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Mold inhalation causes innate immune activation, neural, cognitive and emotional dysfunction

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Abstract

Individuals living or working in moldy buildings complain of a variety of health problems including pain, fatigue, increased anxiety, depression, and cognitive deficits. The ability of mold to cause such symptoms is controversial since no published research has examined the effects of controlled mold exposure on brain function or proposed a plausible mechanism of action. Patient symptoms following mold exposure are indistinguishable from those caused by innate immune activation following bacterial or viral exposure. We tested the hypothesis that repeated, quantified doses of both toxic and nontoxic mold stimuli would cause innate immune activation with concomitant neural effects and cognitive, emotional, and behavioral symptoms. We intranasally administered either 1) intact, toxic *Stachybotrys* spores; 2) extracted, nontoxic *Stachybotrys* spores; or 3) saline vehicle to mice. As predicted, intact spores increased interleukin-1β immunoreactivity in the hippocampus. Both spore types decreased neurogenesis and caused striking contextual memory deficits in young mice, while decreasing pain thresholds and enhancing auditory-cued memory in older mice. Nontoxic spores also increased anxiety-like behavior. Levels of hippocampal immune activation correlated with decreased neurogenesis, contextual memory deficits, and/or enhanced auditory-cued fear memory. Innate-immune

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Author contributions

CFH designed the experiments, oversaw treatments and behavioral testing, analyzed data, and wrote the first draft. CLP analyzed data, helped interpret data and write the manuscript. CLP and KGP supervised immunohistology and tissue analyses. KJR and RDS supervised behavioral testing and treatments. JV assisted in treatments and testing. EN assisted with analysis of neurogenesis. GJR assisted with treatments and proliferation analysis. DBM assisted with treatments and grew spore stock. KGP and LAS did the IL-1β analyses. AL, EPW, and NCA did the immature neuron analyses. All authors read and helped to revise the manuscript.

activation may explain how both toxic mold and nontoxic mold skeletal elements caused cognitive and emotional dysfunction.

Keywords

Sickness behavior; Conditioned fear; Anxiety; Interleukin-1β; Neurogenesis; Microglia; Neuroinflammation; Hippocampus; Memory; Pain

1. Introduction

Individuals who spend time in moldy buildings report numerous adverse health effects, including respiratory problems, chronic fatigue, muscle/joint pain, anxiety, and cognitive problems ("brain fog", Curtis et al., 2004; Ratnaseelan et al., 2018). The ability of mold inhalation to cause respiratory problems is well documented and widely accepted (Heseltine and Rosen, 2009). However, the link between mold and other physiological problems as well as cognitive and emotional impairment is hotly debated. Several small studies (Baldo et al., 2002; Crago et al., 2003; Gordon et al., 2004) concluded neurologists could not differentiate between people with repeated exposure to moldy buildings and people with mild to moderate traumatic brain injury—they had similar neurological and cognitive deficits. Others (Fox et al., 2005; McCaffrey and Yantz, 2005) criticized the conclusion that mold exposure caused serious neurological problems on multiple grounds, including lack of research documenting the effects of specific mold stimuli on brain and behavior and lack of an identified mechanism to cause such effects. Surprisingly, no published research has investigated the effects of controlled doses of characterized mold stimuli on both brain and behavior.

Given the extensive and controversial reports of neurological and behavioral problems among individuals exposed to mold-contaminated environments (Baldo et al., 2002; Crago et al., 2003; Gordon et al., 2004; Shoemaker and House, 2006; Kilburn, 2009; Morris et al., 2016), it is crucial to determine if mold exposure can cause central neural effects and, if so, to identify the underlying mechanisms. We therefore developed a mouse model to assess the effects of known doses of mold spores on brain and behavior. Supported by our data, we propose a plausible mechanism of mold-induced neurological problems: The innate immune response to mold in the periphery leads to immune activation in the brain, triggering neural cytokine release and loss of newly-formed hippocampal neurons with resulting impairment of hippocampal-dependent learning and memory as well as emotional dysfunction.

Our model is based on research on innate immune activation and sickness behavior (Dantzer and Kelley, 2007; McCusker and Kelley, 2013). Animals exposed to bacterial infection or merely to components of bacterial cell walls show strong innate immune responses characterized by high levels of cytokine release at the exposure site. Cytokines released peripherally rapidly activate microglia, the most numerous brain-resident immune cells. Activated microglia release cytokines in the central nervous system causing sickness behavior including malaise, pain, fatigue, and social withdrawal. Well-characterized deficits in hippocampal learning and memory tasks often occur (Dantzer and Kelley, 2007). Like bacteria, mold spores and their component toxins and metabolites activate strong innate

immune responses in the lungs leading to peripheral inflammation (Leino et al., 2003; Rand et al., 2003; Yike et al., 2005). In addition, small inhaled mold fragments or metabolites may access the brain directly via olfactory pathways to activate microglia (Tonelli and Postolache, 2010). Symptoms reported by people spending time in moldy environments sound like a recitation of symptoms of cytokine-induced sickness behavior and the cognitive issues that follow. However, no one has previously determined whether mold exposure causes central immune activation with resultant cognitive and emotional problems.

Among those who think that mold exposure adversely affects health, some believe that only toxin-producing molds like the infamous “black mold”, *Stachybotrys chartarum,* are a threat. Others claim that exposure to any mold could be problematic because the innate immune system has multiple receptors that recognize common fungal skeletal elements just as they recognize those of bacteria (Rand et al., 2010). Both nontoxic and toxic spores caused innate immune activation in infant rat lungs, with toxic spores producing stronger effects (Yike et al., 2005). To test whether nontoxic skeletal components as well as toxic spores can cause adverse neural and behavioral effects, we contrasted the effects of toxic and nontoxic mold stimuli with vehicle treatment. Our data demonstrate that controlled exposure to known quantities of both toxic or nontoxic mold stimuli caused innate immune activation in the brain and decreased neurogenesis, with concomitant cognitive and emotional dysfunction.

2. Materials and methods

2.1. Experimental animals

All animal methodology was approved by the Institutional Animal Care & Use Committees of Hunter College and Queens College, CUNY, and met all local and federal guidelines for animal research.

Adult male C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Because group housing can cause confounding effects in mice experiencing immune challenge (Lyte et al., 2005), mice were housed individually in filter-topped shoebox cages with paper bedding, a piece of PVC piping, and a nestlet (Ancare, Bellmore, NY) on a 12:12 light:dark cycle at 22.2 + 1.4 °C. Mice were habituated to their housing, handling, and transport to behavioral testing rooms for at least 2 weeks prior to instillations. Bedding was changed twice per week after behavioral testing was completed.

2.2. Experimental design and mold exposure

All behavioral tests, tissue processing, microscopy, and data analyses were done blind to the animals’ treatments. Mice were run in 11 blocks of 6–18 mice (total N = 122) so that all behavioral testing could be done in a narrow time frame in the morning, and all spore instillations and sacrifices could be done in a narrow time frame in the afternoon. The first six blocks were mice (N = 56) from standard housing at the vendor shipped at 6 wks. These animals were trained/tested on conditioned fear, and numbers of microglia, neuron proliferation, and immature neurons in their dorsomedial hippocampi quantified. Contextual memory is adversely affected by decreased hippocampal neurogenesis (Denny et al., 2012). Since neurogenesis decreases with age (Kempermann et al., 1998), we were interested in

whether older mold-treated mice would have greater deficits in hippocampal-dependent contextual memory than younger mice. Therefore we tested five blocks of mice (N = 66) shipped at 13 wks. These mice were from maximal barrier rooms to minimize prior immune activation. These 5 blocks of animals were tested on conditioned fear, elevated plus maze, and tail flick. We quantified numbers of cells immunoreactive for interleukin-1β (IL-1β) and mature new neurons in their hippocampi.

In the first 10 blocks, mice were divided into 3 treatment groups using a stratified randomized block design controlling for body weight and instilled with: 1) intact *Stachybotrys chartarum* spores (toxic, TX), 2) *Stachybotrys* spores extracted twice with ethanol to remove toxins and denature proteins (Yike et al., 2005; non-toxic, NTX), or 3) non-pyrogenic saline vehicle containing 0.1% TWEEN 20 (VEH). Block 11 included only NTX and VEH groups. All spore handling, instillations, and cage changes of treated mice were done in a Class II biosafety cabinet (ThermoFisher Model 1365).

Mice were briefly anesthetized with isoflurane, their nostrils gently swabbed with 70% ethanol, and nasally instilled (Amuzie et al., 2010; Cooley et al., 2000) 3 times/week with either 400 (block 11), 4000 (blocks 1, 2) or 15,000 (blocks 3–10) spores in 0.25 μl vehicle/g body weight or vehicle alone. Spore doses were prepared using a hemocytometer to count spores to an accuracy of ± 4%. Half of each dose was instilled in each nostril using a Rainin 0.5–10 μl classic pipette. Mice were held in a vertical position, nose up, for 2 min post instillation to maximize spore inhalation. Following treatment, spore-treated mice did not show obvious signs of overt discomfort that are often reported following high doses of lipopolysaccharide (LPS, e.g., squinting, unkempt fur, obvious changes in movement).

2.3. Food restriction

Four blocks of mice were also tested on cheeseboard, a food-motivated behavioral task. They were restricted to 90% of free-feeding weight to motivate them to search for food. Animals were weighed 6 days/week in the morning to determine free-feeding weights. Mice were then given 2.45–2.55 g chow per day until they reached 90% of their free-feeding weights followed by approximately 3 g per day to maintain this weight. If the mouse's weight was between 88.5 and 91.5% of free-feeding weight, it was given the same amount of food as the day before. If not, the amount was adjusted as necessary. Mice did not master the cheeseboard task, so it is not discussed further.

2.4. Behavioral testing

During the fourth week of instillations, mice were tested on elevated plus maze (EPM, anxiety) and/or conditioned fear (contextual and auditory-cued memory), and/or tail flick (pain sensitivity). If EPM was run, it preceded other tests. Instillations always occurred after behavioral testing. In the case of conditioned fear, instillations occurred following the second day's tests. Testing orders for each test were counterbalanced across mice and treatment groups. All apparatus was cleaned with Conflikt (Fisher Scientific), followed by 70% ethanol, and then water after each mouse.

2.4.1. Elevated plus maze—The elevated plus maze is typically used to quantify anxiety-like behavior in rodents, testing animals' willingness to enter open (more anxiety-inducing) versus walled (less anxiety-inducing) arms of the maze. For EPM testing, mice were placed in the center (11.4 × 11.4 cm) of a black plexiglass Kinder Scientific EPM (closed arms 10 cm × 55.9 cm, open arms 11.4 cm × 55.9 cm, 91.4 cm above the floor) and their entries, time spent, and distance moved in the open and closed arms were automatically quantified by Kinder Motor Monitor software (Build #08356–14). Open arm measures were corrected for total entries; total duration spent, or total distance traveled in both arms respectively, to control for possible differences in activity (File, 2001).

2.4.2. Conditioned fear—In rodents, hippocampal memory deficits are often tested using a conditioned fear paradigm, contrasting the animals' behavior on hippocampal-dependent contextual memory tests versus non-hippocampal-dependent auditory-cued memory tests. In rodents, "freezing" or standing motionless, is a natural response to fear. While the usual output measure for conditioned fear is percent time freezing, the animal's memory of the fearful situation can also be evaluated by calculating movement during testing (Curzon et al., 2009).

Mice were habituated to a Kinder Scientific conditioned fear apparatus (42.5 cm long × 20.6 cm wide × 20.3 cm high) for 5 min using plain wallpaper (white paper wrapped around the apparatus' clear plexiglass walls) with the wire grid floor exposed (rods 6.4 mm in diameter set 19 mm apart on center) about a week prior to their first training. Training was divided into a) 2 min of acclimation to Context A (wallpaper pattern A, grid floor), b) initial 15 sec of tone c) final 15 sec of tone during which mice received a 0.5 mA footshock during the final 2 sec, and d) the min following shock. Mice were then returned to their home cages. Thirty min and 24 h after training, mice were returned to the apparatus and given 5 min context tests (Context A, no tone, no shock). Twenty-five hours post training they were tested for auditory-cued conditioning in context B (different wallpaper, grid floor covered with thick, rigid white plastic). As in the training session, 30 sec of tone was presented after 2 min of acclimation, but the animals were not shocked. For training and auditory tests, Kinder Motor Monitor software quantified movements per min during acclimation, the first and second 15 sec of tone presentation, and the min following tone presentation. For the context tests, the software calculated movements per min for each of the five min. Mice were given one conditioned fear training about 12 days before instillations began. One block of mice shipped at 6 wks received a second pre-instillation training. The data reported here are from the animals' second (third in the case of the aforementioned block) training during the fourth week of instillations.

2.4.3. Tail flick—The tail flick test, which measures how long it takes an animal to move its tail away from a hot beam of light, is widely used to assess pain thresholds in rodents (Hole and Tjolsen, 2007). Before testing, mice were habituated to resting quietly in a plexiglass restrainer that was then covered with an insulated box exposing only the tail. On each test day, mice were tested 3 times with a 30-sec intertrial interval. The mouse was placed on a tail-flick apparatus built following the instructions in Otto et al. (2011) with its tail in the tail groove. The light above the tail was turned on and simultaneously a stopwatch

started to determine latency to move the tail to the side out of the groove. The light was turned off as soon as the mouse moved its tail. Maximal latency allowed was one minute.

2.5. BrdU treatments

Mice received intraperitoneal (ip) injections of BrdU (Sigma-Aldrich B5002) in saline timed to quantify either neuron proliferation (one 200 μg/g injection 2 h before sacrifice) or mature neurons (2 injections per day of 50 μg/g for 4 days). The mice were only injected on four days, but because half the mice were sacrificed two days later than the others, across mice injections occurred 31–37 days prior to sacrifice.

2.6. Tissue preparation

All sacrifices were done in the afternoon, approximately 24 h after each mouse's final instillation. Larger blocks of mice were sacrificed over two days so that sacrifices could be done in a narrow time window to control for possible circadian effects. After about 6 weeks of instillations, mice were deeply anesthetized with ketamine:xylazine (200 μg:10 μg/g body weight ip) and then intracardially perfused with 30 ml of 0.1 M phosphate buffered saline (PBS) followed by 30 ml of 4% paraformaldehyde in PBS and post-fixed in 4% paraformaldehyde in PBS for 1 h. Brains were transferred to PBS overnight, dehydrated in increasing concentrations of ethanol and embedded in polyethylene glycol (Polysciences), sectioned sagittally at 6 μm on a rotary microtome, mounted onto Superfrost++ slides, dried overnight and stored at −20 °C. Series of every fifth section throughout the dorsomedial hippocampus were processed for quantification of cells containing Iba-1 (expressed in microglia), the inflammatory cytokine IL-1β, doublecortin (DCX, expressed in immature neurons), BrdU and the neuronal marker Hu (expressed in neurons of all ages) or NeuN (expressed in mature neurons).

All IHC protocols were run with a negative control slide omitting primary antibody, and equivalent numbers of sections for each treatment were run in each assay. All rinses were 10 min unless otherwise stated.

2.7. Immunohistochemistry for microglia

To visualize microglia, we labeled the microglia-specific protein Iba-1. Sections were brought to room temperature, rinsed 3 times with PBS, then sections reacted with 0.06 or 0.09% hydrogen peroxide in PBS for 30 min, followed by three PBS rinses. Sections were then blocked with 5% normal goat serum (Jackson ImmunoResearch) and 0.3% Triton X-100 in PBS for 1 h, followed by exposure to rabbit Iba-1 antibody (1:500, 019–19741, Wako) for 72 h at 4 °C. After three PBS rinses, sections were incubated in biotinylated goat anti-rabbit secondary antibody (1:500, ab97049, ABCAM) in 2% normal goat serum (Jackson ImmunoResearch) in PBS for 2 h. Tissue was then labeled with avidin and biotin following manufacturer's instructions (ABC Elite kit, PK6100, Vector Laboratories) for 2 h followed by three PBS rinses. The secondary antibody was visualized by reaction with 3,3-diamino-benzidine-tetrahydrochloride (DAB) chromogen (peroxidase HRP kit, SK-4100, Vector Laboratories), washed in PBS, dipped in distilled water, dehydrated in increasing concentrations of ethanol and cover slipped with Krystalon (EMD Chemicals).

2.8. Immunohistochemistry for the inflammatory cytokine IL-1β

Sections were brought to room temperature and rinsed with PBS 3 times. Slides were immersed in 0.25% pepsin in 0.1 N HCl at 37 °C for 3 min, then rinsed 3 times with PBS. Sections were exposed to 0.06 or 0.09% hydrogen peroxide + 1% methanol in PBS for 30 min, followed by three 5-min PBS washes. Sections were then blocked with 2% normal donkey serum (Jackson ImmunoResearch) and 0.3% Triton X-100 in PBS for 1 h, followed by exposure to polyclonal antibody to mouse IL-1β made in goat (1:100, AF-401-NA, raised against the mature, cleaved (activated) form of IL-1β, R&D Systems) in 0.3% Triton X in PBS overnight at 4 °C. After three PBS rinses, sections were incubated in biotinylated donkey anti-goat secondary antibody (1:500, 705–066-147, Jackson ImmunoResearch) in 0.3% Triton X in PBS for 2 h. Tissue was rinsed three times for 5 min in PBS, then labeled with avidin and biotin complex following manufacturer's instructions (ABC Elite kit, PK6100, Vector Laboratories) for 1 h followed by three PBS rinses. The secondary antibody was visualized by reaction with DAB chromogen (DAB peroxidase HRP kit with nickel, SK-4100, Vector Laboratories), washed in PBS, dipped in distilled water, dehydrated in increasing concentrations of ethanol and cover slipped with Krystalon (EMD Chemicals).

2.9. Immunohistochemistry for neurogenesis: Proliferation, immature new neurons, and mature new neurons

BrdU, Hu and NeuN were labeled as described in (Tsoi et al., 2014) and in the Protocol Exchange. To label DCX, tissue was brought to room temperature, rinsed 3 times in tris-buffered saline (TBS) and exposed to 0.06 or 0.09% hydrogen peroxide + 1% methanol in TBS for 30 min. Tissue was washed in TBS, blocked in 3% normal horse serum (Jackson Immuno Research) and 2.5% Triton X-100 in TBS for 30 min, and incubated overnight with primary goat polyclonal anti-DCX (1:150 at 4 °C, sc-8066, Santa Cruz Biotechnology). Tissue was then washed in TBS 3 times for 5 min, then incubated in biotinylated horse anti-goat secondary antibody (1:200, BA-9500, Vector Laboratories) in TBS for 3 h. Tissue was washed in TBS 3 times for 5 min, and exposed to avidin and biotin (ABC Elite kit, PK6100, Vector Laboratories) for 1 h. Tissue was washed 3 times for 5 min in TBS, reacted with DAB chromogen (IMMPact DAB, SK-4105, Vector Laboratories), rinsed in TBS, dipped in distilled water, dehydrated in increasing concentrations of ethanol and cover slipped with Krystalon (EMD Chemicals).

2.10. Quantification of labeled cells

Data were collected without knowledge of mouse identity or treatment. All tissue quantified was matched across animals for comparable medial-lateral position. Area and perimeter measurements and cell counts were performed using a computer-yoked microscope and mapping software (Olympus BX51; Lucivid microprojection, Neurolucida, Micro-BrightField, Inc.). The dorsomedial hippocampus, dorsomedial DG, and CA1 were defined using anatomical markers in accordance with the mouse brain atlas (Allen Institute for Brain Science, 2004; Lein et al., 2007). The granular and subgranular zones were identified by packing density and cell morphology forming the boundary with the hilus, and lying deep to the relatively cell-free molecular layer (Amaral et al., 2007). Labeled cells were visualized

using lightfield or fluorescence optics, marked with Neurolucida software, and saved digitally.

2.10.1. Iba-1—The granular cell layer of the DG was traced using darkfield optics in 10 sections per animal. All Iba-1-expressing microglia within the DG in these 10 sections were marked using Neurolucida. Microglia with small somas with thin, delicate, radially projecting processes were classified as ramified while microglia with densely stained, enlarged cell bodies and few short processes were classed as amoeboid (Shapiro et al., 2009). Numbers of ramified and amoeboid microglia per mm^2 sampled were then calculated for each mouse.

2.10.2. BrdU/Hu and BrdU/NeuN—We quantified cells expressing BrdU and the neuronal marker Hu in 10 sections throughout the dorsomedial DG in mice that had received a single injection of BrdU 2 h before sacrifice. As expected, double-labeled cells were seen in the subgranular layer, the proliferative zone of the DG. Hu is expressed in young neurons within 2 h after mitosis (Marusich et al.,1994). BrdU/NeuN double-labeled cells were quantified in mice that received 8 injections of BrdU 31–37 days prior to sacrifice. For this work, a single line was drawn tracing the length of the DG along the boundary between the subgranular zone and hilus in darkfield. Double-labeled cells were visualized using a fluoroscein isothiocyanate (FITC) filter (BrdU) and rhodamine filter (Hu or NeuN), and a dual FITC/rhodamine filter. Double-labeled cells were marked using Neurolucida and their numbers per mm length of DG sampled calculated for each mouse.

2.10.3. DCX—Young neurons expressing doublecortin labeled with DAB were visualized using brightfield microscopy and quantified per mm DG as described above.

2.10.4. IL-1β—Cells expressing IL-1β were visualized using brightfield microscopy and quantified per mm^2 of the dorsomedial DG, CA1, and dorsomedial hippocampus.

2.11. Statistical analysis

Data were analyzed in GraphPad Prism (Version 8.1.2 for Mac). All tests were two-tailed. Data sets that did not have equal variances (Bartlett's test) were transformed as noted and that issue eliminated prior to further analyses. However, all graphs are means + SEMs of untransformed data.

Four outcome measures were tested in mice shipped at 6 wks: conditioned fear, microglia (Iba-1 immunoreactivity, Iba-1-ir), neuron proliferation (BrdU/Hu-ir), and immature neurons (DCX-ir). Before combining blocks for analysis of the effects of mold treatment, we determined that any differences in treatment between blocks (spore dose of 4000 or 15,000/g, food restriction, one additional conditioned fear training/testing prior to mold treatment, or age at sacrifice did not affect the outcome of the analyses of the effects of mold treatment (2-way ANOVAs followed by Tukey's multiple comparisons tests, mold treatment X variable of interest). In all cases, there was either no effect of the variable on the outcome measure or the difference affected all three treatment groups equivalently. There were no significant interactions. These variations in treatment parameters between blocks did not

affect the mold effects we report. All blocks of mice shipped at 13 wks that were combined for analysis were treated identically.

The effects of mold treatment were analyzed by one-way ANOVAs followed by post-hoc Holm Sidak's multiple comparisons tests. Pearson correlation coefficients were used to calculate relationships between variables. When multiple comparisons were made across correlations, the Benjamini-Hochberg procedure (McDonald, 2014) was used to control for false discovery rate (FDR) and determine the appropriate alpha. FDR was set to 0.05.

Three outliers were detected by Rout (Graphpad Prism) using a conservative Q = 0.2% and removed from analysis. One outlier was found in the contextual fear data shown in Fig. 1. This VEH mouse moved significantly more than all others at 30 min, but not at 24 h. A second outlier mouse was identified in the duration and distance data of the NTX group on EPM (Fig. 6). This animal's hippocampus was also eliminated from study. A final outlier was found in the numbers of DCX-ir neurons in the NTX group. In all other cases, all available data were used. One mouse's conditioned fear data was lost due to experimenter error (wrong latency to auditory test). For the proliferation analysis, tissue from 5 mice was not processed. Across all histochemical analyses, tissue from 14 mice did not yield quantifiable cell counts on one assay due to damaged tissue or poor labeling. These issues affected the three groups almost equally.

3. Results

3.1. Mice shipped at 6 weeks

3.1.1. Mold exposure impaired hippocampal memory—Like freezing, movements/min clearly documented that mice learned the association between both 1) context, 2) auditory cue, and footshock. Following one footshock training prior to instillations, mice moved significantly less during both the 30 min and 24 h tests of contextual memory and the 25 h test of auditory-cued memory than they moved prior to footshock (data not shown, all comparisons $P < 0.0001$). There were no differences in movement between the three groups during training following experimental treatment (data not shown). However, mice treated with TX or NTX spores showed striking deficits in contextual memory, moving significantly more than VEH controls both 30 min (Fig. 1A, ANOVA, log-transformed data, F = 9.34, $P = 0.0017$) and 24 h after training (Fig. 1B, ANOVA, log-transformed data, F = 13.96, $P = 0.0002$). NTX and TX mice showed no deficits in auditory-cued memory at hour 25 (Fig. 1C).

3.1.2. No effect on gross microglial morphology in the dentate gyrus—We expected to find more amoeboid microglia in spore-treated mice. However, neither ramified, amoeboid, nor total microglia (ramified + amoeboid) differed across treatments (Fig. 2A, B, Fig. 3A–D). All groups had significantly more amoeboid than ramified microglia (paired t tests, VEH $P = 0.025$, NTX $P = 0.009$, TX $P = 0.013$). Across all mice, numbers of ramified microglia were inversely correlated with numbers of amoeboid microglia. The more amoeboid microglia a mouse had in the dorsomedial DG, the fewer ramified microglia were present (Fig. 2C).

3.1.3. Relation between amoeboid microglia and contextual fear—Across all animals, numbers of amoeboid (Fig. 2D) or ramified microglia (not shown) did not correlate with contextual nor auditory-cued memory. However, within TX mice, the more amoeboid microglia in the dorsomedial DG, the more the animal moved on the 24-h contextual fear test (i.e., the worse the mouse's memory for the context in which it was shocked, Fig. 2E). Interestingly, while NTX and TX mice showed equivalent contextual fear deficits (Fig. 1A, B) and similar numbers of amoeboid microglia (Fig. 2B), the strong relationship between amoeboid microglia and memory deficits was not seen in NTX mice (Fig. 2F). There was also no correlation between amoeboid microglia and memory in VEH mice (not shown).

3.1.4. Mold exposure decreased 2 stages of neurogenesis—To determine if mold exposure decreased neurogenesis, we quantified three stages of hippocampal neurogenesis. Neural proliferation in mice shipped at 6 wks did not differ significantly across treatments (Fig. 4A). NTX-spore treatment significantly decreased numbers of immature neurons expressing doublecortin in mice shipped at 6 wks (Fig. 3H–J, 4B, ANOVA of square-root-transformed data, F = 4.42, $P = 0.0186$) compared to VEH controls. Treatment with TX spores significantly decreased mature new neurons double labeled with antibodies to BrdU/NeuN compared to VEH in mice shipped at 13 wks (Fig. 3K–M, 4C; ANOVA, F = 6.27, $P = 0.0053$). Numbers of mature new neurons were negatively correlated with numbers of IL-1β-ir cells in the granular cell layer of the DG across all mice and in spore-treated mice (all mice: r = −0.60, $P = 0.04$, N = 12, spore-treated mice: r = −0.62, $P = 0.10$, N = 8). The correlations in VEH mice were in the opposite direction (r = 0.23, $P = 0.76$, N = 4).

3.2. Mice shipped at 13 weeks

3.2.1. Mold exposure enhanced auditory-cued memory—Contrary to our prediction, mice shipped at 13 weeks (N = 23) did not show enhanced deficits in contextual fear. In fact, they showed no contextual fear deficits (data not shown). Unexpectedly, mold exposure significantly affected the behavior of these mice on the non-hippocampal auditory-cued portion of the conditioned fear test. Because the effects were unexpected, we tested an additional 24 mice to ensure that this was a replicable effect. After 10 instillations, all treatment groups showed equivalent movements/min immediately following tone/footshock training (Fig. 5A) and when tested for contextual memory 30 min and 24 h later (data not shown). When response to the auditory cue was tested 25 h after training, both NTX and TX mice moved significantly less than VEH mice (Fig. 5B, ANOVA, log-transformed data, F = 7.39, $P = 0.0017$). These differences were more dramatic when the data were examined as change in response from training to auditory testing: for each mouse, movements/min immediately following shock training minus movements/min following tone presentation 25 h later (Fig. 5C, F = 8.571, $P = 0.0007$). Negative values indicate VEH mice moved more following tone presentation at hour 25 than immediately after training.

3.2.2. Mold exposure increased anxiety-like behavior—Increased anxiety-like behavior was only seen in mice treated with NTX, not TX, spores. NTX-treated mice had significantly fewer entries into the more anxiety-inducing open arms compared to total entries into all arms (Fig. 6A, ANOVA, F = 5.23, $P = 0.0149$); spent less time in the open

arms compared to time in all arms (Fig. 6B, ANOVA log-transformed data, F = 11.01, P = 0.0006); and traveled less distance in the open arms compared to all arms (Fig. 6C, ANOVA log-transformed data, F = 16.08, $P < 0.0001$).

3.2.3. Mold exposure increased pain sensitivity—As expected, mold-exposed mice had lower pain thresholds than VEH mice. We ran two tail-flick experiments. In the first, mice were given 1 test with three trials after 13 instillations. Both NTX and TX mice (15,000 spores/g) had significantly lower mean and maximal latencies than VEH mice over the three tail flick trials (Fig. 7A, B, ANOVA, F = 3.87, P = 0.0379; ANOVA square-root-transformed data, F = 3.74, P = 0.0417, respectively). In the second experiment, mice were given four sets of tail flick tests (three trials per test) at weekly intervals from weeks 4 to 7 of treatment comparing the effects of a much lower dose of 400 NTX spores/g to those of VEH. Over the first three tests, even this low dose of NTX spores resulted in significantly lower maximal latencies to tail flick than seen in VEH mice (Fig. 7C, unpaired t test, t = 3.20, df =14, P = 0.0059). By the fourth test, latencies in VEH mice fell to the level seen in NTX mice (data not shown). In both experiments, mold exposure acted primarily by reducing the maximal latencies to tail flick. Minimum latencies were not affected (data not shown).

3.2.4. Mold exposure increased IL-1β-ir cells—We found no significant differences across groups in IL-1β-ir cells in dorsomedial DG as a whole or in its granular or molecular layers quantified separately (not shown). However, in both dorsomedial CA1 and dorsomedial hippocampus as a whole, TX mice had significantly more IL-1β-ir cells than VEH mice (Fig. 8A, B, ANOVA, F = 6.546, P = 0.0062, ANOVA, F = 7.341, P = 0.0047, respectively; Fig. 3E–G). In both areas, TX spores caused larger increases than NTX spores, though this difference was only significant in dorsomedial hippocampus as a whole.

3.2.5. Relation between IL-1β-ir cells and auditory-cued fear—There were no significant correlations between numbers of IL-1β-ir cells and performance on EPM, contextual memory, or auditory-cued memory measured 25 h post training (data not shown). However, there were significant correlations between numbers of IL-1β-ir cells and enhanced response to the auditory cue over time (Fig. 5C). Intriguingly, in both TX and NTX mice, the more IL-1β-ir cells in CA1 and the hippocampus as a whole, the less the mice moved at 25 h compared to training (i.e., difference scores of movement post shock minus movement 25-hour post tone shown in Fig. 5C). Correlations varied by both subregion and treatment (Fig. 8C, D and Table 1A). Correlation coefficients were highest and significant in all brain areas in mice treated with TX spores. The correlations across all animals in CA1 and hippocampus were driven by the TX and NTX data (Table 1A, Fig. 8C). In VEH mice, all correlations between IL-1β-ir cells in any region and change in fear were nonsignificant and in the opposite direction (e.g., Fig. 8D). Significant correlations between IL-1β-ir cells in the DG (both as a whole and its granular and molecular subregions separately) and increased fear over time were only found in TX mice (Table 1A).

TX mice also showed strong positive correlations between numbers of IL-1β-ir cells in the hippocampus and movements per min immediately following shock during training. The more IL-1β-ir cells in all five hippocampal areas, the more TX mice moved immediately

following the training shock (Table 1B). TX mice did not move significantly more than the other groups at this time point (Fig. 5A). However, their frequency of movement correlated with their numbers of IL-1β-ir cells. NTX mice showed similar correlations to those of TX mice between IL-1β-ir cells in dorsomedial hippocampus and CA1 and the change in auditory-cued fear over time (Table 1A), but did not show the initial positive correlations between IL-1β-ir cells and movement following training (Table 1B). The significant correlations in the TX group were sufficient to result in the correlations across all animals following training also being significant in most brain areas (Table 1B). It is notable there was a delay of 16 or 18 days between behavioral testing and brain tissue collection. The significant correlations suggest that repeated mold exposure established brain changes by the time of behavioral testing that were maintained until perfusion.

4. Discussion

There is now compelling evidence that mold exposure causes serious multi-system health problems in humans including peripheral immune activation and behavioral dysregulation that implies central effects (Shoemaker and House, 2006; Kilburn, 2009; Morris et al., 2016). In addition to documenting a variety of cognitive and emotional problems, Kilburn (2009) quantified wide-ranging health problems in mold-exposed individuals including increased reaction times, visual abnormalities, decreased grip strengths, balance problems, and peripheral neuropathies. Shoemaker et al. (2014) found changes in blood inflammatory markers in mold-exposed patients were correlated with significant structural differences in six of eleven brain areas studied. Mechanisms by which mold exposure caused neurological or behavioral problems were not identified.

We hypothesized that mold inhalation causes these diverse neural and behavioral problems by the same mechanism of action as bacterial or viral exposure: innate immune recognition of a pathogen causes immune activation at the site of exposure, ultimately causing innate immune activation in the brain with resultant changes in behavior. We predicted that the neural and behavioral effects of mold exposure would match those caused by innate-immune activation following bacterial infection or merely treatment with LPS, a noninfective component of gram-negative bacterial cell walls (Dantzer and Kelley, 2007; Yirmiya and Goshen, 2011). We also predicted that nontoxic mold skeletal elements, like bacterial skeletal elements, would cause innate immune activation.

Our results are consistent with this model. Like animals exposed to bacterial stimuli, exposure to TX spores increased numbers of hippocampal cells immunoreactive for the proinflammatory cytokine IL-1β and decreased numbers of mature adult-born neurons in the dorsomedial hippocampus. Mice shipped at 6 wks and treated with either NTX or TX spores showed significant deficits in contextual memory 30 min and 24 h after training. Mice shipped at 13 wks and treated with NTX or TX spores showed increased anxiety-like behavior, increased pain sensitivity, and unexpected enhanced auditory-cued memory. Levels of hippocampal immune activation (i.e., activated microglial morphology or numbers of cells immunoreactive for IL-1β) correlated with contextual memory deficits, decreased neurogenesis, and/or enhanced auditory-cued fear memory. These changes match the mechanisms and symptoms of innate immune activation in laboratory animals and people in

response to bacterial or viral challenge (Dantzer and Kelley, 2007; McCusker and Kelley, 2013). Critics of the idea that mold inhalation can cause a wide variety of health problems often invoke the nonspecific nature of reported symptoms (Fox et al., 2005; Pettigrew et al., 2010). But a variety of innate immune challenges activate similar changes in physiology and behavior (Dantzer and Kelley, 2007).

There are three generally-accepted mechanisms through which mold exposure affects human health: 1) allergy, 2) infection, and 3) toxicity (Council on Scientific Affairs, 2003). We propose that innate immune activation should be added to the list. While NTX spores induced less innate immune activation in the brain as measured by IL-1β-ir cells than toxic TX spores, NTX spores induced serious neural and behavioral symptoms. Infection or toxicity clearly cannot explain these effects since NTX spores are neither viable nor toxic. Previous studies (Beijer et al., 2002; Leino et al., 2003; Yike et al., 2005; Rand et al., 2010) documented that respiratory exposure to nontoxic, noninfective spores or merely a skeletal component, beta-glucan, induced peripheral immune activation and inflammation. This is not surprising given that there are at least nineteen pattern recognition receptors (PRRs) that recognize carbohydrates in fungal cell walls or RNA/DNA and activate immune responses (Dambuza et al., 2017). All four of the major classes of PRRs are involved in fungal recognition with the C-type lectin family believed to be the most important (Dambuza et al., 2017; Vacher et al., 2015). Although we cannot rule out an allergic response to mold, and certainly there are people for whom mold is an allergen, the responses observed in this study fit the innate-immune-activation model.

TX spores have a greater inflammatory potential (Yike et al., 2005) and caused stronger effects than NTX spores on numbers of IL-1β-ir cells in the dorsomedial hippocampus, maturation of adult-born neurons in the dorsomedial DG, increased pain sensitivity, and enhanced auditory-cued memory. There were also stronger correlations between measures of brain immune activation and changes in behavior in mice exposed to TX spores. However, intriguingly, NTX spores caused deficits in contextual memory similar to those seen in TX mice. Moreover, only NTX spores increased anxiety-like behavior as quantified by EPM. Perhaps this can be explained by differential PRR activation of TX mice. The precise effects of immune activation depend on integration of the effects of all activated PRRs (Rosenberger et al., 2014). The extraction process used to remove toxins and denature proteins may have altered antigen availability on the spore surface activating differential PRR responses. It is also probable that TX spores, because of their many additional chemical components, interact with more PRRs than NTX spores. Activation of additional receptors may have blocked anxiety-like responses. Alternatively, since TX spores cause more rapid inflammatory effects than NTX spores (Yike et al., 2005), perhaps TX spores increased anxiety during the first 3 weeks of treatment when no testing was done.

After running 6 blocks of mice that arrived in the lab at six weeks of age, we decided to move to 13-week-old mice. Since contextual memory is supported by hippocampal neurogenesis (Huckleberry et al., 2018), we wanted to study mold’s effects in older mice that had significantly lower basal levels of neurogenesis (Kempermann et al., 1998). At the same time, we switched from mice coming from standard housing at the vendor to mice from maximum barrier rooms to minimize subjects’ prior pathogen exposure. Blocks of

mice from standard housing shipped at 6 wks and then mold exposed had clear deficits in contextual memory, but mice from maximal barrier rooms shipped at 13 wks tested using the same conditioned fear paradigm showed no deficits in hippocampal-dependent memory. Instead these mold-exposed mice showed enhanced responses to the auditory cue previously paired with shock, a nonhippocampal task. A likely explanation for mice shipped at 6 wks showing strong deficits in contextual memory, while mice shipped at 13 wks did not is innate immune priming. Early immune challenge can prime the immune system, increasing responses to immune challenges later in life (Bilbo and Schwarz, 2012). Five-to-six-week-old C57Bl/6 mice are very sensitive to shipping stress (Laroche et al., 2009; Blaustein et al., 2016). Both males and females shipped at this age showed deficits in adult behavior not seen in mice shipped at younger or older ages. Extensive studies in females found that such behavioral deficits were highly replicable. As adults, mice shipped during this sensitive period performed more poorly on hippocampal-dependent cognitive tasks (Blaustein et al., 2016). Interestingly, immune challenge with LPS during this sensitive period caused similar cognitive deficits (Blaustein et al., 2016). Thus, shipping C57Bl/6 mice during week 6 appears to alter brain immune function long term, making mice more vulnerable to later immune challenge and hippocampal deficits. The fact that these mice came from standard rooms also increased the likelihood that they experienced greater immune challenge than the mice shipped at 13 weeks from barrier rooms. Mice shipped at 6 wks and tested on conditioned fear were also mildly food restricted which might have contributed to this difference between age groups.

When mice shipped at 13 wks were trained/tested on the conditioned fear task, they showed enhanced responses to the auditory cue. Enhanced fear of the auditory cue is not a classic symptom of innate immune activation but is consistent with mold exposure being stressful. A variety of stressors enhance consolidation of fear memories (Roozendaal and McGaugh, 2011; Schwabe et al., 2012). The fact that we trained mice with two cue/shock pairings a month apart (one before instillations and one after 3 wks of instillations) probably contributed to increased fear of the auditory cue. Most studies use multiple closely-spaced training trials causing a ceiling effect—mice show maximal levels of freezing by the final training trial. Because our mice had only two widely-separated training trials with a mild shock, their responses could strengthen over time.

Intriguingly, enhanced fear of the auditory cue over time in NTX and TX mice correlated strongly with numbers of IL-1β-ir cells in various areas of the dorsomedial hippocampus. The dorsomedial hippocampus is not thought to be involved in the circuit mediating auditory fear (Fanselow and Dong, 2010). However, this does not imply that it may not modulate the process. One recent study found that IL-1β-ir increased in the dorsal hippocampus following footshock in a stress-enhanced learning paradigm, causing enhanced fear responding on later tests (Jones et al., 2018).

Small increases in hippocampal IL-1β are crucial for long-term potentiation and forming long-term memories, and IL-1β has been implicated in improved memory in other circumstances (Yirmiya and Goshen, 2011). We found the strongest relationships between IL-1β and auditory-cued fear memory in TX mice which had significantly higher numbers of IL-1β-ir cells than the other groups. In TX mice, the greater the number of IL-1β-ir cells

in all hippocampal areas, the more mice moved immediately after being shocked during training, but the less they moved 25 h later following presentation of the auditory cue (i.e., the stronger their memory or increase in fear). NTX mice showed smaller nonsignificant increases in hippocampal IL-1β-ir cells, and only numbers of IL-1β-ir cells in CA1 and dorsal hippocampus as a whole correlated significantly with increased response to the auditory cue over time in NTX mice. There were no significant correlations with movement post shock. Increased response to the auditory cue may also be related to the fact that TX and NTX mice had lower pain thresholds at the time this testing was done, with TX mice being the most sensitive. Thus, mold-treated mice may have perceived the shock to be more painful, causing enhanced responses over time.

Animals or people exposed to peripheral immune challenge show increased sensitivity to pain. Data strongly implicate both peripheral and central IL-1β in development as well as maintenance of increased pain sensitivity (Ren and Torres, 2009). In animal models, increased pain sensitivity has been associated with increased IL-1β expression in the hippocampus (del Rey et al., 2012). As predicted, mold exposure increased pain sensitivity in both NTX and TX mice, with TX mice being more sensitive. The most common effect was a decrease in maximal latencies mold-exposed mice waited before moving their tails away from the heat source. With repeated testing, VEH mice also became more sensitive, and their latencies to respond fell to levels seen in NTX and TX mice.

To control for possible confounding circadian effects, this research was run treating and testing small blocks of mice, so that all of the behavioral testing was done during the morning and all treatments done during a fairly narrow time frame in the afternoon. Data from two or more blocks were combined for analysis. While we attempted to control as many variables as possible, some characteristics and/or treatments varied across blocks. Blocks were run at different times of the year when space was available, and different individuals were involved in handling, treating, and testing the various blocks. Experimenter sex significantly affects the outcome of behavioral tests in mice (Sorge et al., 2014; Bohlen et al., 2014). All of our mice were handled by both sexes, but the sex of individuals performing specific tasks varied block to block, instillation to instillation, test to test. We think that the clear effects of mold exposure despite some variation in treatments between blocks, and the consistency of these effects with previous research on innate immune activation, strengthen our conclusions.

While our results document adverse effects of repeated exposure to toxic and nontoxic mold spores on brain and behavior, our stimuli are not representative of typical human mold exposure. People are typically exposed to complex mixtures of molds, bacteria, and dust that activate stronger, synergistic immune responses than predicted by adding the effects of the individual stimuli (Ratnaseelan et al., 2018; Heseltine and Rosen, 2009; Morris et al., 2016). Our spore doses were substantially higher than reported human exposures. However, mold contamination in water-damaged buildings is generally underestimated (Eduard, 2009; Nevalainen et al., 2015). Additionally, most mold exposure is not to spores but to nanoparticles that are often hundreds to as much as a million times more numerous, penetrate much deeper into the lungs than whole spores (Heseltine and Rosen, 2009; Miller and McMullin, 2014; Nevalainen et al., 2015), and are more likely to cause adverse health

effects (Gorny et al., 2002; Adhikari et al., 2013). Inhaled mold nanoparticles carry the additional risk of directly accessing the brain via olfactory pathways (Tonelli and Postolache, 2010). Another issue is that a single species of mold may produce over 90 bioactive metabolites (Peitzsch et al., 2012) which are rarely quantified, even though they may pose major health risks (Eduard, 2009). For example, Inamdar and coworkers (2013) identified a volatile semiochemical that helps give mold its distinctive musty odor. Exposing drosophila to 0.5 ppm of this chemical caused significant loss of dopamine neurons, decreased dopamine levels, and initiated onset of Parkinson's-like symptoms, suggesting that exposure to molds producing this chemical may be another environmental risk factor for Parkinson's disease. Furthermore, roughly 25% of Americans carry major histocompatibility complex gene variants that make them susceptible to long-term inflammation following mold exposure, including the initiation of autoimmune problems and changes in brain structure/function (see Table 3 in Shoemaker et al., 2017). Since exposure to mold often persists for months or even years, it might be expected to cause prolonged innate immune activation of the type already demonstrated to be neurotoxic in a variety of diseases (Ransohoff, 2016). But even if mold exposure is terminated in a timely fashion, the effects of brain immune activation often persist beyond resolution of the initial activation (Bilbo, 2013). Moreover, chronic exposure to moldy workplaces changed the immune response to later mold or mycotoxin exposure (Rosenblum Lichtenstein et al., 2015). Peripheral blood mononuclear cells from mold-exposed people showed significantly different patterns of cytokine and chemokine secretion than those of unexposed controls.

Mold exposure is clearly a problem. Floods, building and plumbing leaks result in widespread mold contamination. Our data document for the first time that exposure to known quantities of both toxic and nontoxic mold spores activated a central neural immune response with concomitant cognitive and emotional dysfunction. In addition, we identified a mechanism, innate immune activation, which aptly explains how mold exposure may cause such a diverse array of problems. The extent of the contribution of mold exposure to neural and behavioral dysfunction in humans under ecologically-relevant conditions remains to be determined. However, mold exposure, both toxic and nontoxic, must be considered another factor, like pesticide exposure or smoking, that can add to an individual's burden of inflammation with possible serious consequences for health and behavior.

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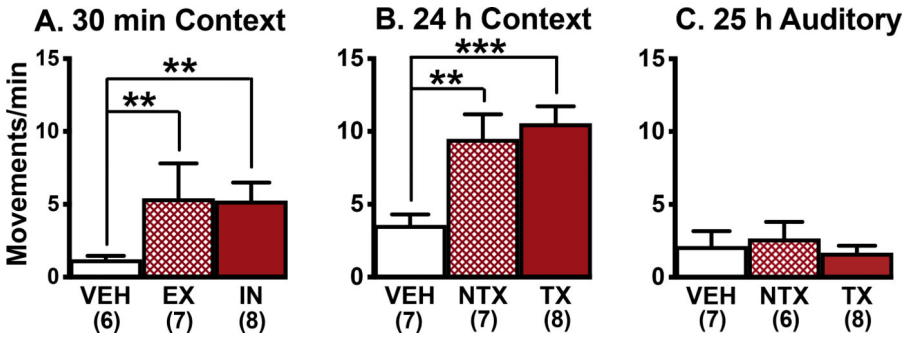

Fig. 1.

Both TX and NTX spores caused significant deficits in hippocampal-dependent contextual (A, B), but not non-hippocampal auditory-cued memory (C) compared to vehicle treatment. $^{**}P < 0.01$, $^{***}P < 0.001$, (Ns).

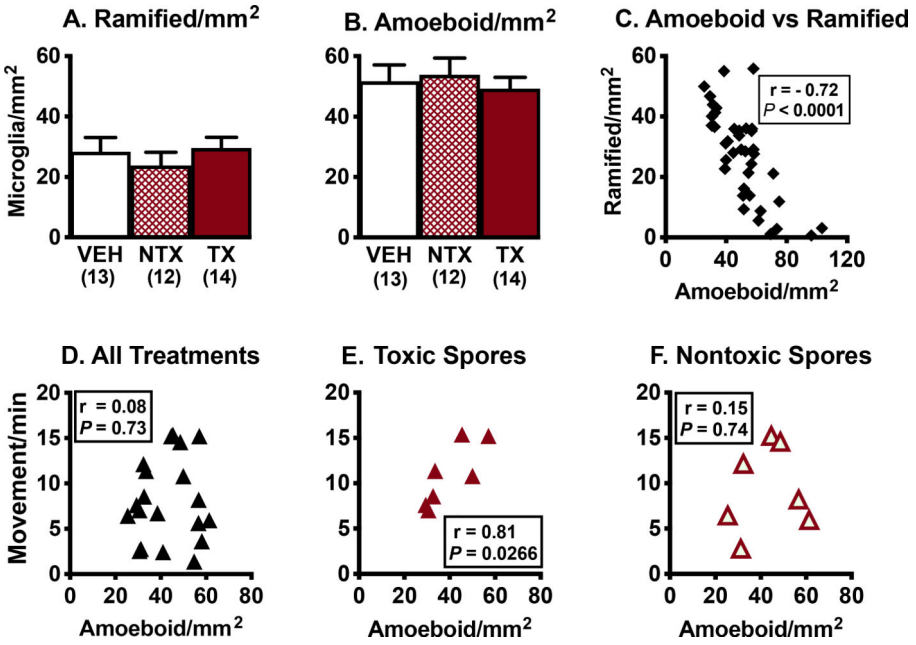

Fig. 2.

Exposure to either NTX or TX mold spores did not affect numbers of A. ramified or B. amoeboid microglia in the dorsomedial DG. C. Numbers of amoeboid and ramified microglia were inversely related. D. There was no relationship between numbers of amoeboid microglia and contextual memory deficits across all treatments. E. However, in mice treated with TX spores, the more amoeboid microglia in the DG, the more the animal moved on the test of contextual memory 24 h after training (i.e., the larger the memory deficit). F. This relationship was not seen in mice treated with NTX spores.

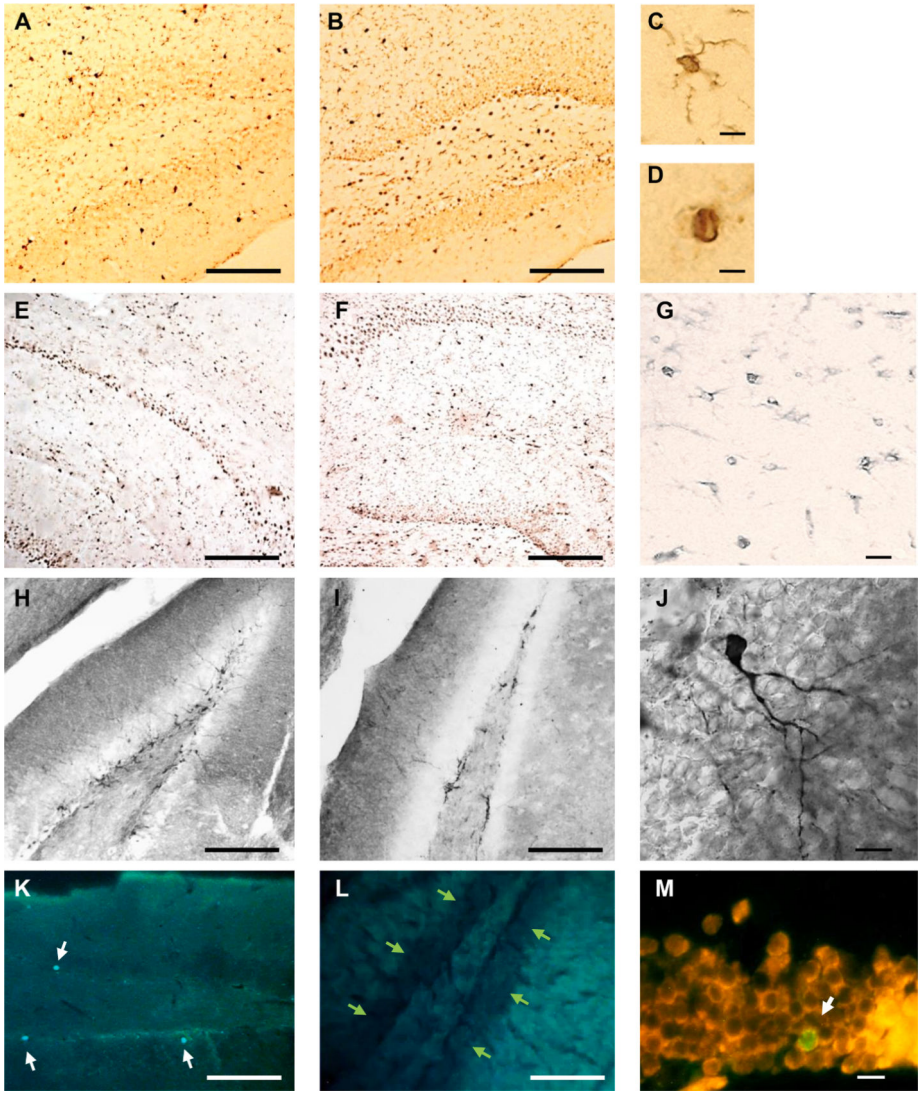

Fig. 3.

Representative IHC. Iba-1+ cells in A. VEH-treated and B. TX-treated mouse, C. Ramified, and D. Amoeboid microglia in a TX-treated mouse. E. IL-1β + cells in VEH-treated and F, G, TX-treated mice. H. DCX+ cells in the dentate gyrus of VEH-treated and I. NTX-treated mouse. J. DCX+ cell from H. K. DG viewed under FITC filter showing 3 BrdU+ cells in VEH-treated mouse (white arrows) and L. lack of BrdU+ cells in TX-treated mouse. Green arrows indicate the granular layer of the dentate gyrus. M. BrdU + /NeuN+ cell (white arrow) in the granule cell layer of the DG shown with a double FITC/rhodamine filter in VEH-treated mouse. All sections are dorsal hippocampus. Scale bars A, B, E, F, H, I, K, L 250 μm; C,D = 10 μm; G = 25 μm; J,M = 20 μm. A, B, E, F, H, I, K, L viewed under 10x magnification; C, D, G, J, M, viewed under 60x magnification.

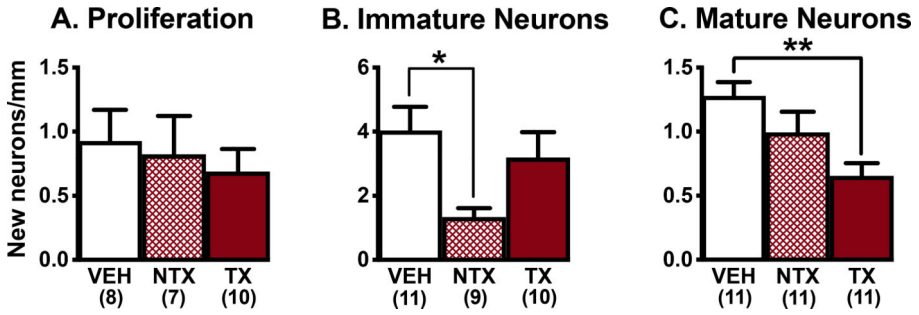

Fig. 4.

Mold exposure decreased two out of three stages of neurogenesis in the dentate gyrus. A. Mold exposure did not affect new neuron proliferation as measured by BrdU/Hu-labeled cells produced in 2 h following BrdU injection. B. NTX spore treatment significantly decreased numbers of immature doublecortin-expressing neurons. C. TX spore treatment significantly decreased numbers of mature neurons double-labeled for BrdU/NeuN. $*P < 0.05$, $**P < 0.01$, (Ns).

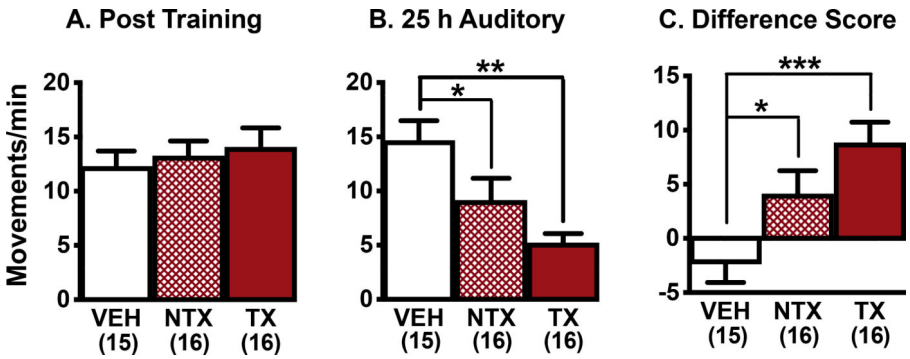

Fig. 5.

Mice treated with NTX and TX spores moved less in response to the auditory cue. A. All groups showed equivalent movements/min immediately following tone/footshock training. B. Twenty-five hours later, mice treated with NTX or TX spores showed significantly stronger responses to the auditory cue. C. The same data plotted as difference scores. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, (Ns).

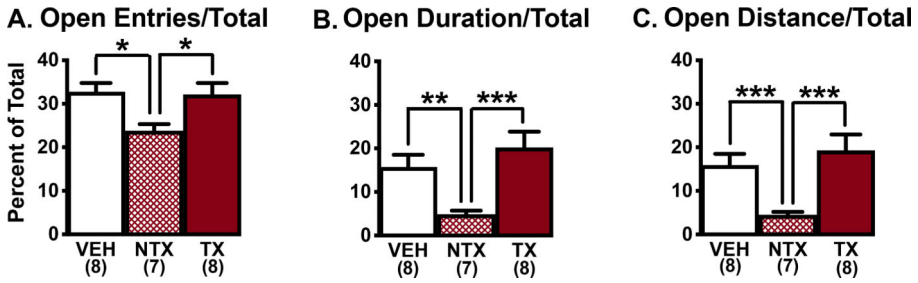

Fig. 6.

NTX spores increased anxiety-like behavior. A. NTX-treated mice entered the open arms significantly less often, B. spent significantly less time in the open arms, and C. traveled less distance in the open arms compared to both VEH and TX mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, (Ns).

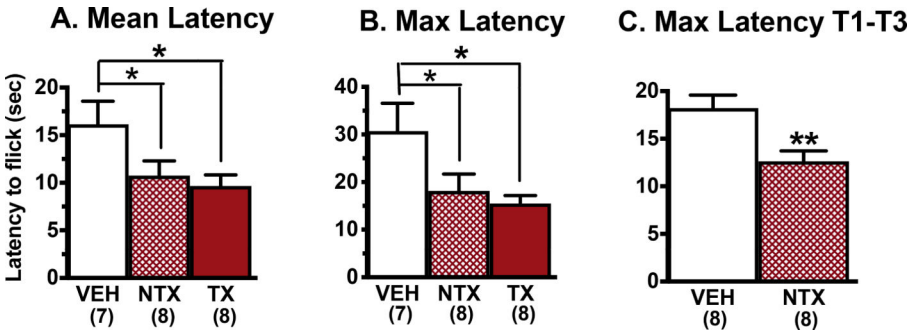

Fig. 7.

Exposure to both NTX and TX mold spores significantly decreased pain thresholds as measured by latency to tail flick. After 13 instillations, mice exposed to 15,000 NTX or TX spores/g had significantly lower A. mean and B. maximal pain thresholds over the three trials than VEH-treated mice. A second experiment contrasted the effects of 400 NTX spores/g with VEH. C. NTX-treated mice had lower maximal pain thresholds than VEH mice over the first three tail-flick tests after 9, 12, and 15 instillations. $*P < 0.05$, $**P < 0.01$, (Ns).

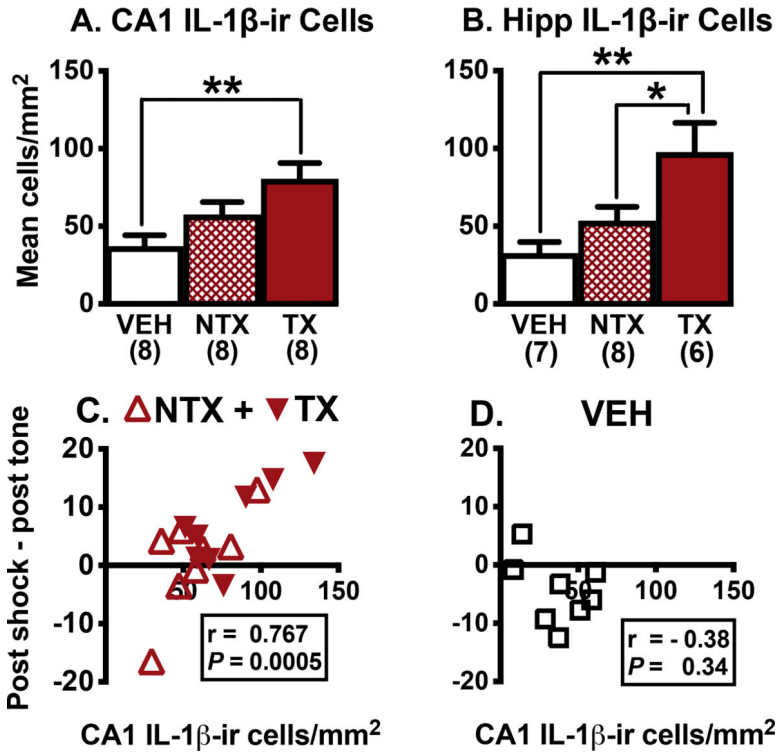

Fig. 8.

TX spore exposure increased numbers of cells producing the inflammatory cytokine IL-1β in dorsomedial hippocampus. Numbers of IL-1β-ir cells in CA1 were significantly correlated with increased memory of the auditory cue in NTX and TX mice. TX spore exposure significantly increased IL-1β-ir cells in A. dorsomedial CA1 and B. dorsomedial hippocampus. In hippocampus (B), TX mice also had significantly more IL-1β-ir cells than NTX mice. $*P < 0.05$, $**P < 0.01$, (Ns). C. Numbers of IL-1β-ir cells in CA1 in spore-treated mice (NTX + TX) correlated strongly with developing greater fear of the auditory cue previously paired with footshock. D. VEH mice did not show this relationship.

Table 1

Relationship between numbers of IL-1β-ir cells in the dorsomedial hippocampus and conditioned fear. A. Pearson correlation coefficients between numbers of IL-1β-ir cells in five hippocampal areas and change in the animal's response over time, corresponding *P* values, and Ns. Significant correlations are bolded. B. Correlation coefficients between numbers of IL-1β-ir cells and response to the tone/shock pairing during training. Different Ns across groups were caused by tissue loss from some animals.

	DG Granular	DG Molecular	DG	CA1	Hippocampus
A. Post Shock – Post Tone					
ALL	0.34, 0.11, 23	0.35, 0.10, 23	0.34, 0.12, 23	**0.67, 0.0004, 24**	**0.67, 0.0004, 24**
VEH	−0.21, 0.62, 8	−0.44, 0.28, 8	−0.52, 0.18, 8	−0.38, 0.35, 8	−0.36, 0.38, 8
NTX	0.01, 0.99, 8	0.39, 0.34, 8	0.48, 0.23, 8	**0.71, 0.05, 8**	**0.71, 0.048, 8**
TX	**0.86, 0.01, 7**	**0.83, 0.02, 7**	**0.81, 0.026, 7**	**0.77, 0.024, 8**	**0.73, 0.042, 8**
B. Post Shock					
ALL	0.40, 0.06, 23	**0.44, 0.035, 23**	**0.45, 0.03, 23**	**0.46, 0.025, 24**	**0.50, 0.014, 24**
VEH	0.15, 0.73, 8	0.13, 0.76, 8	0.13, 0.75, 8	0.49, 0.21, 8	0.33, 0.43, 8
NTX	−0.16, 0.70, 8	0.004, 0.99, 8	0.02, 0.97, 8	0.07, 0.87, 8	0.14, 0.75, 8
TX	**0.83, 0.022, 7**	**0.84, 0.019, 7**	**0.84 0.018, 7**	**0.86, 0.006, 8**	**0.84, 0.009, 8**