first dose, and again 90 min after the fourth dose.

NAC Measurements

Rat, mouse, or human CSF was immediately aliquoted into samples for separate analyses of total NAC and reduced NAC.

Samples for total NAC determinations were treated for 30 min with 2 mM Tris (2-carboxyethyl)phosphine hydrochloride to convert NAC disulfides to reduced NAC. All samples were then derivitized with 100 μM *N*-(9-acridinyl)maleimide, stabilized with 40 mM formic acid, and stored at –80 °C. Standards of NAC were prepared in water and treated exactly as the samples. A separate set of internal standards was prepared by spiking NAC into CSF collected from untreated humans and mice, to exclude the possibility that factors present in CSF might affect the NAC quantification. Samples and standards were analyzed in parallel by liquid chromatography–tandem mass spectrometry (Integrated Analytical Solutions, Berkeley, CA, USA) as previously described [24].

Data Analysis

All data quantification was performed by individuals who were blinded to experimental conditions, and the mouse GSH determinations and antioxidant study results were confirmed by independent observers in 2 different laboratories. NAC measurements are means±SEM. All other data are presented as medians±interquartile range, with the "*n*" denoting the number of animals or human subjects in each experimental group. Group values were compared with the nonparametric Kruskal–Wallis test followed by Dunnett's test for comparison of multiple groups to a single control group.

Results

We first determined the dose of NAC required to influence neuronal GSH synthesis and antioxidant capacity in mice. Hippocampal sections, in which GSH was quantified by the GSH–NEM immunohistochemical method [21, 22], showed a strong GSH signal in the soma and processes of pyramidal neurons, with less of a signal in the adjacent neuropil and glia (Fig. 2A). NAC administered to normal mice did not produce a detectable increase in neuronal GSH (Fig. 2B). This result is not unexpected as GSH synthesis is limited by product inhibition at normal intracellular GSH concentrations [11, 25]. For this reason we turned to the EAAC1$^{-/-}$ mouse, which has a neuron-specific reduction in cysteine and GSH levels [26]. Immunostaining for GSH confirmed reduced levels in EAAC1$^{-/-}$ neurons, and NAC administered 5 h prior to brain harvest raised these levels (Fig. 2B), as previously described [6]. Evaluation of NAC over a range of doses showed that doses of 7.5 mg/kg were sufficient to raise GHS levels in EAAC1$^{-/-}$ neurons to values comparable with those of wild-type neurons. There was no large difference in results obtained by oral or i.p. drug administration. The effect of NAC delivered by either route was negated in mice that had been co-treated with BSO, which inhibits the enzymatic step at which cysteine is incorporated into GSH [25]. Biochemical

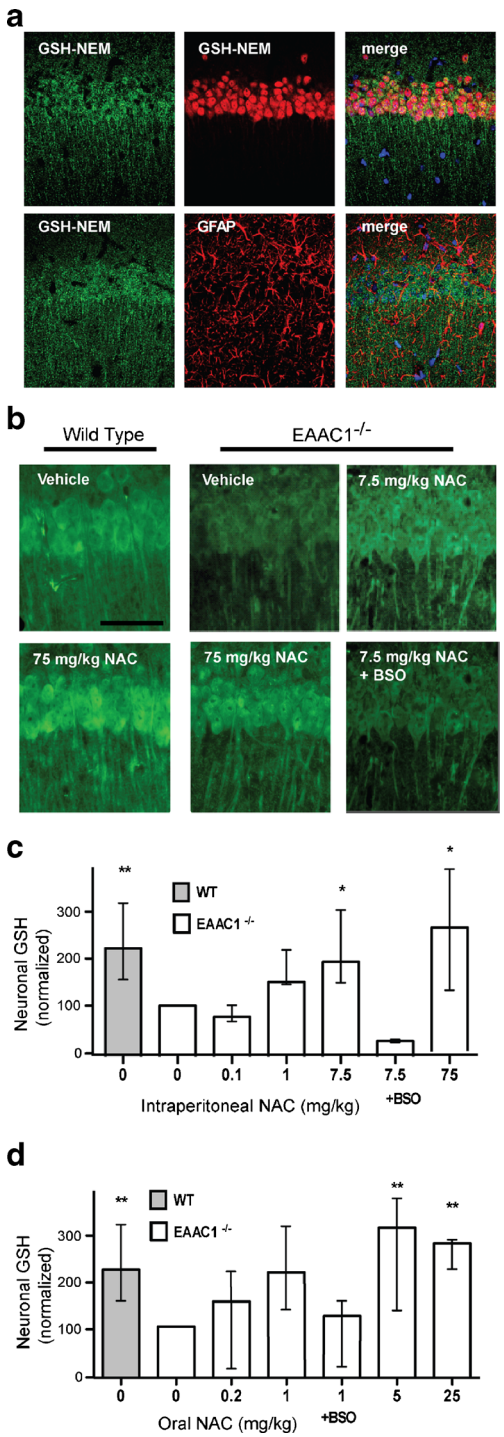

Fig. 2 *N*-Acetyl cysteine (NAC) raises glutathione content in EAAC1$^{-/-}$ neurons. (**A**) Immuonstaining for glutathione–*N*-ethylmaleimide (GSH–NEM) adducts in hippocampal sections treated with NEM. Co-labeling with the neuronal nuclear marker NeuN and the astrocyte marker glial fibrillary acidic protein (GFAP) show the signal to be primarily localized to neurons. (**B**) Neuronal GSH in mice treated with NAC 5 h prior to brain harvest. The increase induced by 7.5 mg/kg NAC was blocked by co-treatment with buthionine sulfoximine (BSO). (**C**, **D**) Quantified measures neuronal GSH content after intraperitoneal and oral treatment with NAC. Results in each of 7 experiments were normalized to values obtained in untreated (0 NAC) EAAC1$^{-/-}$ mice. n=3–5 mice in each condition; *p<0.05; **p<0.01 *vs* EAAC1$^{-/-}$, 0 NAC. WT = wild-type

determinations of GSH showed no significant reduction in overall brain GSH content in mice treated with BSO alone for the 5-h interval (not shown), consistent with the slow rate of *de novo* GSH synthesis in normal brain [27].

We next established a method for evaluating the functional antioxidant capacity of mouse neurons *in situ*. In this method, acutely prepared brain slices are incubated in artificial CSF containing the peroxynitrite-generating compound SIN-1 and subsequently analyzed for the formation of 3-nitrotyrosine. EAAC1$^{-/-}$ slices incubated with SIN-1 exhibited nitrotyrosine formation in neurons, and this was attenuated in slices from mice that had been treated with NAC prior to brain harvest (Fig. 3A, B). This effect of NAC was also negated by co-treatment with BSO, indicating that NAC acts primarily by supporting GSH production rather than as a free radical scavenger per se. Measures of the lipid peroxidation product 4-hydroxynonenal as a second, independent marker of oxidative stress showed the same pattern: the neuronal 4-hydroxynonenal signal induced by SIN-1 was attenuated in treated with NAC, and the effect of NAC was negated by BSO (Fig. 3A, C). Nitrotyrosine formation also could be induced in wild-type hippocampal slices when incubated with higher SIN-1 concentrations, confirming this effect was not unique to EAAC1$^{-/-}$ cells (Fig. 4A). Likewise, neurons in cerebral cortex and likewise showed nitrotyrosine formation after incubation with SIN-1 (Fig. 4B), but for technical reasons this was easier to quantify in the hippocampal pyramidal layer.

Using this approach, we then evaluated neuronal antioxidant capacity in mice that had been treated with NAC over a range of doses. Results showed that dosing with as little as 1 mg/kg NAC i.p. was able to reduce significantly SIN-1-induced nitrotyrosine formation in EAAC1$^{-/-}$ neurons

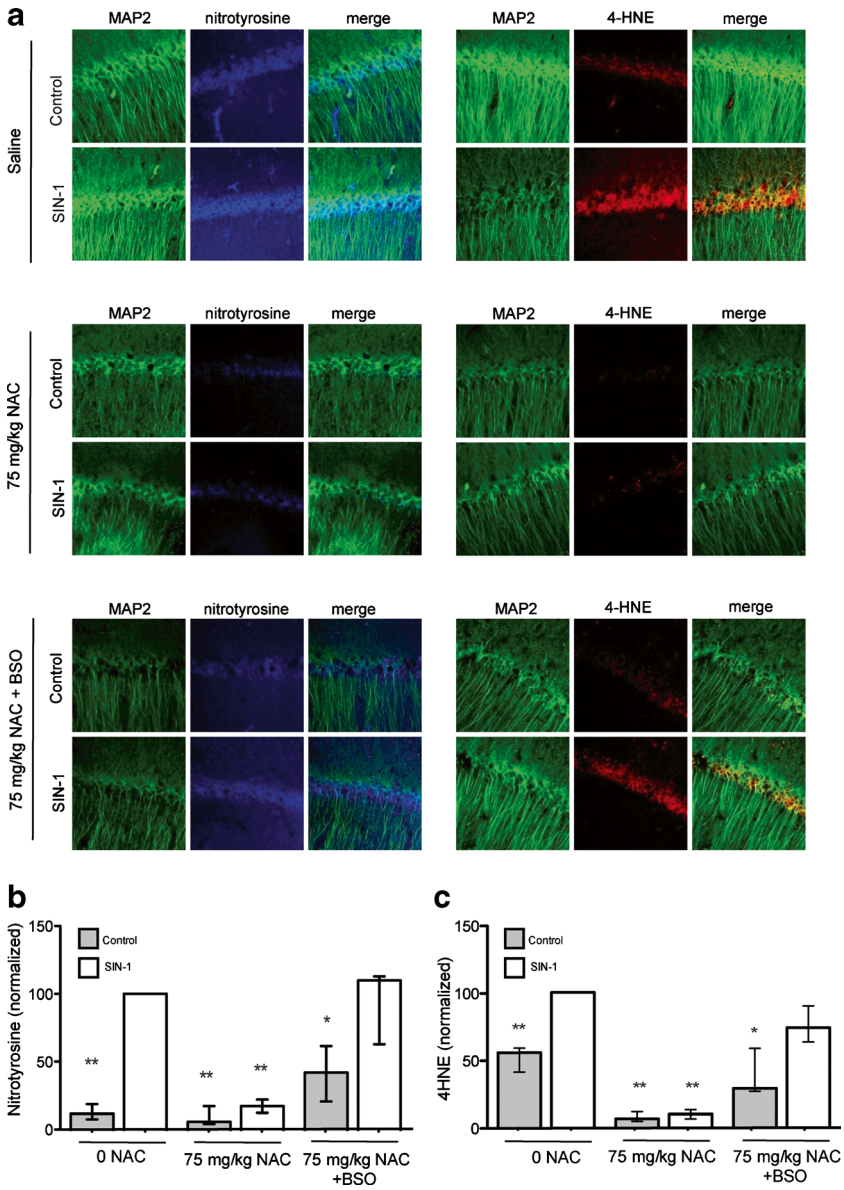

Fig. 3 *N*-Acetyl cysteine (NAC) administered to mice restores antioxidant capacity in glutathione (GSH)-deficient neurons. (A) EAAC1$^{-/-}$ hippocampal neurons formed nitrotyrosine (blue) and 4-hydroxynonenal (4-HNE; red) during exposure to 3-morpholinosydonimide (SIN-1). Neuronal cell bodies and processes are labeled with microtubule-associated protein 2 (MAP2; green). Nitrotyrosine and 4-HNE formation were attenuated in neurons of mice treated with NAC, and the effect of NAC was blocked in mice co-treated with buthionine sulfoximine (BSO). (**B**, **C**) Quantified data, pooled from 6 experiments. Results in each experiment were normalized to values measured in EAAC1$^{-/-}$, 0 NAC mice. n=4 mice in each condition; $^{**}p<0.01$ *vs* EAAC1$^{-/-}$, 0 NAC

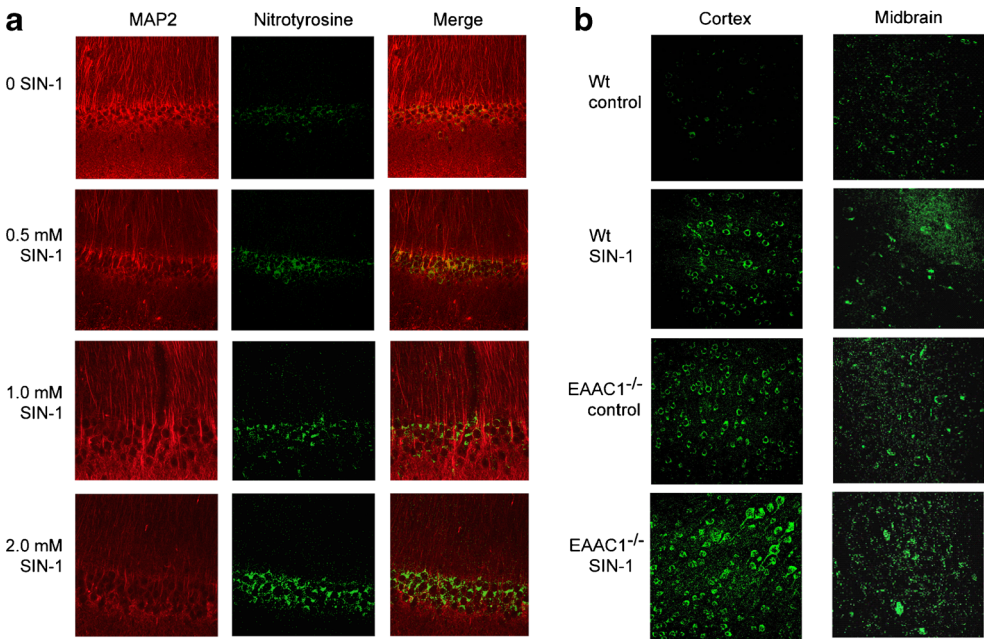

Fig. 4 3-Morpholinosydonimide (SIN-1)-induced nitrotyrosine formation. (**A**) Effects of increasing SIN-1 concentrations on nitrotyrosine formation in wild-type (Wt) hippocampal neurons. (**B**) SIN-1 (1 mM) also induced nitrotyrosine formation in cortical and midbrain neurons. Images are representative of sections from at least 2 mice in each condition. MAP2 = microtubule-associated protein 2

(Fig. 5A). NAC administered by oral gavage showed comparable potency (Fig. 5B).

We next measured the concentrations of NAC present in mouse CSF under these conditions. We chose a dose, 25 mg/kg, that was substantially higher than required for maximal effects on both neuronal GSH repletion and antioxidant capacity, in order to ensure that the corresponding CSF NAC measurements would identify the concentrations at which NAC has biological activity in neurons. We measured both native (reduced) NAC and total NAC (NAC present in both its reduced form and in reversible disulfide linkages with other thiols), and found that the vast majority of NAC was present in its reduced form at all doses and time points examined (Fig. 6). The peak (20 min) NAC concentration after 25 mg/kg i.p. dosing was 126±15 nM. Oral dosing produced a lower peak level but longer duration of elevation. Experiments performed using rats gave a very similar pattern but with somewhat higher CSF concentrations achieved with a lower (15 mg/kg) NAC dose (Fig. 6D).

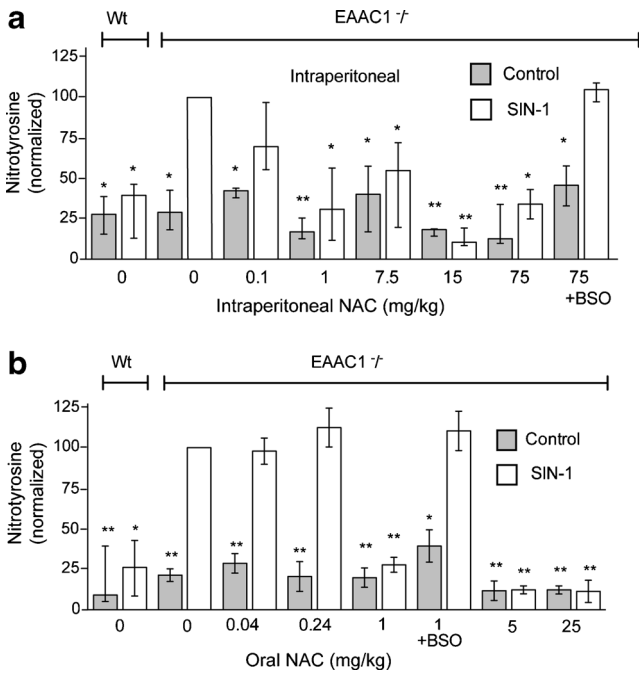

Fig. 5 Dose–response effects of *N*-acetyl cysteine (NAC) on neuronal antioxidant capacity. Mice were treated with NAC at the indicated doses by (**A**) intraperitoneal injection or (**B**) oral gavage 5 h prior to slice harvest, and co-treated with buthionine sulfoximine (BSO) where indicated. Results in each of 12 experiments were normalized to values obtained in 3-morpholinosydonimide (SIN-1)-treated, EAAC1$^{-/-}$, 0 NAC mice. n=3–5 mice in each condition; *p<0.05; **p<0.01 *vs* SIN-1-treated, EAAC1$^{-/-}$, 0 NAC. Wt = wild-type

Last, we administered NAC to humans with PD at several doses and formulations to determine if the CSF levels of NAC shown to have biological activity in mouse brain could be achieved in human CSF. CSF was obtained by lumbar puncture in each subject prior to the first dose, and again 90 min after the fourth dose. Thirteen patients were enrolled, and 12 completed the study procedures [24]. Oral NAC administration produced a dose-dependent increase in CSF NAC concentrations, with the highest dose producing a peak total NAC concentration of 10.1 ±0.8 μM (Fig. 6E), a concentration > 80 times higher than the CSF NAC concentration found to have robust biological activity in mouse neurons (Fig. 6A–C). The liquid and capsule forms of NAC had comparable effects on human CSF NAC levels. As in the mouse and rat, most of the NAC in human CSF was found in its reduced (free thiol) form. The highest dose used in the human studies was 70 mg/kg twice daily, a value chosen

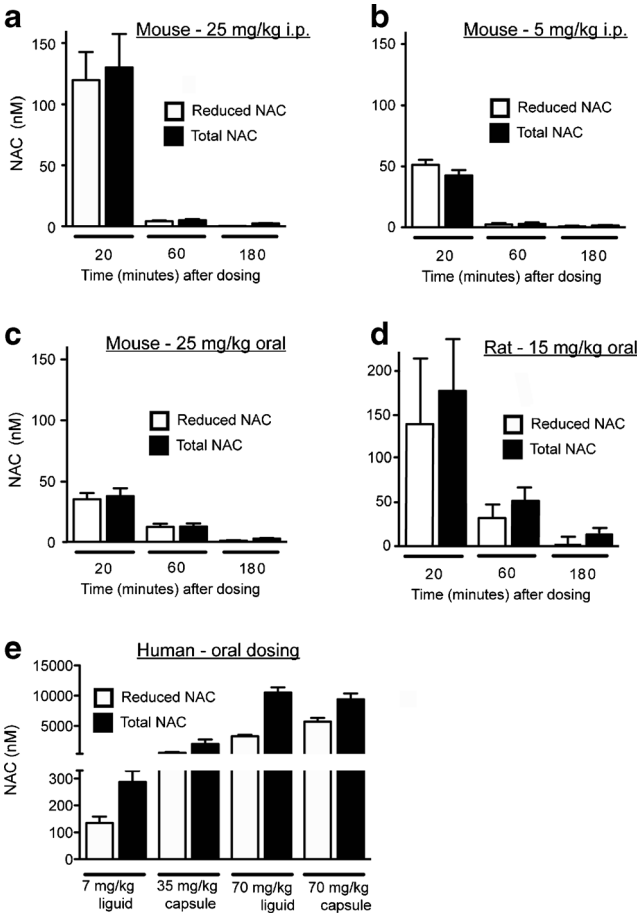

Fig. 6 Total and reduced *N*-acetyl cysteine (NAC) levels measured in cerebrospinal fluid (CSF) in mice, rats, and humans. (**A**, **B**) NAC in EAAC1$^{-/-}$ mouse CSF at time points after 25 mg/kg or 5 mg/kg intraperitoneally (i.p.). (**C**) NAC in EAAC1$^{-/-}$ mouse CSF after 25 mg/kg orally. (**D**) NAC in rat CSF at time points after 15 mg/kg orally. (**E**) NAC in human CSF, 90 min after the designated oral dose. Data are mean±SEM. Data from human subjects are re-plotted from [24]

because it is at the upper end of published clinical trials [15, 16]. The dose of 35 mg/kg also produced CSF levels that were far higher than the comparable 25 mg/kg oral dose in mice, whereas values measured in rat CSF were intermediate between those in mice and humans (Fig. 6).

Discussion

It is often difficult to validate drug target engagement in clinical studies of neurodegenerative disorders. The therapeutic goal of NAC in PD is to normalize neuronal GSH levels and thereby restore neuronal antioxidant capacity [13, 14]. Here we employed a strategy in which we first used an animal model to identify the CSF concentration of the drug required to have a biological effect in neurons, and then enrolled human subjects to identify the dose required to achieve this drug concentration in human CSF. Our findings show that NAC is able to both normalize neuronal GSH content and functional antioxidant capacity at doses producing peak CSF levels of 50 nM or higher in mice, and that NAC concentrations in human CSF far exceed these levels when administered at well-tolerated oral doses.

A novel aspect of this study was the use of *ex vivo* acute brain slice cultures for quantitative assessment of neuronal antioxidant capacity. This approach allowed us to administer pharmacological treatment to intact mice, and then subsequently analyze the capacity of the mouse brain neurons to counter an exogenous oxidative stress under controlled conditions. SIN-1 generates superoxide and NO, which, in turn, form the highly ROS peroxynitrite. The oxidation of tyrosine to nitrotyrosine provides a marker of peroxynitrite chemical activity in cells [28], and the formation of this product in cells exposed to peroxynitrite thus provides an index of cellular antioxidant capacity. 4-Hydroxynonenal similarly serves as a measure of functional antioxidant capacity, as it is generated by the interaction of superoxide (or superoxide metabolites) and cell lipids [29].

There are several mechanism by which neuronal GSH depletion could contribute to neuronal loss or injury in PD, including impaired mitochondrial respiratory function, accelerated apoptosis, and impaired scavenging of ROS [30–33] . Superoxide, NO, and peroxynitrite are all scavenged by GSH-dependent mechanisms [3, 34]. GSH is also a substrate for glutaredoxin-mediated repair of proteins that have been oxidatively modified, a process that may be of particular importance in disorders such as PD that are associated with toxic protein aggregates. These enzyme-catalyzed reactions ultimately generate GSH disulfide (GSSG) from GSH. GSSG can either be recycled to GSH in the GSH reductase reaction, or exported from cells. GSSG has intrinsic cytotoxicity; consequently, cells export GSSG when formation exceeds capacity for recycling to GSH [35], and the GSH moieties lost through GSSG export or other means must be replaced by *de novo* GSH synthesis.

Cysteine availability is usually the rate-liming factor for GSH synthesis [12], and consequently the ability of NAC to support GSH synthesis and antioxidant capacity provides indicators of intracellular biological activity. Here we used a previously validated immunohistochemical method for quantifying GSH levels specifically in neurons because biochemical determinations do not distinguish between neuronal GSH and GSH present in astrocytes or other cell types [21, 22]. We found that NAC doses required to normalize antioxidant function were generally lower than required to normalize neuronal GSH content, presumably because GSH content need not be fully restored to improve oxidant scavenging. In addition to providing a substrate for GSH synthesis, NAC can itself react with ROS at its cysteine residue. This property raises the possibility that observed effects of NAC could be attributable to direct antioxidant effects rather than, or in addition to, its role

in supporting GSH synthesis. However, the rate of direct NAC reaction with ROS is orders of magnitude slower than the rates of GSH-coupled enzymatic scavenging [3, 11, 36]. Accordingly, the results presented here show that NAC at the doses employed failed to increase neuronal antioxidant capacity when GSH synthesis was prevented by BSO.

It may be surprising that NAC was able to affect neuronal GSH content significantly at submicromolar CSF levels, given that the normal intracellular GSH concentration is about 1 mM. However, intracellular cysteine levels are much lower, about 25 μM in normal cells [37], and presumably far lower than this in cells with cysteine-responsive GSH depletion. Moreover, CSF concentrations do not indicate the rate of NAC flux from plasma to the neuronal intracellular space. There is a dynamic flux of NAC down its concentration gradients, plasma > CSF > intracellular space, that is maintained, in part, by deacetylation of NAC to cysteine in the intracellular space. NAC can also act more indirectly to increase cysteine availability by displacing cysteine from protein binding sites and labile disulfides (e.g., NAC + Cys–Cys ⟷ NAC–Cys + Cys) [38, 39], and NAC that is present in reversible linkages such as these may subsequently be liberated to regenerate free NAC or cysteine.

In the mouse studies, CSF NAC levels fell to negligible values within 1 h of NAC administration, but the effect of NAC on neuronal GSH levels and antioxidant capacity were robustly evident when assessed 5 h after dosing. These findings suggest a rapid NAC uptake and intracellular GSH synthesis, followed by a much slower decline in neuronal GSH content. This is consistent with a normally slow loss of GSH in brain [27], and with our present finding that BSO had no significant effect on GSH levels over a 5-h observation interval. These results also suggest that beneficial effects of NAC in the CNS can be achieved with spaced rather than continuous dosing.

We have proposed here that the NAC concentrations measured in mouse CSF under conditions in which NAC has demonstrated effects on mouse neurons can be used to provide a target human CSF concentration for guiding human dosing. A useful feature of this approach is that species differences in NAC absorption, liver metabolism, and CSF transport all become moot, as the only measure of consequence is the CSF NAC level. However, this approach does involve certain assumptions: that neuronal metabolism of NAC to cysteine and GSH is comparable in humans and mouse; that the relationships between NAC concentrations in CSF and brain extracellular fluid are comparable in human and mouse; that NAC can enter human and mouse neurons at comparable rates; and that this rate is not significantly altered with chronic NAC treatment. Moreover, the studies presented do not provide an indication of how frequently dosing must occur to maintain normal GSH levels, as this is likely dependent on the underlying cause of GSH depletion. Given these assumptions and limitations, it may be prudent in human clinical studies to use a dosing strategy that raises NAC in human CSF to levels that are several fold higher than those required for effects in the mouse. The findings of our human CSF studies indicate this is feasible.

Despite the use of NAC in numerous clinical studies, there has been uncertainty about doses required to achieve biological effect in brain. The present findings indicate that NAC can normalize GSH levels and antioxidant capacity in mouse brain, and that the CSF concentration of NAC associated with this effect can easily be achieved in humans with oral dosing. These findings should help remove a significant barrier to the use of NAC in fully powered, randomized clinical trials. Variations of this approach, pairing measures of drug efficacy in *ex vivo* brain slices with CSF drug level measurements, may be useful in rational drug dosing for other agents targeting central nervous system disorders.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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