Early Failure of N-Methyl-d-aspartate Receptors and Deficient Spine Formation Induced by Reduction of Regulatory Heme in Neurons

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ABSTRACT
An initial stage of many neurodegenerative processes is associated with compromised synaptic function and precedes synapse loss, neurite fragmentation, and neuronal death. We showed previously that deficiency of heme, regulating many proteins of pharmacological importance, causes neurodegeneration of primary cortical neurons via N-methyl-D-aspartate receptor (NMDAR)-dependent suppression of the extracellular signal-regulated kinase 1/2 pathway. Here, we asked whether the reduction of heme causes synaptic perturbation before neurite fragmentation in neuronal cultures and investigated molecular mechanisms of synaptic dysfunction in these cells. We showed the change in the NR2B subunit phosphorylation that correlates with compromised NMDAR function after the reduction of regulatory heme and a rapid rescue of NR2B phosphorylation and NMDAR function by exogenous heme. Electrophysiological recordings demonstrated diminished NMDAR currents and NMDAR-mediated calcium influx after 24 h of inhibition of heme synthesis. These effects were reversed by treatment with heme; however, inhibition of the Src family kinases abolished the rescue effect of heme on NMDA-evoked currents. Diminished NMDAR current and Ca2+ influx resulted in suppressed cGMP production and impairment of spine formation. Exogenous heme exerted rescue effects on NR2B tyrosine phosphorylation and NMDA-evoked currents within minutes, suggesting direct interactions within the NMDAR complex. These synaptic changes after inhibition of heme synthesis occurred at this stage without apparent dysfunction of major hemoproteins. We conclude that regulatory heme is necessary in maintaining NR2B phosphorylation and NMDAR function. NMDAR failure occurs before neurite fragmentation and may be a causal factor in neurodegeneration; this could suggest a route for an early pharmacological intervention.

Introduction
Neurodegeneration progresses from reversible down-regulation of synaptic function to irreversible synapse loss and neuronal death, so rescue at such an early stage of synaptic loss offers an attractive approach for therapeutic intervention (Wishart et al., 2006; Mallucci, 2009). Elucidating mechanisms causing neurodegenerative process requires capturing an earlier synaptic perturbation, which is not always easy in existing in vivo and in vitro models of neurodegeneration (Boekhoorn et al., 2006; Soto and Estrada, 2008).

Here, we focus on early stages of synaptic dysfunction before neurite fragmentation in primary neurons with reduced level of heme and on identifying factors triggering synaptic loss that has greater potential to facilitate pharmacological strategies to reverse neurodegeneration.

Deficiency of heme is detrimental to neurons and is a contributory factor in cell aging (Chernova et al., 2006), Alzheimer’s disease (Atamna and Frey, 2004), and drug-induced neurotoxicity (Meyer et al., 2005). It also diminishes neuron-specific gene expression, alters cellular signaling, and induces apoptosis (Zhu et al., 2002). Heme synthesis is up-regulated during the differentiation of cultured neuronal cells (Shinjyo and Kita, 2006) and exogenous heme promotes outgrowth of neurites (Ishii and Maniatis, 1978). Heme exists in at least two pools in cells: heme bound within hemoproteins, and “free” heme (i.e., not associated with a protein).

ABBREVIATIONS: NMDAR, N-methyl-D-aspartate receptor; ALAS, aminolevulinic acid synthase; nNOS, neuronal nitric-oxide synthase; SFK, Src family kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine; SA, succinyl acetone; PCR, polymerase chain reaction; DIV, days in vitro; aCSF, artificial cerebrospinal fluid; BSA, bovine serum albumin; PSD, postsynaptic density; sGC, soluble guanylate cyclase; HO-1, heme-oxygenase-1.

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A portion of the unbound heme forms a regulatory pool that mediates a signaling role through binding to heme-regulatory motifs in proteins and modulating their functions; this includes ion channels (Tang et al., 2003), transcription factors (Ogawa et al., 2001), and nuclear receptors (Raghuram et al., 2007). We demonstrated previously that heme is necessary to maintain neurite integrity, whereas heme deficiency causes neurodegeneration via NMDA receptor (NMDAR)-dependent suppression of the extracellular signal-regulated kinase 1/2 pathway. Heme-deficient cultured neurons displayed progressive neurite fragmentation followed, eventually, by cell death. The rescue of prosurvival extracellular signal-regulated kinase 1/2 activation by heme was mediated predominantly via the NR2B-containing NMDARs (Chernova et al., 2007). These observations were made in neurons chronically deficient in heme, in which the loss of both bound and regulatory heme pools might have contributed to the progressive fragmentation of neurites.

Here, we tested the hypothesis that heme is necessary for the regulation of NMDAR and that a lack of “free” heme rapidly causes this ion channel dysfunction. We have examined the early stage of heme deficiency in cortical cultures and identified an early NMDAR impairment. Failure of NMDAR function occurred shortly (16–24 h) after reduction of regulatory heme and was reflected by markedly decreased NMDAR currents and Ca<sup>2+</sup> influx. This correlated with a reduction in the phosphorylation of the NR2B subunit of NMDARs. In addition, NMDAR-dependent GMP production was compromised, and dendritic spine formation reduced. Exogenous heme rescued the ion channel function and spine formation, but only if normal levels of NR2B phosphorylation were resumed. We have demonstrated that heme modulates NMDAR function and tyrosine phosphorylation of NR2B subunit within minutes in a signaling mode and requires Src family kinases (SFKs) activity. Heme bound to major hemo-proteins was preserved and the functions of tested proteins were unaffected. These findings suggest that early synaptic impairment after inhibition of heme synthesis is caused by the reduction of regulatory heme. We conclude that NMDAR failure occurs before neurite fragmentation at the early stage of heme deficiency and may be a causative factor in neurodegeneration.

**Materials and Methods**

**Primary Cell Culture.** Primary cortical neurons were prepared from either male or female 14-day-old fetuses of the BALB/c mouse strain bred in-house. The isolated neocortex of embryos was gently dissociated to release the neurons, which were washed twice in ground levels of fluorescence.

**Immunocytochemistry.** After treatment, cells were fixed with 4% paraformaldehyde at room temperature for 20 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline for 5 min. Cells were then incubated with rabbit anti-phospho-NR2B (Tyr1252 or Tyr1336) (R&D Systems, Abingdon, UK) and mouse anti-PSD-95 antibody (NeuroMab, Davis, CA) at room temperature for 1 h. Secondary antibodies (goat anti-rabbit Alexa Fluor 546 and goat anti-mouse Alexa Fluor 488 at 1:500) were then added for 1 h, followed by nuclear staining with 300 nM 4′,6-diamidino-2-phenylindole for 10 min. Secondary antibodies alone were used as specificity controls and uniformly resulted in very low background levels of fluorescence.

**Transfection of Cortical Neurons.** At 7 DIV, neurons were transfected with plasmids expressing FLAG-tagged tTomato as a cell fill provided by Dr. Michael J. Schell (Department of Pharmacology, Uniformed Services University, Bethesda, MD) to visualize dendritic morphology. A total of 0.5 μg of DNA and 1 μl of Lipo-fectamine 2000 (Invitrogen) was premixed according to the manufacturer’s instructions and added to cultures (0.5 × 10<sup>6</sup> cells) growing on glass-bottomed chambers (Thermo Fisher Scientific) in 900 μl of medium. After 6 h, the medium was replaced with a fresh 900 μl of serum-free Neurobasal medium supplemented as described above.

**Inhibition and Readmission of Heme, Measurement of Cellular Heme Content, and Heme Synthesis.** To inhibit heme synthesis, cells were cultured in serum-free medium with 250 μM SA (4,6-dioxoheptanoic acid) (Sigma-Aldrich, Dorset, UK) for the stated durations. For heme readmission experiments, cells were treated daily with 100 nM hemin (iron protoporphyrin IX); stock solution was added to human serum albumin in a 1:1 M ratio before treatment. The heme concentrations were verified spectrophotometrically.

Total cellular heme was measured by using a modified Quanti-Chrom Heme Assay (BioAssays Systems, Hayward, CA). The optical density measurements were taken using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The amount of heme in each sample was expressed as micromoles of heme per milligram of total protein. For measurement of heme synthesis, cells were incubated with 0.4 μCi of [3,5-<sup>3H</sup>]aminolevulinic acid hydrochloride (2.6 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA) for 24 h. Heme was extracted from the cells by acetone-HCl and diethyl ether. The amount of radioactivity in extracted heme was measured by scintillation counting as described previously (Chernova et al., 2007). Total recovery of radioactivity from all fractions was the same for treated and untreated cells.

**RNA Extraction and Quantitative Real-Time PCR Analysis.** Total RNA was isolated from treated and untreated cells at different time points by using TRI reagent (Sigma-Aldrich), and cDNA synthesis was performed using random primers and Superscript II (Invitrogen, Carlsbad, CA). PCR primers were selected using the Primer Express version 2.0 Software program (Applied Biosystems, Foster City, CA). Primers sequences were as follows: β-actin forward primer, 5′-GATTCGGTTCTGCGTACAGG-3′, reverse primer, 5′-GGGACGGATCCAGGATC-3′; 3′-aminolevulinate synthase 1 (ALAS1) forward primer, 5′-TCTTTCGAGAGCCAGTCT-3′, reverse primer, 5′-GAGGCTGAGGGCCCTTCT-3′; CYP4F1 forward primer, 5′-CTGGACATATAATGAAATGTGCC-3′.

Primers were designed to cross-exon boundaries. PCR was performed using SYBR Green PCR Master Mix, primers, and 10 ng of reverse-transcribed cDNA in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described previously (Kannan et al., 2010). Quantification was performed using the comparative CT method (ΔΔCT). Data are presented as the mean ± S.D. (n = 3–8 for each group). Statistical significance was assessed as p < 0.05 using one-way analysis of variance.
Transfection efficiency was estimated to be less than 2%. Neurons were imaged at 13 DIV.

Imaging and Quantification of Spines. Cortical neurons with pyramidal morphology were selected for analysis from the transfected cell population. Images of cultured cells were obtained with a Zeiss LSM 510 META confocal microscope equipped with a 63x oil immersion lens (Carl Zeiss, Thornwood, NJ). Images were collected as a series of Z-sections (approximately 0.5 μm). Images were reconstructed using Velocity software (Improvision, Coventry, UK). Total protrusions from the dendrite (spines) were quantified over unit length and expressed as the number per 10 μm of the neurites. Only fields with a low density of transfected neurons were used to quantify the spine length and number.

Immunoblotting. Proteins were extracted from primary neurons using NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10 mM glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/μl leupeptin, and 10 μg/μl aprotinin) and brief sonication. Microsomal fraction was prepared as described previously (Kapoor et al., 2006). In brief, the cells were scraped in phosphate-buffered saline and then pelleted by centrifuging at 200g for 10 min. The cell pellet was resuspended in microsomal dilution buffer containing 0.1 (v/v) glycerol, 0.25 mM phenylmethylsulfonyl fluoride, 0.01 M EDTA, and 0.1 mM dithiothreitol. After brief sonication, the cells were centrifuged at 9000g for 20 min. The supernatant was then further centrifuged at 105,000g for 60 min to isolate the microsomes. The microsomal pellet was resuspended in microsomal dilution buffer and used for analysis. Separation by SDS-polyacrylamide gel electrophoresis or native polyacrylamide gel electrophoresis [for neuronal nitric-oxide synthase (nNOS) detection] was followed by immunoblotting and enhanced chemiluminescence detection (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Antibodies were from the following sources: NMDAR NR1 and total NR2B were from Santa Cruz Biotechnology (Santa Cruz, CA); NMDAR phospho-NR2B Tyr1252 and Tyr1336 were from R&D Systems; α-tubulin was from Sigma-Aldrich; ALAS1 was from Abcam plc (Cambridge, UK), heme-oxygenase-1 (HO-1) and P450 reductase antibodies were from AKELA Pharma Inc. (Montreal, QC, Canada), CYP1A1 was from Cambridge Bioscience (Cambridge, UK) and CYP4X1 was from Dr. D. Bell (School of Biology, University of Nottingham, Nottingham, UK). Blots were subsequently exposed to a second primary antibody against α-tubulin, to verify equivalent protein loading and transfer. Bands were detected by enhanced chemiluminescence (GE Healthcare), exposed to X-ray films under nonsaturating conditions. Results were quantified using densitometry and ImageQuant 5.2 software (Molecular Devices). A pair of images was obtained every second with 20-ms exposure time at each wavelength. Fluorescence measurements were preformed in the cell soma. Only cells with low resting ratios (<0.6) and cells that returned to basal ratios after stimulation were used for analysis.

Radioimmunoassay of cGMP. Control, NO-donor S-nitroso-N-acetylpenicillamine (Sigma-Aldrich)-stimulated or NMDA-stimulated (10, 100 and 50 μM, respectively) cortical cultures were used for assessing cGMP production. In brief, neutralized cell extracts were diluted 5-fold in 100 mM sodium acetate, pH 6.2, and acetylated by consecutive addition of triethylamine (10 μl) and acetic anhydride (5 μl) and used in the radioimmunoassay (Brooker et al., 1979) within 60 min. cGMP standards (100 μl; 0–4 nM) were treated identically. Acetylated samples (100 μl) were mixed with 2′-O-acetylated 3,3′,4′,5′-tetraethylbenzimidazoyl cationic dye 5,5′-dihydrolongyl methyl ester cGMP (GE Healthcare) [50 μl; ~3000 d.p.m. made up in 50 mM sodium acetate, 0.2% bovine serum albumin (BSA), pH 6.2] and 100 μl of anticyclic GMP antibody (GE Healthcare; diluted in 50 mM sodium acetate and 0.2% BSA, pH 6.2). Samples were intermittently vortex-mixed during a 4-h incubation at 4°C. Free and bound cGMP was separated by charcoal precipitation with 500 μl of a charcoal suspension [1% (w/v) activated charcoal (TILL Photonics, Martinsried, Germany) with excitation at 340, 360, and 380 nm and emission detected at ~505 nm. The ratio of fluorescence at 340 and 380 nm excitation was used as a measure of [cGMP]. Fluorescence was detected using a charge-coupled device PentaMAX camera (Princeton Instruments, Trenton, NJ). Data were recorded and analyzed using Meta Imaging (series 7.0) software (Molecular Devices). A pair of images was obtained every second with 20-ms exposure time at each wavelength. Fluorescence measurements were preformed in the cell soma. Only cells with low resting ratios (<0.6) and cells that returned to basal ratios after stimulation were used for analysis.

Detection of Mitochondrial Membrane Potential. Mitochondrial membrane potential (∆Ψm) was detected using DePsipher Kit for Detection of Mitochondrial Membrane Potential Disruption (R&D Systems) according to the manufacturer’s instructions. Lipophilic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazoly carbocyanine iodide enters the inner mitochondrial matrix in its monomeric form when the mitochondrial membrane is polarized. When the mitochondrial has a high ∆Ψm, the dye crosses the membrane and forms aggregates, which appear red under UV light. Living neurons were stained and observed immediately with a microscope using a long-pass filter (fluorescein and rhodamine). In healthy cells, the mitochondria were stained red after aggregation of the DePsipher within the mitochondria. In cells with disrupted ∆Ψm, the dye remains in its monomeric form in the cytoplasm and appears entirely green.

Measurement of ATP in Neurons. Cultured neurons were treated with 250 μM SA for 1 to 6 days and harvested at 12 DIV. The level of cellular ATP in neurons was determined with a luciferase-based CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer’s recommendations. After the plates were developed, luminescence was measured in a microplate luminometer (Luminoskan; Labsystems, Helsinki, Finland).
land). Each set of data was collected from multiple replicate wells of each experimental group from five independent experiments; the concentrations were normalized against total cellular protein and expressed as nanomoles per milligram of protein (mean ± S.D.).

Results

Inhibition of Heme Synthesis Promptly Reduced Regulator Pool of Intracellular Heme. To examine the chronology of the neuronal deficit with reduced heme, we inhibited its synthesis for 1 to 6 days by treatment with 250 μM SA, a specific inhibitor of aminolevulinic acid dehydratase (Sassa and Nagai, 1996). Heme synthesis was reduced by approximately 50% (Fig. 1A), causing 17 ± 6% decrease of total intracellular heme (Fig. 1B). This reduction was accompanied by a marked increase in the expression of ALAS1 (Fig. 1C), the gene encoding aminolevulinic acid synthase, which is under negative transcriptional feedback control by the regulatory pool of intracellular heme (Sassa and Nagai, 1996; Sassa, 2004). The up-regulation of ALAS1 expression in SA-treated neurons indicated that the decrease in heme was physiologically significant, and this was a rapid process, occurring within hours after inhibition of synthesis. After 16 h of SA treatment ALAS1 mRNA increased 2-fold, similar to the up-regulation observed in continuously deficient neurons (Chernova et al., 2006). An increased amount of ALAS1 protein in response to reduced heme levels was detected in these SA-treated cultures (Fig. 1D), consistent with previously shown regulation by “free” heme via alterations in protein half-lives (Zheng et al., 2008). By contrast, when heme was added back to SA-treated cells, there was a 24 ± 2% increase in intracellular heme, accompanied by a decrease in ALAS1 expression, again consistent with a regulated physiological response (Fig. 1, B and C). Our results suggest that the regulatory pool of heme was compromised within hours if de novo synthesis was inadequate. We examined how a lack of regulatory heme affected neuronal function and tested whether NMDAR was compromised in neurons with reduced regulatory heme. We chose a 24-h time point of heme synthesis inhibition because this corresponded to the depleted regulatory heme pool, reflected by a 2-fold up-regulation of ALAS1 and a relatively small (17%) reduction of total cellular heme.

Reduction of Intracellular Heme Decreases Tyrosine Phosphorylation of NR2B. Although continuously heme-deficient neurons have reduced expression of NMDAR subunits at the stage of neurite loss (Chernova et al., 2007), the initial changes after inhibition of heme synthesis do not involve down-regulation in gene or protein expression for NMDAR subunits NR1 and NR2B (Supplemental Fig. 1, A–D). NR2B is a major tyrosine-phosphorylated protein regulated by SFKs in the postsynaptic density (Moon et al., 1994) and is impaired in heme-deficient neurons (Chernova et al., 2007). To examine the early changes caused by reduced heme availability, we used immunoblotting and immunostaining to monitor NR2B phosphorylation at Tyr1336 and Tyr1252 in the presence or absence of SFKs inhibitor PP2 and heme in neurons treated with SA for 24 h, the time point at which the 2-fold up-regulation of ALAS1 indicated a depletion of the regulatory heme pool (Fig. 2, A–N). Phosphorylation of NR2B at Tyr1336 and Tyr1252 was diminished in neurons by incubation with PP2 and was markedly lower in SA-treated compared with control cultures (Fig. 2, A and B). PP2 and SA treatments had additive effects on the reduction in Tyr1336 and Tyr1252 phosphorylation (Fig. 2, A and B). In cultures cotreated with SA and heme, phosphorylation of Tyr1336 and Tyr1252 was unaffected (Fig. 2, A and B). The rescue effect by exogenous heme was abolished if these cultures were concomitantly treated with PP2 (Fig. 2, A and B). The levels of NR2B protein phosphorylated at Tyr1336 and Tyr1252 were also examined by immunostaining of neuronal cultures under the same conditions (Fig. 2, C–N). Together, these data provide strong evidence that compromised availability of heme impairs NR2B subunit phosphorylation, and this is an early event in heme deficiency-induced neurodegeneration. This raises the question of whether the functional outputs of NMDARs are also affected at this early stage.

NMDAR Current Is Promptly Impaired by Reduction of Intracellular Heme. Phosphorylation of NMDAR regulates the channel kinetics and the Ca2+ permeability and membrane localization and trafficking (Cull-Candy and Leszkiewicz, 2004). After the finding of altered NR2B subunit phosphorylation after reduction of cellular heme, we examined how the NMDAR current was affected by changes...
of heme concentration. Whole-cell patch-clamp recordings were made from single cortical neurons maintained in dissociated culture (12 DIV) and treated with SA for 24 h in the presence or absence of heme. Reduction of intracellular heme for 24 h decreased NMDA-mediated whole-cell currents compared with control cultures [Fig. 3A, top (Ctrl), versus top middle traces (24 h SA)]. Neurons cotreated with SA and heme for the same time exhibited larger responses to NMDA application [Fig. 3A, bottom middle traces (24 h SA + H)]. Incubation of control cultures with 100 nM heme for 24 h did not have an effect on NMDA-evoked whole-cell current [Fig. 3A, bottom traces (24 h + H)]. Data summarized in Fig. 3B show I/V curves for neurons under control conditions, heme treatment, SA treatment, and SA treatment in the presence of heme. NMDA-evoked currents at 40-mV holding potential were suppressed after SA treatment, and this effect was reversed by cotreatment with heme (Fig. 3C). The rescue effect of heme on NMDAR current was abolished in heme-deficient cultures cotreated with SFK inhibitor PP2 (Supplemental Fig. 2, A–C). The results suggest that the impairment of NMDAR function in neurons with reduced heme is associated with compromised phosphorylation of the regulatory NR2B subunit.

**Ca2+ Influx through NMDARs Is Reduced in Neurons with Low Intracellular Heme Levels.** Because NMDARs are highly Ca2+-permeable (Viviani et al., 2006), we monitored changes in intracellular Ca2+ in response to NMDA application, as a further index of channel function. Cultures (12 DIV) were loaded with Fura 2-AM and imaged using a charge-coupled device camera; NMDA was pressure-applied onto control or SA-treated (24–44 h) cultures. The ratio of fluorescence at 340 and 380 nm excitation was used as a measure of intracellular Ca2+ ([Ca2+]i). Basal [Ca2+]i did not change in SA-treated cultures (data not shown), whereas response to NMDA application was affected. SA-treated treated with 250 μM SA for 24 h with or without exogenous heme and with or without Src kinase inhibitor PP2 at 13 DIV. A, phosphorylation levels of NR2B at Tyr1252 and Tyr1336 corresponding to the above conditions were examined by Western blot; images show representative blots. The same blots were reprobed for α-tubulin. B, quantification of band intensities obtained using densitometry and ImageQuant 5.2 software and normalized to α-tubulin. Each column is the mean normalized expression of protein ± S.D. of at least three independent experiments. * , statistically different from control group, p < 0.05; #, statistically different, p < 0.05. C to N, confocal micrographs of maximum projection Z-series of cultured neurons at 13 DIV stained for phospho-NR2B (Tyr1252 and Tyr1336, top and bottom, respectively, red) and for PSD-95 (green), the nuclei were stained with 4′-6-diamidino-2-phenylindole (blue). C and I, control cultures; D and J, cultures treated with 50 nM PP2 for 2 h. E and K, SA treated for 18 h; F and L, treated with SA and PP2 for 18 h; G, H, M, and N, treated with SA and heme for 18 h in the presence (H and N) or absence (G and M) of PP2.
quale, 2005). Given the impaired NMDAR function we have observed in the neurons with reduced heme, we used imaging techniques to examine changes in dendritic spine formation. To visualize dendritic morphology, neurons were transfected with FLAG-tagged tdTomato (red fluorescent cell fill) at 7 DIV and then examined at 13 DIV after treatment with SA or SA and heme for 24 h with or without addition of the SFKs inhibitor PP2. Cortical neurons with pyramidal morphology were selected for analysis from the transected cell population. The number of spines per 10 μM length of dendrite was significantly lower in the neurons with reduced cellular heme, whereas cotreatment of these cultures with heme reversed this phenomenon (Fig. 5, A–C, F). Inhibition of SFKs abolished the rescue effect of heme (Fig. 5,E and F) but did not cause further deterioration in spine formation in SA-treated cultures (Fig. 5, D and E). These findings suggest a mechanistic link between failure of NMDAR and spine formation at early stage of heme deficiency and provide evidence that the rescue by heme is NR2B phosphorylation-dependent.

**Heme Modulates NMDAR Function and NR2B Phosphorylation within Minutes.** To examine whether the potentiation of NMDA-mediated current by heme is a signaling event, we conducted whole-cell patch-clamp recordings simultaneously with reintroducing heme to the heme-deficient neurons and recorded for 13 min, after which currents reached plateau levels under all recording conditions (Fig. 6A). The neurons treated with SA for 24 h showed a 60% reduction in NMDA-evoked currents (Fig. 6A, blue diamonds) over a recording time of 13 min, which is consistent with a dialysis process and a current run down. Heme was introduced into the bath solution after 2 min of recording and caused a 40% increase in the NMDAR current (red square; after an initial 2-min decrease before heme addition). When heme was present in the patching pipette, there was no initial decrease, and the increase of the current started immediately (Fig. 6A, green triangles). At the 13-min time point, NMDA-evoked currents were ~140% of initial currents values if heme was added to the bath and ~110% if heme was present in the pipette. In contrast, in SA-treated cells currents, were only ~40% of initial values after 13 min of recording. Comparison of the three curves showed that by 10-min exposure to heme, the NMDAR current is 2- to 3-fold larger than in the absence of heme.

These alterations of the ion channel function correlated with the changes in NR2B phosphorylation levels upon readmission of heme to heme-deficient neurons (Fig. 6, B and C). At a 15-min time point, NR2B phosphorylation of NR2B at Tyr1252 and Tyr1336 were recovered to control levels. However, if these neurons were pretreated for 2 h with PP2, the rescue effect of heme was abolished, similar to the changes observed in the neurons with reduced heme, where cotreatment of these cultures with heme abolished this phenomenon (Fig. 5, A–C, F). Inhibition of SFKs abolished the rescue effect of heme (Fig. 5,E and F) but did not cause further deterioration in spine formation in SA-treated cultures (Fig. 5, D and E). These findings suggest a mechanistic link between failure of NMDAR and spine formation at early stage of heme deficiency and provide evidence that the rescue by heme is NR2B phosphorylation-dependent.

**Fig. 4.** Heme depletion reduces NMDA-evoked Ca^{2+} influx and Ca^{2+}-dependent cGMP production. The ratio of fluorescence at 340 and 380 nm excitation in the cell soma was used as a measure of [Ca^{2+}]. A, representative ratio traces of NMDA-evoked intracellular Ca^{2+} ([Ca^{2+}]i) in control cells (Ctrl) and in cells treated with SA for 24 and 44 h at 12 to 13 DIV. B, NMDA-evoked [Ca^{2+}]i responses (Δratio, peak – basal) are reduced after 24 h of SA treatment but rescued by exogenous heme. Data denote mean ± S.E.M. of at least three cells from each of the three independent experiments (n shown in each bar). C, Ca^{2+}-dependent production of cGMP is decreased in neurons with reduced intracellular heme. Neurons (12–13 DIV) were treated with 250 μM SA and harvested after 16, 24, 30, or 42 h of treatment, cGMP production was monitored by radioimmunoassay, and measurements are expressed as the percentage of cGMP production in control cultures. Data denote the mean ± S.D. of three independent experiments. *, statistically different from control group, p < 0.05; #, statistically different from SA treatment for 16 h, p < 0.05.
observed in cultures cotreated with heme and SA for 24 h. These data show that regulation of NMDAR by heme is exerted rapidly and suggest a phosphorylation-dependent signaling mechanism.

**Heme Is Preserved in Major Hemoproteins in Neurons Despite Depletion of Regulatory Pool.** Depletion of heme from the regulatory pool clearly occurred within hours of SA treatment. We investigated what effect this treatment had on various hemoprotein functions in neurons.

Many mitochondrial proteins are hemoproteins, including those engaged in the electron transport chain and ATP generation. Mitochondrial membrane potential (ΔΨm) and ATP generation were monitored after inhibition of heme synthesis to determine whether mitochondrial hemoproteins functions were affected in neurons when total cellular heme was reduced by ~17%. Inhibition of heme synthesis for at least 72 h had no effect on ΔΨm (Fig. 7, A and B), consistent with a well-sustained ATP generation in SA-treated neurons. Further measurement of ATP concentration (up to 6 days of heme synthesis inhibition) revealed that mitochondrial function was also unaffected in these cultures (Fig. 7C). These data show that heme was not depleted from mitochondrial hemoproteins under these conditions.

Soluble guanylate cyclase (sGC) is a hemoprotein which, on binding NO to heme, increases cGMP production up to 300-fold (Ballou et al., 2002). sGC activation by the NO donor S-nitroso-N-acetylpenicillamine was measured as the ratio of basal to stimulated cGMP accumulation and was similar in control cultures and those treated with SA for 42 h (Fig. 8A), demonstrating that the capacity of sGC to generate cGMP was unaffected.

The hemoprotein nNOS catalyzes the NADPH-dependent oxidation of L-arginine to citrulline and NO (White and Marletta, 1992). nNOS is a homodimer with heme as a prosthetic group controlling subunit dimerization and the quaternary structure of nNOS (Klatt et al., 1996). In contrast, heme-deficient enzyme (apo-nNOS) is an inactive monomer that degrades rapidly in vitro (Dunbar et al., 2004). The amount of...
nNOS protein in neurons after 72 h inhibition of heme synthesis was similar in heme-depleted and control cultures (Fig. 8B), consistent with nNOS being in a stable and active homodimer in SA-treated cultures at 72 h.

HO-1 heme serves as both the substrate for the enzyme and the prosthetic group. The amount of HO-1 protein in the microsomal fraction of neuronal lysates was not reduced in the SA-treated neurons (Fig. 8C), suggesting that HO-1 basal expression was not affected when total cellular heme was decreased by ~17%.

Cytochromes P450 (P450) are drug- and steroid-metabolizing enzymes expressed in various tissues, including brain. Two isoforms, CYP1A1 and CYP4X1 (Al-Anizy et al., 2006), were detected in the microsomal fraction by immunoblotting (Fig. 8, D and E). No differences in protein expression were seen between control and SA-treated for 24 h cultures. Heme also is a positive modulator of P450 gene transcription, and low heme concentration may be a limiting factor for gene expression (Dwarki et al., 1987). We monitored CYP4X4 gene expression in cultures with inhibited heme synthesis for up to 14 days and did not observe any down-regulation (Fig. 8F).

Together, these data show that heme bound in mitochondrial and other major hemoproteins, such as sGC, nNOS, CYP1a1 and CYP4X1, remained in this state associated with proteins after reduction of heme synthesis by ~50% and depletion of the regulatory heme pool.

Discussion

We showed previously that heme deficiency in primary neurons causes neuronal degeneration (Chernova et al., 2007); however, the mechanisms triggering neuronal damage are not well understood. Here, we explored the mechanisms by which a deficit of heme affects synaptic function at an early stage of neurodegeneration, before neurite fragmentation in primary neurons. We have shown early loss of NMDAR function, which correlated with reduced phosphorylation of NR2B subunits. Reduced NMDAR currents and Ca²⁺ influx affected NMDAR-dependent cGMP production and were accompanied by deficient formation of dendritic spines. In this study, we used selective and specific inhibition of the second enzyme aminolevulinic acid dehydratase in the heme synthetic pathway, which remains the optimal means.
of modulating intracellular heme in vivo and in vitro (Zhu et al., 2002). ALAS1 is the first and rate-limiting enzyme, and consistent with depletion of the regulatory heme pool, we monitored the increased expression of this gene (Sassa and Nagai, 1996) and protein (Zheng et al., 2008), which is the generally accepted approach to reflect “free” heme levels (Raghuram et al., 2007). In our model, the rate of heme synthesis was reduced by ~50%, whereas total intracellular heme was reduced by ~17%, suggesting strong homeostatic mechanisms and supporting previous studies of heme turnover and transport (Maines and Gibbs, 2005). However, the reduction of heme cannot be attributed to reduction of the regulatory pool alone, which has been estimated as ~100 nM (Sassa and Nagai, 1996). This decrease could be explained by the depletion of the regulatory pool and heme from cellular storage. Such heme storage was demonstrated in astrocytes and involved a reputed heme transporter HCP1 (Dang et al., 2010). It is plausible that core functions using cofactor heme (e.g., respiratory chain in mitochondrial cytochromes) would be protected by homeostatic mechanisms.

The dynamics of heme depletion in neurons were reflected by apparent reduction of regulatory heme within hours, whereas heme bound in hemoproteins remained at sufficient levels. Mitochondrial hemoproteins were not affected in the neurons with impaired synthesis, and mitochondrial function was not compromised for up to 6 days. This is consistent with the fact that the dissociation constant of mitochondrial hemoprotein heme is <10^{-13} M, and “free” heme concentration is estimated at 10^{-7} M (Sassa, 2004). Therefore, “free” heme could be lowered by several orders of magnitude before the depletion of bound heme occurred. Furthermore, heme is covalently bound in some hemoproteins, and depletion would probably only occur with turnover of the protein. Heme was not depleted from sGC because cGMP production did not change when Ca^{2+}-dependent part of the pathway was bypassed. nNOS protein amounts were unaltered in SA-treated cultures, indicating sufficient heme levels to form nNOS homodimers and prevent protein degradation. The amounts of CYP1A1 and CYP4X1 proteins in microsomal fraction remained similar in control and treated cells. Impaired heme levels are believed to decrease CYP450 function via incomplete saturation of apoprotein (Jover et al., 2000), cytosolic...
Deficiency of Regulatory Heme Causes NMDA Receptor Failure

853

Deficiency of Regulatory Heme Causes NMDA Receptor Failure

853

persistence of the protein (Meyer et al., 2005), and involvement of heme in transcriptional regulation (Dwarki et al., 1987). Our data did not contradict these suggestions but indicated that more time is required to develop heme deficiency in these functional compartments.

In contrast to hemoproteins, the regulatory pool of heme was depleted promptly after starting treatment and resulted in NMDAR dysfunction. After 24 h of SA treatment, NMDA-evoked current was decreased by more than 50%, similar to the reduction monitored in the continuous model after 12 days of inhibition. This reduction cannot be related to a direct effect of SA on the receptor because in the presence of heme, SA-treated neurons showed a large response to NMDA, similar to control. High permeability of NMDAR for Ca\(^{2+}\) places the receptor in the upstream part of various signaling pathways. Physiological levels of synaptic NMDA receptor activation promote the survival of many types of neurons and increase their resistance to cellular damage (Hardingham and Bading, 2003). A marked decrease of Ca\(^{2+}\) influx in response to NMDA also reflects receptor dysfunction after reduction of heme and is consistent with diminished current. Ca\(^{2+}\) can enter cells through many pathways, including neurotransmitter-gated ion channels (NMDAR), Ca\(^{2+}\)-permeable subtypes of AMPA receptors, and voltage-dependent Ca\(^{2+}\) channels. There was no reduction in basal Ca\(^{2+}\) concentration in treated cells that can be attributed to compensatory Ca\(^{2+}\) flow through other receptors, whereas stimulation of NMDAR failed to produce an adequate response. The more sensitive radioimmunoassay of cGMP production detected reduction of NMDAR function even earlier. NMDAR-dependent cGMP production declined as inhibition of heme synthesis continued, suggesting that reduced cGMP concentration itself could contribute to the development of neurodegeneration in the continuous model. However, NMDAR functional failure was an earlier change in neurons with depleted regulatory heme and may have triggered alterations of many Ca\(^{2+}\)-dependent signaling pathways.

NMDARs play an important role in dendritic spine formation and maintenance. Elongation of existing spines and formation of new filopodia can be blocked by NMDAR antagonists (Shi et al., 2005). Decrease in Ca\(^{2+}\) influx affects the organization of cytoskeleton, trafficking of postsynaptic density (PSD) components, and recycling of proteins (Nimchinsky et al., 2002), thereby affecting spine formation. Reduction of spines after inhibition of heme synthesis could be another consequence of NMDR dysfunction. NR2B phosphorylation-dependent recovery of spine formation by heme indicates that NMDAR failure causes spine formation deficit; however, we cannot exclude the interactions of heme with other proteins. Ca\(^{2+}\) current through the NMDAR is largely regulated by its NR2B subunit (Krapivinsky et al., 2003), and tyrosine phosphorylation of NR2B is essential to sustain the elevation of neuronal Ca\(^{2+}\) current (Viviani et al., 2006). We have shown that a marked decrease in NR2B tyrosine phosphorylation was an early change after the reduction of regulatory heme in neurons. Similar to NMDA-evoked current, NR2B phosphorylation was rescued by exogenous heme, suggesting a coupling of these recovery mechanisms. Inhibition of SFKs abolished the rescue effect of heme on NMDAR currents, providing further evidence of the mechanistic link. Replenishment of heme concentration could reverse early synaptic changes, but only if NR2B phosphorylation was resumed. This and additive effects of PP2 and SA on NR2B phosphorylation suggest that heme is involved in maintaining NMDAR function via its phosphorylation. Heme modulates NMDAR function in an acute signaling mode, suggesting a direct interaction, probably within the NMDAR complex and with involvement of SFKs. Exogenous heme alters phosphorylation status of tyrosine kinases in heme-deficient cells (Yao et al., 2010). Src and Jak2 phosphorylations (Tyr530 and Tyr1007, respectively) were increased by heme, and the first has an inhibitory effect on activity of Src, and the latter activates JAK2. NR2B phosphorylation at Tyr1252 and Tyr1336 monitored in our study is regulated by Fyn member of SFKs (Nakazawa et al., 2001). There is an evidence of JAK2 cross-talk with SFKs (Jiang et al., 2008); however, it requires further clarification of how heme, JAK2, and NMDAR may interact. It is noteworthy that a mitochondrial protein NADH dehydrogenase subunit 2 was found recently to act as an adapter protein anchoring Src to the NMDAR, thereby playing a crucial role for Src regulation of synaptic NMDAR activity. NADH dehydrogenase subunit 2 is a subunit of complex I in mitochondria, but in the brain, it interacts with Src outside of this organelle (Gingrich et al., 2004). Using hemin-agarose affinity chromatography of neuronal lysates followed by proteomics analysis, we identified a number of putative heme-binding proteins, including partners of NMDAR complex and constituents of mitochondrial complexes I and II, which can be potential sites for regulation (Supplemental Table 1). Heme binding resulting in conformational changes of a target protein (e.g., a kinase or a protein facilitating a phosphorylation) is a plausible mechanism of the rapid increase of NMDAR currents by heme. Further systematic examination is required to identify exact targets of heme interactions with synaptic proteins.

Subunit-specific phosphorylation also controls differentially the trafficking and surface expression of NMDARs (Cull-Candy and Leszkiewicz, 2004), and monitoring these phosphorylations in heme-deficient neurons will be addressed in future studies aimed of the relationships in the NMDAR complex in heme-deficiency induced neurodegeneration. The alterations in NMDAR signaling seen in heme-depleted neurons could be the trigger for neurite decay. Functional and molecular connections of NMDARs with other receptors, such as AMPA and metabotropic glutamate receptors, are very complex, and at this stage, we cannot exclude contributions from other receptors and synaptic proteins to neuronal decay in heme-deficient cultures. However, rapid and reversible changes in NMDAR function and in phosphorylation of its regulatory subunit correlated with the availability of heme couple the receptor and heme in a signaling mode.

Together, these findings lead to the conclusion that depletion of regulatory heme rapidly compromises NR2B phosphorylation and causes the dysfunction of NMDARs; this compromises calcium-dependent signaling pathways and triggers changes in cGMP production and dendritic spine formation. Synaptic changes precede the depletion of heme from major hemoproteins. Further exploration of heme interaction with NMDAR complex and understanding how exactly the effects of heme are exerted and could be modulated by drugs may provide new insights into the reversal of neurodegeneration.
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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Chernova, Steinitz, Smith, and Forysthe.

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