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Acute Inhalation of Combustion Smoke Triggers Neuroinflammation and Persistent Anxiety-Like Behavior in the Mouse

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Abstract

Context—Acute inhalation of combustion smoke triggers neurologic sequelae in survivors. Due to challenges posed by heterogeneity of smoke exposures in humans, mechanistic links between acute smoke inhalation and neuropathologic sequelae have not been systematically investigated.

Methods—Here, using mouse model of acute inhalation of combustion smoke, we studied longitudinal neurobehavioral manifestations of smoke exposures and molecular/cellular changes in the mouse brain.

Results—Immunohistochemical analyses at eight months post smoke, revealed hippocampal astrogliosis and microgliosis accompanied by reduced myelination. Elevated expression of proinflammatory cytokines was also detected. Longitudinal testing in different neurobehavioral paradigms in the course of post smoke recovery, revealed lasting anxiety-like behavior. The examined paradigms included the open field exploration/anxiety testing at two, four and six months post smoke, which detected decreases in total distance traveled and time spent in the central arena in the smoke exposed compared to sham control mice, suggestive of dampened exploratory activity and increased anxiety-like behavior. In agreement with reduced open field activity, cued fear conditioning test revealed increased freezing in response to conditioned auditory stimulus in mice after acute smoke inhalation. Similarly, elevated plus maze testing demonstrated lesser presence in open arms of the maze, consistent with anxiety-like behavior, for the post-smoke exposure mice.

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DECLARATION OF INTEREST:

The authors declare that there are no conflicts of interest

Conclusions—Taken together, our data demonstrate for the first time persistent neurobehavioral manifestations of acute inhalation of combustion smoke and provide new insights into long-term progression of events initiated by disrupted brain oxygenation that might contribute to lasting adverse sequelae in survivors of smoke inhalation injuries.

Keywords

smoke inhalation injury; brain; neuroinflammation; myelination; longitudinal behavioral testing; anxiety-like behavior

INTRODUCTION

Emerging evidence suggests that disruptions of brain energy metabolism such as occur in ischemia and reperfusion, traumatic brain injuries and other brain insults trigger the development of delayed manifestations, which include neuroinflammation, neurodegeneration and neurologic disorders that often can be monitored through temporal appearance of biomarkers in cerebrospinal fluid and blood (Zhang Z et al. 2011; Papa and Wang 2017). Disruption of brain homeostasis and energy crisis occurs also in acute inhalation of combustion smoke leading to early as well as delayed neurological sequelae in survivors (Zikria et al. 1975; Hartzell 1996; Rossi et al. 1996; Smith et al. 1996; Alarie 2002; Stuhmiller et al. 2006). The injurious components of smoke include carbon monoxide, hypoxia, hydrogen cyanide and mixtures of other toxic gases and volatile organic compounds, which synergize to perturb energy metabolism and initiate progression of events that precipitate brain injury (Baud et al. 2011; Dries and Endorf 2013; Geldner et al. 2013). However, due to complexity and very few systematic investigations, the actual progression of events that contribute to development of neurological sequelae following acute inhalation of smoke remains understudied when compared for example, to incidents of carbon monoxide poisoning that are driven by failure of brain oxygenation (Weaver 2009; Huang et al. 2014; Mizuno et al. 2014; Pages et al. 2014; Pang et al. 2014; Tsai et al. 2014; Yeh et al. 2014). To bridge this gap of knowledge and delineate the progression of changes triggered by acute exposure to combustion smoke, we have developed a long term-survival model of acute smoke inhalation for the awake rodent (Lee et al. 2005; Chen L et al. 2007; Lee et al. 2009; Lee et al. 2010; Lee et al. 2011). Our previous studies that focused on the early recovery times after smoke, revealed severe disruptions of energy homeostasis in the brain, which include mitochondrial perturbations, reduced ATP and formation of oxidative DNA damage (Chen L et al. 2007; Lee et al. 2009; Lee et al. 2010), while studies by others documented induction of inflammatory processes in the rat brain by smoke exposures (Zou et al. 2013; Zou et al. 2014). In this current study, we extended the timeline of investigation to characterize long-term cellular/molecular and morphologic manifestations of smoke inhalation in the mouse brain and longitudinally monitored the smoke exposed mice for neurobehavioral manifestations. We found that smoke inhalation leads to chronic neuroinflammation and compromised myelination in the brain, as well as persistent anxiety-like behavior.

METHODS

Mouse model of fire smoke inhalation - experimental design

CB57BL/6 mice were purchased (ENVIGO RMS, Inc) and housed in the Animal Resource Center Facilities at the University of Texas Medical Branch (UTMB). UTMB Animal Resource Center Facilities operate in compliance with the USDA Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, under OLAW accreditation, and IACUC approved protocols. All procedures were conducted in accordance with mandated standards of humane care and were approved by the UTMB Animal Care and Use Committee. We have previously developed a survival model of acute inhalation of wood smoke for the un-anesthetized rodent (Lee et al. 2005; Chen L et al. 2007; Lee et al. 2009; Lee et al. 2010; Lee et al. 2011; Gorgun FM et al. 2014). Briefly, smoke was generated by smoldering aspen wood shavings (1.3 gram/min) and dispersed by a tube-mounted fan into connected 20-liter transparent mouse exposure chamber. Smoke composition in the exposure chamber was determined (Lee et al. 2010). Measurements revealed 3,500 ppm carbon monoxide (0.35%), 11,500 ppm (1.15%) carbon dioxide and 14.5% oxygen, while volatile organic compounds included, 57 ppm propene, 15 ppm chloromethane, 10 ppm 1,3-butadiene, 11 ppm 2-butanone, 7 ppm benzene and several other organic compounds at lower concentrations, as we previously reported (Lee et al. 2010). Un-anesthetized CB57BL/6 male mice (24-27 gram) were exposed to smoke for 60 min with 10–20 seconds venting to ambient air at approximately 10 min intervals. Mice that were handled identically, except for the omission of smoke, served as sham-controls (n=18). Survival rate in the course of exposure and within the first 48-hour recovery was > 95%. Hemodynamic parameters measured in venous blood were 5% carboxyhemoglobin in controls and 62% carboxyhemoglobin immediately following smoke exposure, with a drop to 11% after 2-hour recovery, indicative of efficient clearance of carbon monoxide, as we reported previously (Lee et al. 2011). Similarly, oxygen saturation in blood, which was reduced to 29% immediately after exposure (compared to 80% oxygen saturation in controls), was restored to 72% after 2-hour recovery (Lee et al. 2011). In the course of 2-hour recovery following transfer to home cages, mice huddled together, with return to normal feeding, drinking and grooming behavior thereafter. Subsequently, mice were maintained for eight months with weekly inspections of general health, weight and sensory perceptiveness. Apparent impairments or differences between the groups were not detected throughout the duration of study.

Neurobehavioral testing

All neurobehavioral testing was carried out at the UTMB Rodent In Vivo Assessment Core Facility (directed by Dr. Dineley) using standard validated protocols as we previously described (Dineley et al. 2002; Dineley et al. 2007; Taglialatela et al. 2009). Timeline of neurobehavioral testing procedures is shown in Figure 1. Over the course of the 8-month study, one smoke-exposed and two sham-control mice died, which is reflected in a lower number of mice tested in the open field paradigm at the four (n=14) and six (n=12) months post smoke time points, as described below. Rotating rod testing was restricted to a subset of randomly assigned eight mice from each group, so that all testing was completed within the 12 hr light cycle (06:00–18:00 hr) for return to home cages prior to the 12 hr dark cycle.

Open Field

The Open Field test serves to assess baseline locomotor and exploratory activity as well as an initial screen for anxiety like behavior (Russell 1973; Graham et al. 2002; Bailey and Crawley 2009; Taglialatela et al. 2009). Briefly, mice were transported to the test room approximately 60 min for acclimation in advance of testing. Testing was done using a 38×38 cm opaque wall chamber. Four identical open field boxes were recorded simultaneously using TopScan (Clever Sys. Inc) software. This software allows delineation of the chamber's central (12.4×12.4 cm) and peripheral arenas. Each mouse was placed gently in the arena center and movement was recorded for 10 min using the TopScan video tracking system with automated analysis software, which quantifies locomotor parameters determining the total distance traveled and travel speed in the arena center and in periphery. Between tests, chambers were cleaned with 70% ethanol to remove any substances or scents that might affect the next cohort (Crawley and Paylor 1997). Longitudinal testing was done at two-month intervals to avoid unwanted effects of habituation (Bailey and Crawley 2009). Open field performance parameters of smoke exposed versus sham control group was analyzed by repeated measures ANOVA followed by Sidak's multiple comparisons test.

Rotating rod (Rota-rod)

Accelerating rotating rod test was used to assess balance and motor coordination as well as motor skill acquisition as we previously described (Dineley et al. 2002). The rotarod (Dual Species Economex Rota-Rod, Columbus Instruments) speed accelerated from 4 to 40 rpm over the course of 5-min trial period. The time to fall was recorded and improvement was observed upon task repetition. Performance criteria screen was set as 60 sec at 4 rpm achieved following a 30-sec training period. Mice were then subjected to four trials per day for three consecutive days with a 45 min rest interval between trials (Dineley et al. 2002; Graham et al. 2002). Rotarod testing was done five months post smoke; latency to fall was recorded and performance of smoke exposed versus sham control groups was analyzed by repeated measures ANOVA followed by Sidak's multiple comparisons test.

Cued Fear Conditioning

Six and a half months post smoke exposure mice were tested in the cued fear paradigm (n=10). Each mouse was placed in the fear conditioning device and given two pairings of conditioned stimulus (auditory cue) and an aversive unconditioned stimulus (electric mild foot shock) (Phillips and LeDoux 1992; LeDoux 2000; Maren 2001). The training was using standard fear conditioning equipment (Med Associates) for a total of 7 min following our standard fear-conditioning protocol (Dineley et al. 2002; Rodriguez-Rivera et al. 2011). Briefly, mice were let explore the chamber for the first 3 min, then a 30-sec auditory-conditioned stimulus (CS, white noise, 80 db) was delivered (McShane et al. 2006)). Prior to the end of the conditioned stimulus, a 2-sec electric shock (0.5 mA) was applied to the grid floor (unconditioned stimulus - US). The CS-US pairing was given again at the 5-min mark and at the 7-min mark, mice were removed and returned to home cages. Cued-fear learning was tested 30 hrs after training, by placing the mice in a different context (novel odor, lighting, cage floor and visual cues). Baseline behavior was recorded for 3 min, and then the CS was delivered for 3 min. The Actimetrics Freeze Frame video capture software and

analysis program (Coulbourn Instruments) was used for recording and analysis. Data were expressed as percent freezing time (defined as absence of movement except for movement required for respiration and heartbeat) within 12 consecutive 30 sec epochs (Dineley et al. 2002; Dineley et al. 2007; Shoji et al. 2014).

Elevated Plus Maze (EPM)

EPM testing was used to assess anxiety-like behavior in the smoke exposed and sham control mice (n=10), at seven and a half months post smoke exposure. The EPM includes a center platform branching into two open and two enclosed arms (30 cm long $\times$ 6 cm wide with 13 cm high walls for the enclosed arms), which is elevated 50 cm above floor level (Komada et al. 2008; Cortez et al. 2017; Lei et al. 2017). Each tested mouse was gently placed on the center platform facing an open arm and let explore the maze for 5 min. The distance, time, and speed parameters for each arm and center platform were calculated using the animal Clever Syn TopScan digital video-camera recording data capture and behavior analysis software.

Tissue collection

Eight months after smoke exposure and completion of behavioral studies, sham-control and smoke exposed mice were sacrificed for tissue collection (n=10-12). Brains were either fixed in 10% formalin, paraffin embedded and cut to obtain 5 μ m coronal sections through Bregma -3.14 region (n=4), or immediately dissected to retrieve hippocampal, cortices and other regions of interest (n=6) and snap frozen for further analyses.

Immunohistochemistry and image analysis

Paraffin sections were dewaxed in xylene and rehydrated through graded ethanol series, steamed 25 min with antigen retrieval solution (#S1700, DAKO) and blocked for 1 hr in PBS with 3% goat serum and 1% BSA. Primary antibodies used were anti-glial fibrillary acidic protein (GFAP, #AB5804, Millipore, at 1:1000), anti-ionized calcium-binding adapter molecule 1 (Iba-1, #019-19741, Wako-Chem, at 1:400) and anti-myelin basic protein (MBP, #AB134018, Abcam, at 1:400). Slides were incubated 2 hr at RT, washed 3 $\times$ with PBS and incubated 30 min with biotinylated secondary antibody (Anti-mouse #BA9200 or anti-rabbit #BA1000, Vector Laboratories at 1:400), washed and incubated 30 min with horseradish peroxidase streptavidin (#SA5004 Vector Laboratories at 1:400). Staining was developed by 3, 3'-diaminobenzidine (DAB) solution (#K3466, DAKO), counter stained by hematoxylin (#TA060MH, ThermoScientific) and mounted with permanent mounting medium (#H5000, Vector Laboratories). Images were captured using Nikon Eclipse 600 microscope with 40 $\times$ Plan Apo objective. To quantify Iba-1 and GFAP positive cells in CA1 and Dentate Gyrus regions, ROIs in CA1 and DG were delineated and their dimensions measured using ImageJ software. Immunoreactive cells in the ROIs were counted using ImageJ (n=3). Data are expressed as mean $\pm$ SEM cell number per millimeter square. To compare myelination patterns, MBP immunoreactivity was evaluated. For quantification purposes sections were processed without counter stain as previously described (Tu et al. 2013; Hammelrath et al. 2016; Zhang H et al. 2016) and images were captured with the 40 $\times$ objective. Images were converted to gray scale, threshold intensity was set at 225 and gray scale images were transformed into binary images using the 'make binary' function of ImageJ. Black areas

were defined as positive for myelin. The CA3 region was divided into three contiguous areas for scoring and positive area percent was calculated. Eighteen areas were scored per group (n=3); data are presented as mean±SEM percent of area positive for MBP.

Real-time qPCR

Total RNA was isolated from snap frozen hippocampi and cortices using the RNeasy plus mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions and as we described (Gorgun MF et al. 2016; Zhuo et al. 2016, 2017). Total RNA (1 µg) was reverse transcribed using iScript RT supermix (Biorad) which contains random and oligo dT primers using the T100 thermal cycler (Biorad). Real-time PCR analyses were done using the CFX96 Real-Time System (Biorad). PCR reactions were assembled with SSO FAST Evagreen supermix (Bio-Rad). PCR amplification settings were 95°C 2 min, 40 cycles of 95°C 5 sec, 55°C 15 sec with plate reading and subsequent melting curve analysis using 18S ribosomal RNA gene as reference gene. Data represent the mean±SEM (n=6). The relative amount of target gene RNA was calculated according to Schmittgen and Livak (Schmittgen and Livak 2008) using the formula: $-\text{Ct} = [(\text{CT gene of interest} - \text{CT internal control}) \text{ sample} - (\text{CT gene of interest} - \text{CT internal control}) \text{ control}]$. Primer sequences are given in Table 1.

Statistical analysis

All data are reported as mean ± standard error of the mean (SEM). Student-t test or repeated measures analysis of variance (ANOVA) followed by Sidak's multiple comparison test was used to analyze differences between post-smoke and sham-control groups. Data were analyzed using Excel or GraphPad Prism software. Statistical significance was determined using a *P* value 0.05.

RESULTS

Astrogliosis, microgliosis and reduced myelin immunoreactivity persist in mouse hippocampus eight months after acute inhalation of combustion smoke

To determine whether acute inhalation of combustion smoke exerts long-term changes in the mouse brain, brains were collected eight months after smoke exposure and processed for immunohistochemical analyses. Astrogliosis and microgliosis in hippocampal regions were assessed by immunoreactivity of astrocytic glial fibrillary acidic protein (GFAP) and the microglial marker, ionized calcium binding protein (Iba-1), respectively (Xu et al. 2012; Prokop et al. 2013), whereas changes in myelination patterns were evaluated by analyzing immunoreactivity patterns of myelin basic protein (MBP), a major structural protein in myelin sheath (Schregel et al. 2012). GFAP immunoreactivity analyses revealed increases in the number of GFAP positive cells in dentate gyrus and CA1 hippocampal regions of mice maintained for eight months after exposure to smoke when compared to age matched sham-controls (Figure 2 and Table 2). Post-smoke increases in number of Iba-1 positive microglia were also detected at this post-smoke recovery time (Figure 3 and Table 2). Density patterns of myelinated fibers were evaluated by immunoreactivity of MBP (Figure 4). For quantification purposes, adjacent hippocampal sections stained for MBP were processed without hematoxylin (Figure 4A, bottom) and MBP positive staining was transformed into

binary images (Figure 4B). Quantification revealed significantly reduced density of MBP positive fibers in CA3 of mice maintained for eight months post smoke exposure when compared to age matched sham controls. Taken together, immunohistochemical analyses demonstrate that long-term inflammatory changes manifest in the mouse brain after acute exposure to combustion smoke.

Upregulation of proinflammatory gene expression in the mouse brain eight months after acute inhalation of combustion smoke

To further assess long-term proinflammatory changes in the mouse brain, expression patterns of genes associated with inflammatory and stress responses were analyzed by Real Time-qPCR. RNA extracted from hippocampi and cortices of mice maintained for eight months after smoke exposure was analyzed, with target gene selection focused on proteins associated with inflammatory processes (Waisman et al. 2015; Becher et al. 2017; Kumar et al. 2017; Yin F et al. 2017) and stress responses (Cohen et al. 2017). Elevated expression of genes encoding proteins considered markers of astrogliosis and microgliosis was observed (Figure 5). Specifically, mRNAs for glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) encoded by the AIF-1 gene (allograft inflammatory factor 1, which is located within the major histocompatibility complex class III region) were elevated. Likewise, increased expression of genes encoding the inflammatory cytokines, interleukin-1 beta (IL- β 1), interferon gamma (IFN- γ), tumor necrosis factor- α (TNF- α), the chemokine C-C motif ligand 2 (CCL2), but not IL-6 was detected. Increased expression of c-Fos gene, a key sensor of neuronal stress and of heme oxygenase 1 (HMOX1), a phase II antioxidant enzyme, was also measured. In contrast, changes in expression of genes encoding enzymes involved in energy metabolism, that were upregulated at the early time points after smoke (Lee et al. 2005; Lee et al. 2009; Lee et al. 2010), were not observed at the eight months post smoke recovery time (not shown).

Neurobehavioral changes suggestive of anxiety persist in mice after acute inhalation of combustion smoke

To characterize the extent and type of behavioral changes that mice develop following acute smoke inhalation, smoke exposed and age matched sham-control mice were subjected to longitudinal testing in different behavioral paradigms starting at two through eight months post smoke exposure.

Open Field

Open Field testing serves to gauge locomotor performance, exploratory behavior and anxiety-related behavioral manifestations in rodents. We subjected age matched sham control and smoke exposed mice to longitudinal Open Field (OF) testing at two, four, and six months (n=15, 14, 12, respectively) post-acute exposure to combustion smoke. Distance traveled and travel speed in periphery and center arenas were recorded and compared between sham-control and smoke groups (Figure 6). Performance was recorded (Figure 6A) using TopScan video tracking system and automated software (Clever Sys. Inc) was used to quantify locomotor parameters. Reduced total distance traveled was observed in mice two, four, and six months after smoke when compared to sham-controls, whereas significantly reduced distance traveled in the central arena was measured at four and six months post

smoke (Figure 6B). Similarly, reduced travel speed in periphery was recorded in smoke exposed mice at all time points examined. Interestingly, a significant increase in travel speed in the central arena was observed six months post smoke (Figure 6B bottom). In combination, longitudinal open field testing sessions revealed long term dampening of exploratory behavior in smoke exposed mice. Center distance traveled was reduced at four and six but not at two months post smoke, plausibly suggesting escalation of anxiety-like behavior with time. Interestingly, while all analyses showed reduced speed in periphery, travel speed in center arena significantly increased at six months after smoke, potentially reflecting worsening of anxiety-like behavior.

Rotating-rod (rotarod)

To assess whether motor impairments could contribute to differences observed between the groups in open field-testing, motor coordination, balance and motor learning were assessed using the accelerating rotating-rod (rotarod) test (Coulbourn Instruments) as we previously described (Dineley et al. 2002). Testing was done five months post smoke between the second and third open field testing. The rotarod task consisted of four trials per day over a three-day period (Figure 7). Each trial lasted five minutes. The duration of time the mouse remained on the rod in the initial trials was used to assess general coordination. The second parameter, motor skill acquisition was evaluated by the rate of improvement on the task with repeated trials. Latency to fall was recorded by integrated software and mean $\pm$ SEM values were analyzed to compare the groups (n=8). Sidak's multiple comparisons test of daily average performance (4 sessions per day over 3 days) of smoke-exposed versus sham-control mice revealed no significant difference (Figure 7A). With repeated measures ANOVA, there was an overall effect of days indicative of improvement in performance over days for both groups; however, there was no interaction or effect of smoke. Pairwise comparison of each session between smoke exposed and sham-control mice found significant difference only in session 2 of day 3 (Figure 7B) that did not affect the overall daily average performance (Figure 7A). In sum, equivalent performance with respect to both parameters that was observed for the sham control and post-smoke groups suggests that motor deficits are not responsible for the observed differences in total distance travelled between sham control and smoke exposed mice. These data, in addition to the data for velocity in the periphery versus center arenas of the open field, further support the conclusion that smoke exposed mice manifest anxiety-like behavior that may worsen with time.

Fear conditioning

The cued fear-conditioning paradigm was used to corroborate the anxiety-like behavior results obtained from longitudinal open field-testing following smoke exposure. Fear conditioning paradigm is a learning and memory task that is amygdala dependent (Phillips and LeDoux 1992; LeDoux 2000; Maren 2001). The outcome measure for cued fear conditioning is freezing in response to re-exposure to the auditory cue (conditioned stimulus-CS), paired with the aversive stimulus, i.e., a mild foot shock (unconditioned stimulus-US). Digital video data capture and analysis with FreezeFrame (Coulbourn Instruments, Inc.) were used to quantify freezing behavior. On training day, each mouse was placed in a standard fear-conditioning chamber. A sound preceded a single mild shock that

was delivered through electrified grid floor. On ensuing day, testing was carried out in a different context where the auditory cue was produced but no shock was delivered. The expected cued freezing behavior was observed in both groups, with significantly increased freezing during the first 60 seconds of the auditory cue delivery recorded in the post smoke inhalation group of mice compared to sham-controls (n=10) (Figure 8).

Elevated Plus Maze

To further probe our postulate that acute inhalation of combustion smoke precipitates long term anxiety-like behavior in mice, seven and a half months after smoke exposure, mice were evaluated using the elevated plus maze paradigm (Figure 9). The elevated plus maze paradigm serves to assess exploratory and anxiety-like behaviors in rodents by measuring the time spent in closed and open arms, as well as in the central arena of the elevated maze. Activity traces (Figure 9A) were analyzed and percent of time in open arms, closed arms and in center arena was determined. Distances traveled and time spent in open arms were calculated and compared between sham controls and smoke exposed mice (n=10). We found that mice after smoke exposure, traveled a lesser distance and spent less time in open arms compared to sham controls (Figure 9B), providing further support for smoke exposure triggered anxiety-like behavior.

DISCUSSION

Long-term sequelae of smoke inhalation injury to the brain have not been systematically investigated. Acute inhalation of combustion smoke is a complex and severe insult, which involves chemical hypoxia, toxic compounds and oxygen deprivation that synergize to produce an instant energy crisis in the brain. While the early molecular, cellular and metabolic signatures of smoke inhalation in the rodent brain have been described in relative detail (Lee et al. 2005; Chen L et al. 2007; Lee et al. 2009; Lee et al. 2010; Lee et al. 2011; Zou et al. 2013; Gorgun FM et al. 2014; Zou et al. 2014), characterization of long-term progression of smoke inhalation initiated events is still lacking. We sought to longitudinally monitor progression of neurobehavioral changes and evaluate the long-term molecular and cellular signatures of acute exposure to combustion smoke in the mouse brain. We report behavioral modulations suggestive of temporal intensification of anxiety-like behavior in the mouse, as well as proinflammatory changes and compromised hippocampal myelination following long-term recovery after acute exposure to combustion smoke.

Our study was carried for eight months following smoke exposure, which is the maximal time span limited by the mouse age, to avoid confounding overlap with onset of age-associated inflammatory changes and increased propensity for the development of neurodegenerative disease states (Cortez et al. 2017; Mangold et al. 2017; Raj et al. 2017; Yin F et al. 2017). At termination of study, eight months after smoke exposure, we detected significantly greater numbers of GFAP and Iba-1 positive cells that are salient indicators of astrogliosis and microgliosis, plausibly consistent with persistent chronic inflammatory condition. In support of this possibility, altered expression patterns of proinflammatory and stress response genes were detected in hippocampal and cortical regions of the smoke exposed mice. Specifically, elevated expression of AIF1 (allograft inflammatory factor 1),

GFAP and the CCL2 that is expressed primarily in astrocytes and microglia, was detected. Elevated expression of inflammatory cytokines including the cytotoxic tumor necrosis factor- α (TNF- α), interferon γ (IFN- γ) and IL- β 1 was also measured. Interestingly, expression of the IL-6 gene remained unchanged, suggesting that delayed manifestations of smoke inhalation, do not involve acute inflammatory responses that are typically associated with marked increases in IL-6 levels (Frugier et al. 2010; Yin M et al. 2017), but rather are consistent with chronic inflammatory condition. Likewise, elevated expression of mRNAs encoding the key sensor of neuronal stress, c-Fos (Cohen et al. 2017) and the phase II antioxidant protein HMOX1 (Ghosh et al. 2011), which is controlled by c-Fos protein binding to the AP-1 response element within its promoter, might reflect response to oxidative stress consistent with inflammatory conditions. Chronic oxidative stress and potentially elevated levels of free radicals might contribute to demyelination (di Penta et al. 2013) that we detect in CA3 hippocampal regions of mice eight months after exposure to smoke. Interestingly, recent studies suggested that in addition to the well recognized role of myelination in enabling efficient signal transmission in the nervous system, myelin also supports axo-myelinic mode of active cell signaling between myelinating oligodendrocytes and neurons. Hence, compromise of myelin sheaths might lead to abnormal neuronal circuitry and potential development of a range of neurologic disorders (Micu et al. 2018).

Many types of brain injuries ranging from mild to severe have been epidemiologically linked to increased risk for lasting behavioral and cognitive deficits (Serra-de-Oliveira et al. 2015; Simon et al. 2017) and proinflammatory changes in the brain at the later stages of life (Waisman et al. 2015; Becher et al. 2017). In injury models, neuroinflammation is commonly seen in secondary injuries initiated by different types of insults (Serra-de-Oliveira et al. 2015; Chen H et al. 2017; Kumar et al. 2017; Zimmermann et al. 2017) and is often accompanied by behavioral changes (Peruga et al. 2011; Lei et al. 2017; Mayerhofer et al. 2017; Yin M et al. 2017). Importantly, neuroinflammatory changes, white matter abnormalities and neurologic disorders have been extensively reported in patients following carbon monoxide exposure (Weaver 2009; Betterman and Patel 2014; Kuroda et al. 2015). We report that acute exposure to combustion smoke leads to chronic inflammation in the mouse brain and initiates behavioral changes that are consistent with increased anxiety. We subjected the cohorts to longitudinal as well as one-time behavioral tests scheduled at adequate intervals to avoid unwanted habituation and potential interference among the different test paradigms. The open field serves to assess exploratory and anxiety-like behavior. We assessed open field at two months intervals, starting at two months after exposure to smoke. For the smoke exposed group, reduced distance and travel speed in the periphery were consistently measured at the three separate testing events. Reduced travel may reflect dampened exploratory behavior potentially due to lower motivation, as well as greater anxiety upon placement in novel seemingly less secure environment. Interestingly, the readout for the second parameter of open field-testing, namely changed performance in the center arena appeared to intensify with time. Specifically, while distance traveled in the central arena at first open field-testing event was equivalent in both groups, the distance was temporally reduced at the second and third testing events post smoke. Moreover, the center travel speed, which was found equivalent during the two initial testing events, at two and four months post smoke, significantly increased in the smoke exposed group in the third

testing at six months post smoke. Considered together, the findings from longitudinal open field-testing suggest that anxiety like behavior reflected in avoidance of center arena and increased in travel speed in the center intensifies with time. To confirm that differences observed in the open field task do not stem from impaired locomotor activity, mice were assessed by the rotating-rod task and in fact, equivalent performance was recorded for both groups, indicating that general coordination and motor skill acquisition, the two parameters assessed in this test, were not affected by exposure to smoke. This prompted us to further probe cohorts performance in additional paradigms designed to glean anxiety-like behavioral changes. Fear freezing testing, which is typically used to distinguish amygdala-mediated behavioral modulations from those governed by hippocampal function (Dineley et al. 2002; Burman et al. 2014; Fadok et al. 2017; Yin M et al. 2017), revealed increased freezing in response to cued conditioned stimulus in the smoke exposed group of mice. To further substantiate these observations elevated plus maze (EPM) was utilized as the final testing paradigm prior to termination of study. EPM serves to identify anxiety like-behavior by comparing exploratory activities in open/unsheltered versus closed/sheltered arms of elevated maze. EPM testing revealed reduced distance traveled and reduced time spent in open arms for the post-smoke exposure group when compared to sham control mice, providing additional support for persistent anxiety-like behavior following acute exposure to smoke.

Taken together, the current set of data shows for the first time that acute inhalation of combustion smoke leads to persistent chronic inflammation and compromised myelination in the mouse brain. In terms of neurobehavioral manifestations, our longitudinal open field studies suggested persistence of anxiety like-behavior following smoke exposure. To substantiate this finding, we utilized additional tests that specifically assess anxiety-associated behaviors and obtained independent supporting data from conditioned fear freezing and EPM paradigms that corroborated the longitudinal open field findings. Combined, our data suggest that akin to other traumatic brain insults that trigger secondary injuries and deficits, acute inhalation of smoke might fall in similar category. These new insights into the majorly understudied area of smoke inhalation injury to the brain may help guide long-term management of survivors of acute inhalation of combustion smoke.

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Figure 1.
Timeline of neurobehavioral testing procedures.

Figure 2.

GFAP positive cell number increases in mouse hippocampus after acute inhalation of smoke. Immunohistochemical detection of GFAP positive astrocytes in CA1 (top) and dentate gyrus (bottom) hippocampal regions eight months post smoke. Representative images of ROIs in coronal sections of sham controls (left) and smoke exposed mice (right) are shown; hematoxylin counter stain, scale bar = 50 μ m. Comparison of mean $\pm$ SEM number of GFAP positive cells in ROIs (Table 2) was by Student-t test (n=3); $P<0.05$ was considered significant.

Figure 3.

Iba-1 positive microglia cell number increases in mouse hippocampus after acute inhalation of smoke. Representative images of Iba-1 positive microglia in CA1 (top) and dentate gyrus (bottom) hippocampal regions in coronal sections of sham control (left) and smoke exposed mice (right) eight months after exposure (hematoxylin counter stain; scale bar = 50 μ m) are shown. Comparison of mean $\pm$ SEM number of Iba-1 positive cells in ROIs (Table 2) was by Student-t test (n=3); $P<0.05$ was considered significant.

Figure 4.

Compromised myelination in mouse hippocampus eight months after acute inhalation of smoke. Representative images of coronal sections immunostained for myelin basic protein (MBP) show decreased MBP immunoreactivity in the CA3 of smoke exposed mice (right) compared to sham-controls (left). For quantification purposes, adjacent sections were immunostained for MBP without hematoxylin counterstain (A, bottom). Images were converted to gray scale and assigned positivity threshold. (B) Quantifiable images of CA3 regions, which were generated by the 'make binary function' of ImageJ depict MBP positive areas in black. Bar graphs show mean $\pm$ SEM percent for MBP positive areas calculated from 18 ROIs scored per group (n=3).

Figure 5.

Increased gene expression of proinflammatory mediators in mouse hippocampi and cortices eight months after acute inhalation of smoke. mRNA levels of proinflammatory and stress response genes were measured by Real Time-qPCR. Data are presented as mean $\pm$ SEM (n=6); *different from sham-control P<0.05.

Figure 6.

Longitudinal performance in the Open Field paradigm differs between smoke exposed and sham control mice. (A) Representative images of locomotor activity traces (pink) of simultaneously recorded chambers with four sham-controls (left) and four chambers with post-smoke (right) mice. (B) Comparison of performance between groups in the periphery, revealed reduced distance and travel speed at all time points for smoke exposed compared to control mice. In the center arena, reduced travel distance was measured only at the four and six months post smoke time points, while travel speed in central arena increased in post smoke mice at the six months post smoke testing (bottom, right) with no significant travel speed changes at earlier time points. Bars represent mean $\pm$ SEM values, $P < 0.05$; NS-not significant (testing at two months $n=15$, four months $n=14$, six months $n=12$).

Figure 7.

Equivalent performance in rotating-rod between smoke exposed and sham control mice.

Mice were subjected to four trials per day over three consecutive days. (A) Repeated measures ANOVA confirmed overall effects of day indicative of improved performance for both groups (n=8). (B) Equivalent performance in rotarod by trial number as measured with latency to fall with the exception of trial #2 on day 3.

Figure 8.

Increased cued freezing in mice post smoke exposure compared to sham controls. Sham control and post smoke mice were tested in the cued fear conditioning paradigm. Testing done 30 hours post training revealed increased freezing time in response to auditory conditioned stimulus in the post smoke exposure group compared to sham control mice. Percent freezing time in 30 seconds epochs, is given as mean $\pm$ SEM; $P < 0.05$ ($n=10$).

Figure 9.

Elevated Plus Maze (EPM) testing at seven and a half months post smoke exposure reveals anxiety-like behavior. (A) Representative images of activity traces (pink) in EPM for two sham-control (left) and two post-smoke mice (right) are shown. (B) Percent time, distance traveled and time spent in open arms was reduced in smoke exposed mice when compared to sham-controls. Data are shown as mean $\pm$ SEM of percent time, distance traveled and time spent. Student-t tests were used to compare between sham control and smoke exposed mice (n=10); P<0.05 was considered significant; NS-not significant.

Table 1

IDs and sequences and of primers used in study.

Gene Symbol	Sequence		Accession #
	Forward	Reverse	
18S	gtaacccgttgaacccatt	ccatccaatcggtagtagcg	NR_003278.3, 18S ribosomal RNA
AIF1	cttcacacctctcttccatcc	tcactccacatcagctttga	NM_019467.2 allograft inflammatory factor 1 (Aif1)
CCL2	catccacgtgttggtca	gatcatcttgctggtgaatgagt	NM_011333.3 chemokine (C-C motif) ligand 2 (Ccl2)
CFOS	gggacagccttctactacc	gatctgcgcaaaagtcctgt	NM_010234.2 FBJ osteosarcoma oncogene
GFAP	acagacttttccaacctccag	ccttctgacacggatttggt	NM_010277.3 glial fibrillary acidic protein
HMOX1	gtcaagcacagggtgacaga	atcacctgcagctcctcaaa	NM_010442 heme oxygenase 1
IFNG	atctggaggaactggcaaaa	ttcaagactcaaagagtctgagg	NM_008337.4 interferon gamma (Ifng)
IL1B	agttgacggaccccaaaag	agctggatgctctcatcagg	NM_008361.3 interleukin 1 beta (Il1b)
IL6	gaggataccactccaacagac	aagtgcacatcgttgttcatac	NM_031168.1 interleukin 6 (Il6)
NOS2	ctttgccacggacgagac	tcattgtactctgaggctgac	NM_010927.3 nitric oxide synthase 2, inducible (Nos2)
TNFA	gcctctctcattcctgcttg	ctgatgagaggaggccatt	NM_013693.3 tumor necrosis factor (Tnf), variant 1,

Table 2

Post smoke changes in number of GFAP and Iba-1 positive cells in hippocampal ROIs

	Dentate Gyrus		CA1	
	Sham control	Post-smoke	Sham control	Post-smoke
GFAP positive cell number/mm²	211 ± 5	383 ± 5 *	139 ± 22	228 ± 27 *
Iba-1 positive cell number/mm²	83 ± 5	89 ± 5	55 ± 1	105 ± 11 *

Values are mean±SEM;

* $P < 0.05$ post smoke versus sham-control.