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TRANSGENIC OVEREXPRESSION OF NEUROGLOBIN ATTENUATES FORMATION OF SMOKE INHALATION-INDUCED OXIDATIVE DNA DAMAGE, IN VIVO, IN THE MOUSE BRAIN

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Abstract

Acute inhalation of combustion smoke causes neurological deficits in survivors. Inhaled smoke includes carbon monoxide, noxious gases and hypoxic environment, which disrupt oxygenation and generate free radicals. To replicate a smoke inhalation scenario, we developed experimental model of acute exposure to smoke for the awake mouse/rat and detected induction of biomarkers of oxidative stress. These include inhibition of mitochondrial respiratory complexes and formation of oxidative DNA damage in the brain. DNA damage is likely to contribute to neuronal dysfunction and progression of brain injury. In search for strategies to attenuate the smoke initiated brain injury, we produced a transgenic mouse overexpressing the neuronal globin protein, neuroglobin. Neuroglobin was found neuroprotective in diverse models of ischemic/hypoxic/toxic brain injuries. Here, we report lesser inhibition of respiratory complex I and reduced formation of smoke-induced DNA damage in neuroglobin transgene when compared to the wild-type mouse brain. DNA damage was assessed using the standard comet assay, as well as a modified comet assay done in conjunction with an enzyme, which excises oxidized guanines that form readily under conditions of oxidative stress. Both comet assays revealed that overexpressed neuroglobin attenuates the formation of oxidative DNA damage, in vivo, in the brain. These findings suggest that elevated neuroglobin exerts neuroprotection in part, by decreasing the impact of acute smoke inhalation on integrity of neuronal DNA.

Keywords

brain; comet assay; neuroglobin; oxidative DNA damage; smoke inhalation; transgenic mouse

INTRODUCTION

In civilian and military scenarios, acute inhalation of combustion smoke causes mortality and morbidity, with immediate and delayed neurological deficits in survivors [1–5]. The major toxicants in combustion-smoke including, carbon monoxide, noxious gases, organic irritants, free radical-generators and hypoxia, combine and synergize to impede oxygenation, disrupt energy metabolism and initiate brain injury. Molecular mechanisms responsible for delayed neurological sequelae following acute inhalation of smoke, however, are not well

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defined, slowing development of targeted intervention strategies. To capture real life complexities of smoke exposures and help understand progression of smoke injury, we developed an awake mouse/rat model of combustion-smoke inhalation, and characterized early and delayed molecular manifestations of smoke inhalation in the brain including, lipid peroxidation, protein nitration and oxidative DNA damage [6–8].

Since neuroglobin (Ngb), a recently discovered oxygen-binding heme protein [9], was found neuroprotective in hypoxic/ischemic/noxious injuries via improved oxygen delivery and neutralization of free radicals [10–13], we asked whether it might also protect targets, which are adversely affected by exposure to combustion smoke. To assess the value of elevated neuroglobin in the context of smoke exposure, a transgenic mouse overexpressing neuroglobin was produced. To ascertain physiologically relevant expression, neuroglobin gene was placed under the control of synapsin I promoter, which limits transcription to neuronal cells. Our goal was to identify among the many molecular targets, which are adversely affected by smoke, those that might be protected by elevated neuroglobin.

In this current study we compared the extent of smoke-induced inhibition of respiratory complex I and formation of DNA damage in the wild-type and transgenic mouse brain. Complex I activity was measured spectrophotometrically and formation of DNA damage was assessed using alkaline comet assays, which support detection of DNA damage at the single-cell level [14, 15]. Overexpressed neuroglobin appreciably attenuated the smoke-induced oxidation of guanines and formation of strand breaks in the transgene compared to wild-type mouse brain. Thus, neuroglobin transgene (Ngb-tg) has potential to mitigate severity of brain injury by decreasing the initial impact of insults on integrity of neuronal DNA.

MATERIALS AND METHODS

Cloning rat neuroglobin cDNA and construction of expression vector for production of transgenic mouse

Rat neuroglobin cDNA was cloned by RT-PCR using rat cerebellum poly(A⁺) RNA. First strand cDNA was synthesized (GeneRacer, Invitrogen); primers based on homology with mouse and human Ngb cDNAs were used to amplify an internal segment of rat cDNA. Based on the rat sequence, primers for 5' and 3' RACE were designed were designed to generate the ends and subsequently, the full-length 1553 nucleotides rat Ngb cDNA was cloned (GeneBank submission accession # AY066001). The coding region of Ngb (209–662 nt) translates into 151 amino acids which share 96% identity with mouse and 94% with human Ngb, while the coding region shares 87% nucleotide identity with human and 96% with mouse sequence. Rat Ngb fragment spanning 209–933 nucleotides corresponding to ATG through 250 nucleotides 3' to the stop codon was amplified (primers: 5'- TTC TAC AAA GCT TAT GGA GCG CCT AGA GTC AG –3' and 5'- AAA GCC TAG ATC TGA AGA ACC CCC ATG CCT CC –3'), digested and ligated into a pCMV vector (Sigma, St. Louis, MO). pCMV-Ngb was cut with XbaI/NcoI and ligated to the pGL3-Basic-vector (Promega, Madison, WI) to generate the pGL3-Basic-CMV-Ngb expression vector; expression of Ngb was verified by detection of the protein in transfected HEK293 cells. The construct was digested with NheI and ligated to NheI-digested pSYNN plasmid, carrying the synapsin I rat promoter [16]. The PSYNN plasmid was a generous gift from Dr. Manfred W. Kilimann. The resultant synapsin I-neuroglobin fusion construct pSyn-Ngb was used for production of the neuroglobin-transgenic (Ngb-tg) mouse.

Production of the Ngb-tg mouse

Ngb-tg mice were produced through the UTMB transgenic core using a standard protocol [17]. All procedures were conducted in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committee. Briefly, the pSyn-Ngb construct was digested to release the 5.4 kb fragment containing synapsin I promoter (4.3 kb) ligated to neuroglobin coding sequence; the 5.4 kb fragment was separated by gel electrophoresis and purified ahead of microinjection into the male pronucleus of fertilized eggs of C57BL/6 $\times$ C3H/He mice; microinjected embryos were incubated overnight to the two-cell stage and then transferred into foster mothers as previously described [18].

Progenies were screened using tail DNA PCR with a forward primer located within the rat synapsin I promoter and a reverse primer within the Ngb cDNA sequence, 5'- CGG TGA GTC CAG TCG GGC -3' and 5'- CCC CGT CCC AGC CTC G-3', respectively, yielding a PCR product, which is unique to the transgene (Fig 1). Ngb positive mice were mated with wild-type mice and positive litters were identified by tail screening; positive littermates were screened for neuroglobin RNA and protein expression levels. A founder mouse was selected and subsequent positive progeny was identified by PCR screening and back-crossed to C57BL/6 wild-type mice for 6 generations with continued screening. Mendelian transmission pattern was observed. A homozygous line was established and used in subsequent experiments.

Northern blot analysis

Ngb mRNA levels in tissues were evaluated by Northern blotting as we described [19]. Total RNA was prepared and 40-microgram samples were resolved in 1% agarose gel. Ngb coding sequence (AY066001) amplified by 5' - GGAGCGCCTAGAGTCAGAGCTG-3' forward and 5'-CCCCGTCCCAGCCTCG-3' reverse primer was used as probe.

Western blot and immunohistochemical analyses

Protein extracts from cerebra of wild-type and transgenic mice were resolved in 15% acrylamide SDS gel and probed with anti neuroglobin chicken antibody (cat# RD181043050, Biovendor). Uniform loading was confirmed by reprobing for actin (Sigma, St. Louis, MO) and differential expression was demonstrated by probing for the mitochondrial enzyme F0-F1 ATPase (Mitosciences, OR). For immunohistochemical analyses brains were immersion fixed in 4% paraformaldehyde, paraffin embedded and sagittal sections through the midline were prepared from wild-type and Ngb-tg mice. Sections were dewaxed in xylene and rehydrated through graded ethanol series. Blocking was for 1 h with 1% BSA followed by 30 min with 3% goat serum. Sections were incubated with the anti neuroglobin chicken antibody at 1:1200 for 1 h at room temperature, followed by three 5-min washes and incubation with biotinylated goat anti-chicken antibody; the avidin:biotinylated enzyme complex (ABC reagent) was used, developed with diaminobenzamine (Dako, CA) as the chromagen and lightly counter-stained with hematoxylin. Hematoxylin Gill #2 (#S401-1D) and Permount (SP15-100) were from Fisher Scientific (Hampton, NH). Sections were observed with a Nikon Eclipse 600 microscope. Images were captured by Nikon DXM1200 digital camera with ACT-1 software.

Mouse smoke-inhalation model

The combustion smoke-inhalation model, which we initially developed for the rat [6–8], was adapted for the mouse (C57BL/6). All procedures were conducted in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committee. Briefly, awake male mice (25–30 gram) were exposed to combustion smoke generated by smoldering wood shavings in a smoke generating container connected

to a 20-liter transparent exposure chamber. Mice were subjected to successive 5-min periods of smoke separated by ~10-sec venting to ambient air for the total duration of 60 min. Survival rate was $\geq 95\%$. Blood was collected from the jugular vein [20]. Carboxyhemoglobin (COHb) and oxygen saturation (O_2 Sat) were measured with oximeter (482 CO-Oximeter) and gas tensions, pH and base excess (BE) with System 1302 (Instrumentation Laboratory, Lexington, MA). Readings were obtained for controls and mice after inhalation of smoke, at 0 h (immediately) and 2 h after exposure (Table 1). The pattern of hemodynamic changes agreed with that previously obtained for the rat [6] and the wood smoke inhalation model reported for anesthetized mice [21].

Isolation of brain cells for comet assay

At the indicated recovery times after exposure to smoke, mice were euthanized by cervical dislocation; brains swiftly collected and cerebra sagittally dissected through the midline. Half of each cerebrum was immediately processed to isolate cells for the comet assay while remaining tissue was snap frozen for storage at -80°C . Cell isolation protocol was based on procedures described by Brewer et al. [22–24] with modifications. Briefly, half cerebrum was homogenized in 1 ml PBS by eight gentle strokes of loose pestle. Homogenates were gently washed in PBS and collected by centrifugation at 200 g. Pellets were incubated 35 min at 37°C with 0.125% Trypsin (Invitrogen; Carlsbad, CA) and 50 $\mu\text{g/ml}$ DNase I (Cat. #10104159001, Roche Applied Science). Treatment was terminated by incubation with Soybean Trypsin Inhibitor (Cat. #T-9128, Sigma) in 10% fetal bovine serum (Thermo Scientific, Waltham, MA). Homogenates were gently triturated on ice and resultant suspension passed through a 70 μm nylon mesh strainer (BD Biosciences; Bedford, MA). Cells in suspension were washed in PBS with 20% FBS. Trypan blue exclusion served to monitor membrane integrity; cells were counted using hemocytometer and adjusted to 10^6 ml^{-1} for immediate processing for the comet assay.

Alkaline Comet assay

The comet assay was carried out as we described previously [25] with modifications. Briefly, cells isolated from cerebrum were counted, mixed with low melting agarose, spread onto multiple slides for each time point (2×10^4 cells per slide), and incubated 16 h at 4°C in alkaline cell lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris pH 10 [set with NaOH], 1% Triton X-100). Throughout the procedure slides were sheltered from light. To facilitate detection of oxidized guanines in addition to DNA strand breaks, slides were incubated with *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) prior to electrophoresis. Fpg readily cleaves 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and converts resultant abasic sites into strand breaks, which are detectable by the alkaline comet assay. Accordingly, duplicate slide sets were used: all slides were equilibrated with the Fpg reaction buffer (10 mM HEPES-KOH, 100 mM KCl, 0.1 mg/ml BSA, pH 7.4) and then incubated either with or without Fpg (cat#4040-100-01, Gaithersburg, Trevigen, MD). Subsequently slides were incubated 40 min in alkaline buffer (0.3 M NaOH, 1 mM EDTA), electrophoresed at 1.2 V/cm (300 mA, 25 V), washed 3 times in neutralization buffer (0.4 M Tris, pH 7.5) and stained with 20 $\mu\text{g/ml}$ ethidium bromide. Slides were viewed using 20 $\times$ objective on IX71 Olympus fluorescence microscope and images of nuclei captured with QIC-F-M-12-C cooled digital camera. Comet tail moments for individual nuclei were determined using the CASP comet analysis software [26] at www.casp.of.pl. Tail moments were determined for nuclei of wild-type and Ngb-tg non-treated mice, and mice at 0, 2, 4, 6 and 24 h recovery after exposure to smoke. Duplicate slides for each experimental set were processed in parallel with and without Fpg digest. At least 30 nuclei for either combination (ie greater than 60 nuclei) were analyzed per mouse ($n=5$). Values are expressed as means $\pm$ SEM. Differences in tail moments for treatment groups versus their respective controls were determined using the Student's t-test and a value of $P \leq 0.05$ was considered significant.

Isolation of brain mitochondria and measurement of Complex I activity

Mitochondrial pellets were prepared according to Sims and Anderson [27] with modifications [28]. Cerebra (~100 mg) were delicately cut and homogenized by 4 strokes of loose pestle in 0.65 ml ice-cold mannitol-sucrose-EDTA (MSE) isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.1 mM EDTA; pH 7.5, fatty acid free BSA 5 mg/ml), followed by 15 strokes of tight-fitting pestle, and centrifuged at 4°C for 8 min at 2000 g to pellet nuclei and debris; pellets were washed with MSE buffer and supernatants centrifuged for 12 min at 15,000 g at 4°C to obtain crude mitochondrial pellets. Mitochondrial pellets were washed twice with 0.25 M sucrose and pelleted at 8,000 g; pellets were re-suspended in small volumes of MSE buffer. Protein concentrations were determined by the Lowry method, using BSA as standard. Activity of complex I was measured spectrophotometrically using Beckman DU 650 spectrophotometer (Fullerton, CA). Assays were done as described [29, 30] with some modifications [28]. Mitochondria from cerebra of wild type and Ngb-tg controls and mice sacrificed at specified recovery times after smoke exposures were analyzed (n=5). Complex I (NADH: ubiquinone oxidoreductase) activity was measured as a decrease in absorbance following the oxidation of NADH at 340 nm. Prior to measurements, mitochondria (40 µg) were frozen/thawed 3-times and preincubated (5 min 30°C) with 25 mM potassium phosphate pH 7.4, 0.1 mM ubiquinone₁, 5 mM KCN, 5 µg antimycin A. The reaction was initiated by addition of 0.2 mM NADH; decrease in absorbance was measured for 90 sec. Activity was calculated using an extinction coefficient of 6.22 mM⁻¹cm⁻¹. Assays were done in duplicate. Activities were calculated as nmol min⁻¹ mg⁻¹, expressed as mean±SEM (n=5) and presented as percent of the respective control, which was assigned the value of 100%. Differences in relative complex I activities for wild type versus Ngb-tg for each recovery group were determined using Student's t-test. * indicates different from control and † indicates that Ngb-tg complex I activity is different than that of wild type at the specified recovery time point (*P*<0.05).

RESULTS

Production of Ngb-tg mouse

To augment neuroglobin levels in neurons, transgenic mouse overexpressing neuroglobin under the control of rat synapsin 1 promoter was produced. Synapsin promoter [16] is predicted to drive robust transcription, limited to neuronal cells [31]. Overexpression of neuroglobin in neuronal tissues was confirmed by Northern and Western blotting analyses and by immunohistochemistry. Northern blotting revealed robust Ngb overexpression in cerebrum and cerebellum, but not in the liver or pancreas of the Ngb-tg mouse (Fig 2). Synapsin promoter driven transcription, was significantly higher in the adult compared to the Ngb-tg pup (Fig 2, lanes 5–6 vs. 13–14) and embryonic E16 brain (lane 10). Very low endogenous expression was detected in wild-type cerebrum and cerebellum after a prolonged exposure (not shown). Western blotting analysis of cerebral extracts revealed robust levels of the Ngb protein in the transgene, with a higher level in the homozygote compared to heterozygote mouse cerebrum (Fig 3, lane 5 vs 2–4). As expected based on developmentally regulated expression of synapsin, higher Ngb levels were observed in the adult compared to pup brain (Fig 3, lanes 5 vs. 6). Endogenous neuroglobin was below detection limit of standard Western analysis (Fig 3, lanes 1, 7). This is expected, as in the striatum, for example, Ngb positive neurons represent less than 0.05% of total neuronal population [32]. Here, analyses revealed narrowly localized, weak neuroglobin immunoreactivity in the wild-type mouse brain (Fig 4A). This is also expected based on *in situ* hybridization analyses from Allen Institute for Brain Science [33], which reveal narrowly distributed Ngb mRNA expression pattern in the mouse brain. To compare endogenous and transgenic Ngb expression, we focused on the mid brain region, where narrowly localized, yet significant endogenous expression of Ngb protein has been reported

[34]. In line with that report, in the current study endogenous Ngb immunoreactivity was detected only in some mid brain neurons (Fig 4B), which is also consistent with earlier reports on neuron-type specific expression of Ngb [35, 36]. In contrast, markedly stronger and broadly distributed immunoreactivity was detected in this region in the Ngb-tg brain (Fig 4B) consistent with the broad neuronal expression pattern of synapsin 1.

Differential inhibition of respiratory Complex I in the wild type and Ngb-tg brain

We reported previously that activities of mitochondrial respiratory complexes were significantly decreased following acute exposure to combustion smoke [28]. Here, we compared the extent of smoke induced inhibition of complex I in the wild type and Ngb-transgenic mouse brain. Complex I activities were measured for controls and at 0, 2, 6 and 24 h recovery after exposure to smoke. A significantly lesser inhibition of respiratory complex I activity was measured in the Ngb-tg when compared to wild type mouse brain at 0, 2 and 6 h but not at 24 h recovery after smoke (Table 2).

Smoke-induced DNA damage: guanine oxidation and strand break formation

To compare formation of smoke-induced DNA damage in the wild-type and Ngb-tg mouse brain, mice were exposed to combustion smoke, generated by smoldering wood shavings as we have described [6, 8]. After exposure, mice were sacrificed either immediately (0 h) or let recover for 2, 4, 6 and 24 h. Brains were processed for immediate isolation of intact cells from brain tissue (see methods) for analysis of DNA integrity by alkaline comet assays. Standard alkaline comet assay as well as a modified assay, which involves pre-treatment of nuclei with the DNA repair enzyme, Fpg, were employed. Fpg excises oxidized guanines from DNA and its lyase activity converts resultant abasic sites into strand breaks, which are then registered by the comet assay. Both assays revealed oxidative DNA damage in the brain following acute exposure to combustion smoke. Importantly, however, the extent of damage was greater in the wild type when compared to the Ngb-tg brain: the standard comet revealed higher levels of strand breaks after smoke in the wild-type brain (Fig 5A). The Fpg digest-involving assay revealed presence of oxidized guanines (Fig 5B). In the wild-type brain, 8-oxoguanine levels peaked immediately after the 60-min exposure to smoke and were significantly greater when compared to Ngb-tg as reflected in formation of comets (Fig 5C). A gradual decline in 8-oxoguanine levels was seen in the course of recovery. Interestingly, strand break formation lagged behind formation of 8-oxodG, and peaked by 2–4 h after smoke, when 8-oxodG levels began to decline. Hence, formation of strand breaks may reflect to some extent the base excision repair process initiated by DNA glycosylases. As expected, strand break levels detectable by the standard alkaline comet assay, were several fold lower than those detectable after Fpg treatment. This is consistent with the capacity of the standard comet assay to directly detect strand breaks but not oxidatively modified DNA bases. Presence of oxidized guanines, which are readily formed under oxidative stress, is revealed by preincubation with the Fpg enzyme.

DISCUSSION

In this study, we report that after acute exposure to smoke, transgenic overexpression of neuroglobin attenuates inhibition of mitochondrial complex I and lessens formation of genomic DNA damage in the mouse brain. Because earlier studies linked inhibition of mitochondrial complex I, to formation of genomic DNA damage [37, 38], it is plausible that in this experimental model of smoke, inhibition of respiratory complex I contributes to generation of genomic DNA damage. We show that transgenic overexpression of Ngb attenuates the smoke-induced inhibition of complex I activity and propose that the lesser extent of complex I inhibition, accounts in part, for reduced formation of oxidative DNA damage in the Ngb-tg mouse brain.

Although neuroglobin, the new member of the globin superfamily has been implicated in diverse modes of neuroprotection, its physiological function and endogenous ligand remain to be elucidated [13]. Initially, neuroglobin was thought to be primarily involved in oxygen transport and temporary storage [39]. Several reports had suggested this role based on its hexacoordinated heme and low dissociation rate for oxygen, reflecting high intrinsic affinity, which could prevent oxygen release under normoxic conditions and facilitate its release in hypoxic environment [39, 40]. Furthermore, the hexacoordinated heme structure and competition with distal histidine prevent rapid oxygen rebinding, which might augment tissue oxygenation and consequently oxygen availability for mitochondrial respiration [41]. In addition, because acidity decreases exogenous ligand binding to pentacoordinated hemes, heme hexacoordination might be advantageous [42] and of biological significance in the context of ischemic/hypoxic conditions that are accompanied by acidosis [43]. Interestingly, Ngb affinity for oxygen increases with declining pH [44].

Similarly to other globins, Ngb has been implicated in detoxification of reactive oxygen and nitrogen species [10, 45]. In addition, involvement of Ngb in G-protein signaling [46], in shifting the threshold of apoptosis in favor of cellular survival via reduction of ferric cytochrome c [47] and in preventing apoptosis via mechanisms independent of cytochrome c reduction [48], have also been proposed. Interestingly, a recent study reported that Ngb helps preserve ATP production in vitro in a neuronal cell line [49]. Most ATP production in respiring cells occurs in mitochondria and is crucial in neurons in view of their high energy demands and limited glycolytic capacity [50, 51]. Since we found that activities of mitochondrial respiratory complexes in the rat brain are markedly reduced by acute exposure to combustion smoke [28], it was also of much interest to determine to what extent overexpression of Ngb might protect respiratory complexes in a setting of smoke. Notwithstanding, since acute exposure to smoke generates broad spectrum of adverse effects [6–8], the overall objective was to identify among the endpoints examined, targets which might be differentially protected in the Ngb-tg brain. To this end, we produced the Ngb transgenic mouse with overexpression limited to physiologically relevant neuronal cells, and assessed elevated Ngb in a setting of acute exposure to combustion smoke.

We have previously proposed that the smoke-induced formation and incomplete repair of oxidative DNA damage, in vivo, in the brain contributes to development of progressive neuronal injury/dysfunction [7]. Here, we compared the integrity of genomic DNA in the Ngb-tg and wild-type mouse brain, after acute exposure to combustion smoke. Quantitative alkaline comet assay revealed that smoke-induced formation of oxidative DNA adducts was significantly attenuated in the Ngb-tg mouse brain. This is important, because under conditions of excessive oxidative stress, neuronal capacity for DNA repair might become overwhelmed by an overload of complex oxidative adducts and fail to maintain the integrity of neuronal DNA [52, 53]. Because in the long term, ill-repaired DNA is likely to compromise neuronal function, an initial reduction in formation of DNA damage in the Ngb-tg brain, might effectively reduce the risk for subsequent accumulation of damaged/ill-repaired DNA and thereby, the risk of delayed neuronal dysfunction after acute inhalation of combustion smoke.

Importantly, a new clinical study revealed that a two nucleotide polymorphism in the Ngb gene correlates with worse outcomes of traumatic brain injury (TBI) in a cohort of 196 patients [54], which might be associated with a decreased capacity for sustenance of energy metabolism after TBI in surviving patients. Thus, we expect that availability of our new transgenic mouse with neuron-targeted overexpression of neuroglobin, will advance the ability to dissect and potentially constrain molecular mechanisms by which acute exposure to smoke promotes the development of neuronal injury.

Highlights

- Neuroglobin (Ngb) a heme protein is protective in diverse models of brain injury.
- A new transgenic mouse with neuron-specific overexpression of Ngb was produced.
- Neuroprotection by Ngb was assessed in a model of acute smoke inhalation.
- Overexpressed Ngb attenuated smoke-induced oxidative DNA damage in the mouse brain.

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Figure 1.

PCR Screening of tail DNA: Screening a batch of 19 pups tails revealed one positive (#18) for the fused Syn-Ngb sequence. PCR reaction set with the Syn-Ngb construct DNA served as positive control. Number #18 was the founder of transgenic line that afforded robust expression and consistent transmission and was used for subsequent studies.

Figure 2.

Northern blot analysis of Ngf mRNA levels in Ngf transgene compared to the wild-type mouse. Strong overexpression of Ngf transcript was detected in cerebrum and cerebellum but not in the liver or pancreas of the Ngf-tg (lanes 5, 6 vs. 7, 8). Lower levels of the Ngf transcript were detected in Ngf-tg embryonic brain (E16), as well as in pup (P1) cerebrum and cerebellum (lanes 13, 14). Ribosomal RNA is visualized by EtBr (lower panel) and serves as loading and quality control. Note, somewhat lesser quality of pancreas RNA (lanes 4 and 8).

Figure 3.

Western blotting analysis of transgenic neuroglobin expression: Wild type (+/+), Ngb-tg heterozygote (+/tg) and homozygote (tg/tg), adult and pup cerebral protein extracts were examined (40 μ g). Overexpressed neuroglobin protein migrating at apparent molecular weight of ~17 kDa is indicated. Differential expression of the mitochondrial protein F0-F1 ATPase, is consistent with developmentally regulated expression. Reprobing for actin served as a loading control; higher actin levels are seen in P1 pup compared to adult brain.

Figure 4.

Neuroglobin immunoreactivity in the mouse brain. Images of sagittal midline sections of the mouse brain are shown (4× objective). A) Localized weak staining (brown) is observed in selected areas, including hypothalamus and mid brain in the wild-type mouse, compared to broad distribution and strong staining observed in the transgene. Major brain structures are visualized by lightly counterstaining of nuclei with hematoxylin (blue). Frontal cortex region shows edge effect and does not reflect specific Ngb-tg immunoreactivity. B) Higher magnification images (20× objective) of the mid brain show individual neurons with cytoplasmic Ngb immunoreactivity (left), compared to significantly stronger Ngb immunoreactivity in the same region in the transgenic mouse brain (right).

Figure 5.

Comet assay reveals attenuated formation of smoke-induced DNA damage in the Ngb-tg compared to wild-type mouse brain. The extent of DNA damage was quantified and expressed as comet tail moment: A) Standard comet assay revealed smoke-induced formation of strand breaks in the WT brain (blank bars). In contrast, the increase in smoke-induced strand break levels (solid bars) did not reach statistical significance in the Ngb-tg brain. B) Comet assay carried out in conjunction with the Fpg enzyme, which excises oxidized guanines and generates strand breaks, revealed lesser guanine oxidation in the Ngb-tg when compared to wild-type mouse brain. Tail moments are expressed in arbitrary units and presented as mean $\pm$ SEM for controls and for specified recovery time points (n=5). Tail

moment value of zero corresponds to intact round nuclei. * indicates significantly different tail moment versus the respective control ($P<0.05$). C) Representative images of comet assays carried out in conjunction with the Fpg enzyme: cells isolated from controls and from mice immediately after exposure to smoke (0-h), or mice recovered for 2 or 4 h, are shown. Nuclei from control mice without Fpg treatment are shown as reference for intact cell nuclei (left panel). Images reveal more compact nuclei for Ngb-tg, indicating that Ngb-tg brains were less affected compared to wild type after exposure to smoke.

Table 1**Smoke Inhalation-Induced Temporal Changes in Mouse Hemodynamics**

Parameter	Control	0 h Post Smoke	2 h Post Smoke
COHb (%)	5.2±0.4	62±7.7*	10±6.7
PvO ₂ (mm Hg)	59±6.2	11.0±2.8*	29±5.5*
O ₂ Sat (%)	80±3.7	29.5±6.5*	72.4±6.6
PvCO ₂ (mm Hg)	40±3.8	55.8±4.1*	48.2±13.2
BE (mEq/L)	-5±2.7	-15.2.1±3.0*	-9.1±3.9
pH	7.32±0.04	7.05±0.05*	7.21±0.07

The mean±SD is shown;

* indicates that the mean is significantly ($P<0.05$) different from control mean (n=6).

BE, base excess; COHb, carboxyhemoglobin.

Table 2

Differential inhibition of complex I in wild type and Ngb-tg brain mitochondria after exposure to smoke.

Complex I % of control activity	Time after smoke exposure			
	0 hours	2 hours	6 hours	24 hours
Wild type	78.2±4.4 *	55.2±6.3 *	68.6±7.1 *	82.5±8.1 *
Ngb-tg	92.5±7.2 †	76.7±7.4 *†	83.3±6.5 *†	80.5±9.3 *

The mean±SEM is shown (n=5);

* indicates different from respective control;

† indicates different from wild type at specified time point ($P<0.05$).