A common fungal volatile organic compound induces a nitric oxide mediated inflammatory response in *Drosophila melanogaster*

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Using a *Drosophila* model, we previously demonstrated truncated life span and neurotoxicity with exposure to 1-octen-3-ol, the volatile organic compound (VOC) responsible for much of the musty odor found in mold-contaminated indoor spaces. In this report, using biochemical and immunological assays, we show that exposure to 0.5 ppm 1-octen-3-ol induces a nitric oxide (NO) mediated inflammatory response in hemocytes, *Drosophila* innate immune cells. Moreover, exposed *Drosophila* brains show increased peroxynitrite expression. An increase in nitrite levels is observed with toluene and 1-octen-3-ol but not with 1-butanol. Pharmacological inhibitors of nitric oxide synthase (NOS) namely, L-NAME, D-NAME and minocycline, and NOS mutants show improvements of life span among 1-octen-3-ol exposed flies. Exposure to 1-octen-3-ol also induces NOS expression in larval tracheal tissues and remodels tracheal epithelial lining. These findings suggest a possible mechanistic basis for some of the reported adverse health effects attributed to mold exposure and demonstrates the utility of this *in vivo* *Drosophila* model to complement existing model systems for understanding the role of inflammation in VOC-mediated toxicity.

Inflammation via NO plays a crucial role in the development and progression of neurodegenerative diseases and asthma. Macrophages and their mediators, including NO, are implemented in xenobiotic induced tissue injury and toxicity, where activation of macrophages protects the host from toxins and pathogens and serves essential functions for the survival of organisms. Conversely, excessive and unregulated activation of macrophages including microglia, the resident immune cells of brain, act as agents of destruction and thus promote tissue injury and disease. Indoor air-VOCs and other chemical exposures are associated with neurogenic inflammation. Similarly, NO induction is detected in lung linings of rodent models after exposure to VOCs. Peroxynitrite, derived from the combination of reactive oxygen species, especially superoxide and NO, interacts with cellular components, lipids, DNA, and proteins and triggers cellular responses that range from harmless cell signaling to overwhelming oxidative injury leading to necrosis or apoptosis.

Invertebrate hemocytes have been used as a model to study and measure the impact of chemicals on the immune system, including pesticides and heavy materials. *Drosophila* hemocytes act as a surveillance system and respond to foreign agents like bacteria and parasites via stimulation of nitric oxide synthase (NOS), an enzyme for NO production. These hemocytes are present in both larval and adult *Drosophila*, and are considered morphologically and functionally similar to mammalian microglia.

Exposure to fungal contamination in water damaged buildings is correlated with adverse human health effects. Fungal VOCs associated with moldy odors are hypothesized to contribute to such health effects. Several of these compounds are toxic to mammalian models and cell cultures. One of the major components of mold VOC mixtures is 1-octen-3-ol, a compound known to be ubiquitously produced by fungi, which is commonly detected reported in moldy, water damaged office buildings, residences and classrooms.

We have pioneered a *Drosophila* model to characterize the toxicity profile of mixtures of VOCs emitted from living fungal cultures. Exposure to 1-octen-3-ol leads to neurotoxicity, stimulated apoptotic signaling pathways in flies and cytotoxicity in human embryonic stem cells. Furthermore, human volunteers exposed to 1.9 ppm of 1-octen-3-ol for 2 hours showed an increase in inflammatory markers in nasal secretions.

Induction of the *Drosophila* innate immune response has been demonstrated against parasites and bacteria. There has been no study with respect to a possible toxic chemical mediated induction of such innate immune responses.
response in flies that is comparable to microglial activation in mammals. In this report, we demonstrate that exposure to low concentrations (0.5 ppm) of 1-octen-3-ol vapors results in induction of NOS in Drosophila adult brain and larval tracheal linings. The exposure to 1-octen-3-ol appears to activate Drosophila innate immune cells. Taken together, these data demonstrate that a common fungal VOC leads to excessive stimulation of the inflammatory response and subsequent toxicity, thereby providing a possible mechanistic basis for some of the reported adverse health effects attributed to mold exposure.

Results
To evaluate if 1-octen-3-ol exposure leads to activation of the inflammatory marker, NO; we quantified the level of nitrites, the product of NO breakdown, after exposure to 0.5 ppm of 1-octen-3-ol, using a modified Griess reagent 24. An increase in nitrite levels was found in extracts of 0.5 ppm 1-octen-3-ol-exposed head, body and whole flies but not in unexposed flies (Fig. 1a). The increased nitrite levels from exposed whole flies were further verified by performing qRT-PCR (Fig. 1b). After 6 hr, a more intense purple discoloration (indicative of elevated diaphorase activity) was detected in the anterior lobe and mushroom body regions of adult brains from exposed brains than in unexposed brains (Fig. 1c, d). The increase in the nitrite levels and diaphorase activity in the exposed flies suggests the activation of NO in response to 1-octen-3-ol.

Since we have previously showed that exposure to 1-octen-3-ol induces reactive oxygen species in Drosophila head extracts 25, we hypothesized that peroxynitrite is also involved in 1-octen-3-ol mediated toxicity. We performed immunostaining using anti-nitrotyrosine antibody and found increased expression in the exposed adult brains as compared to non-exposed control brains (Fig 1e, f, g, h).

Furthermore, we also previously showed that exposure to 1-octen-3-ol is associated with the shortening of the fly survival span 18-21. In order to assess the role of NO/peroxynitrite in 1-octen-3-ol mediated truncation of life span, we exposed 0.5 ppm 1-octen-3-ol to adult flies in the presence of the NO inhibitors, D-NAMe, L-NAMe and minocycline at a concentration of 2 mM. The feeding of L-NAME and minocycline led to an improvement in survival span by 6 and 8 days, respectively, indicating that truncation of life span with 1-octen-3-ol exposure is at least partially due to NO/peroxynitrite-induced damage and that prevention of 1-octen-3-ol-mediated NOS induction could be neuroprotective (Fig. 2a). Furthermore, the heterozygous mutant flies for NOS, NOS24283 exposed to 0.5 ppm of 1-octen-3-ol had a 3 day improvement in survival duration compared to exposed wild type flies (Fig 2b). To determine if the induction of NO/peroxynitrite is specific to 1-octen-3-ol, we tested tolune, a well-known industrial solvent, and 1-butanol another fungal VOC. Previously, we have found that exposure of 2.8 ppm of tolune, but not 1-butanol, led to significant truncation of survival span of flies 18. Here we assessed the level of nitrites in the extracts of head, body and whole flies exposed to 2.8 ppm of tolune and 1-butanol. There was a significant increase in the nitrite levels in the extracts of flies exposed to tolune as reported in mammalian model 25 but not in those exposed to 1-butanol when compared with the control extracts (Fig. 2c). This suggests that the phenomenon of induction of NO/peroxynitrite is common to 1-octen-3-ol and tolune but not to 1-butanol.

The activation of NO and peroxynitrite in response to 1-octen-3-ol exposure led us to seek for the source of the Drosophila NO. In Drosophila, glial cells are known to perform immune-like functions during development and remodeling of the nervous system 26 and in a genetic model of neurodegeneration 27. Therefore, we used glial reporter, Repo-GALA; UAS-eGFP transgenic lines to determine if glial cells are the source of NOS positive signal in response to 1-octen-3-ol exposure. Although 1-octen-3-ol exposure intensified the GFP signal of the repo-positive glial cells in the adult brain but no co-localization between NOS signal and GFP labelled glial cells was observed (Fig. 3a,b). Since Drosophila responded to infection by gram negative bacteria and parasitoids via stimulation of NO in hemocytes 8,9, we then sought to investigate if hemocytes were involved in our observed activation of NO. We extracted hemocytes from control and exposed 3rd instar larvae of He-GALA; UAS-eGFP and performed co-immunostaining for NOS and larval hemocytes using universal anti-NOS and anti-H2 antibodies. We detected co-immunostaining of NOS and H2 with GFP-labeled larval hemocytes, thus confirming that larval hemocytes were the source of NOS (Fig. 3 c,d,e,f). There was also increase in the expression of NOS in the exposed hemocytes (Fig. 3g).

Our earlier studies showed that 1-octen-3-ol exposure causes neurotoxicity in adult Drosophila brain 9,28. In order to determine if the activation of NOS was via adult hemocytes in response to 1-octen-3-ol induced toxicity, we performed co-immunostaining to detect the expression of NOS and anti-P1 (plasmatocyte-specific) antibodies in adult Drosophila brain in control (unexposed) and exposed (0.5 ppm for 6 hr) adult flies. Unexposed control adult brain showed few discrete NOS positive cells, while exposed adult brain showed an increased number of NOS positive cells that exhibited rounded to amoeboïd cell shape (ranging from 0.5 μm to 2 μm in size) that co-localized with anti-P1 positive cells (Fig. 4 a,b,c,d). Since fly-hemocytes are considered morphologically and functionally similar to mammalian microglia, the co-localization of expression of NOS and P1 positive plasmatocytes in adult brain, along with an increase in the expression of NOS and P1 positive cells in the exposed brains, indicate stimulation of a microglial-like response against 1-octen-3-ol in adult brain (Fig. 4 e,f).

In rats, exposure of volatile industrial compounds is associated with up regulation of NO and other inflammatory markers in airway linings 8. Upon exposure to 0.5 ppm of fungal VOC, 1-octen-3-ol for 6 hr, induction of NOS was seen in primary and secondary branches of larval tracheal linings where the source of NOS was confirmed as larval hemocytes (Fig 5 a,b,c,d). Furthermore, increased numbers of nuclei were detected in the distal branches of tracheal linings of exposed larvae indicating that exposure to 1-octen-3-ol possibly led to remodeling of the epithelial lining (Fig 5 e,f). Similar changes in the Drosophila tracheal lining have been observed in flies challenged with gram negative bacteria 29 and such remodeling is a common pathological feature of asthma 30.

Discussion
Exposure to environmental agents is a major risk factor for various pathological conditions 32. Although industrial chemicals have received the most intense research focus, epidemiological studies have correlated the presence of fungi and their metabolic products with adverse health consequences in indoor environments 11,12. Most of the research on mold has focused on mycotoxins 31 while fungal volatile organic compounds (VOCs) have received less attention 13,14. These VOCs are low-molecular-weight compounds found in the gaseous state under normal atmospheric temperature and pressure, that are emitted by growing molds as a mixture of aldehydes, alcohols, esters, ethers, terpenoids and other compounds 4. The C-8 compound, 1-octen-3-ol, commonly called “mushroom alcohol”, is a major VOC produced by fungi and is also one of the major fungal VOCs emitted by molds commonly found in moldy and water damaged buildings 15-17. Our previous reports have demonstrated that exposure of flies to 1-octen-3-ol causes neurotoxicity by means of a selective loss of dopaminergic neurons 19,32, and it stimulates the caspase-3 dependent apoptotic signaling pathway 32. Furthermore, 1-octen-3-ol and its enantiomers display cytotoxicity to human embryonic stem cells 32.

Nitric oxide (NO), a fundamental signaling agent, regulates various cellular functions and serves as a potent mediator of inflammation and cellular damage/cytotoxicity 8,22. In a Scandinavian study, using human volunteers, 2 hr exposure to 1.9 ppm of 1-octen-3-ol induced activation of the inflammatory markers; eosinophil cationic protein,
myeloperoxidase, lysozyme and albumin in nasal secretions. The NO signaling pathway is well-conserved among different species, including Drosophila. The enzyme nitric oxide synthase (NOS) mediates the oxidation of L-arginine into citrulline and NO in the presence of NADPH and other cofactors. Drosophila possesses a single NOS gene (dNOS) that shares 47% sequence similarity to mammalian neuronal NOS. dNOS activity is reported in fly brains, larval tracheal linings and other tissues. Like mammalian NOS, dNOS regulates diverse biological processes including host immune response. Up-regulation of other components of the Drosophila immune response has been reported in fly models of neurodegenerative diseases. Moreover, in a fashion similar to mammals, Drosophila airway linings express NOS, one of the known asthma susceptibility genes.

In this report, we used Drosophila to investigate if exposure of fungal VOC, 1-octen-3-ol induces an inflammatory response via...
activation nitric oxide. Foley and O’Farrell and Carton et al. found that injections of NOS inhibitor, L-NAME into the Drosophila body cavity prior to infection with gram-negative bacteria and parasites decreased survival of flies, thereby suggesting that NO functions as an important component of the host immune response that counteract the cytotoxicity associated with infectious agents. Thus, NO seems to be a protective mechanism in responding to microbial infections. However, inflammation is a Janus-faced mechanism. It is well known that many of the volatile industrial solvents, environmental chemicals/pollutants, and xenobiotics also induce NOS-mediated inflammatory response in different organ systems including brain and airway linings. In our study, exposure to the industrial solvent, toluene, and the fungal VOC, 1-octen-3-ol, led to an increase in the nitrite levels in the head, body and whole fly extracts while another fungal VOC, 1-butanol, failed to alter nitrite levels. Exposure to toluene is known to induce activation of NO/cGMP in mammalian models and in rat synaptosomes and bronchoalveolar lavage. In our study, the pharmacological inactivation of NOS via inhibitors of NOS, L-NAME and minocycline also lead to improvement in 1-octen-3-ol mediated truncation of survival span. In addition, upon exposure to 1-octen-3-ol, the heterozygous mutant strain for NOS survived 3 days longer than the wild type flies. The heterozygous NOS mutant flies survival was comparable to that of wild type flies under unexposed conditions (data not shown). The immunostaining of adult brains with anti-peroxynitrite antibodies showed the increase in peroxynitrite levels in the 1-octen-3-ol exposed brains indicating that 1-octen-3-ol mediated truncation of survival span is due to production of peroxynitrite as a result of possible interaction of NO with reactive oxygen species generated upon exposure of 1-octen-3-ol. In summary, our data collectively suggest that 1-octen-3-ol mediated toxicity is at least partly mediated via activation of NOS and peroxynitrite in Drosophila.

In mammals, glial cells are non-neuronal cells constituting about 50% of the volume of the CNS and play a key role in support and nutrition to CNS neurons, formation of myelin sheaths and in signal transmission in the CNS. The Drosophila nervous system possesses counterparts for mammalian glia. Further, the glial cell specific transcription factor, repo (reverse polarity), is expressed in nearly all fly glial cells. Recently, glial cells were implicated in the induction of immune response and subsequent neurodegeneration in the Drosophila model of the human disease, Ataxia-Telangiectasia. In contrast, in our study, failure of co-localization between the 1-octen-3-ol induced NOS positive cells and GFP labelled repo-positive glial cells demonstrates that glial cells are not the source of the NOS signal we detected.

Drosophila hemocytes are immune surveillance cells and form crucial components of the cellular immune response. There are mainly three different types of Drosophila hemocytes: plasmatocytes, lamellocytes and crystal cells. The plasmatocytes comprise 90–95% of all mature hemocytes which appear as small rounded cells capable of altering their sizes in response to foreign agents and resemble the mammalian monocyte/macrophage lineage. The plasmatocytes

![Figure 2](image-url)
are the only class of hemocytes known to be present in adult *Drosophila* where hemese-specific (H2) and plasmatocyte-specific (P1) antibodies have been reported. Infection with gram-negative bacteria and parasites induce elevated expression of NOS in hemocytes of *Drosophila* and other invertebrates. In mammalian models for neurodegeneration and xenobiotic toxicity, there is an up-regulation of NOS in macrophages/microglia in response to MPTP, paraquat and other xenobiotics.

Using 3rd instar larvae of transgenic line for hemese, a blood-cell-specific transmembrane protein, *He-GAL4; UAS-eGFP*, we performed co-immunostaining with anti-hemese monoclonal (H2) and anti-NOS antibodies, and confirmed that larval hemocytes indeed express NOS. The quantification of mean fluorescent intensity (MFI) of NOS was elevated in hemocytes exposed to 1-octen-3-ol. (n = 100–150 hemocytes; ** = P < 0.01) and represents a significant difference between control and octen-3-ol exposed larvae.

**Figure 3 | 1-octen-3-ol triggers increased induction of NOS in the exposed hemocytes but not in glial cells.** (a,b) The GFP labelled repo-positive glial cells (*repo-GAL4; UAS-eGFP*) failed to show any co-localization with NOS positive cells although exposure of 1-octen-3-ol intensified the GFP signal of repo-positive glial cells (arrowheads). (c,d) The hemocytes from the 3rd instar stage larvae of *He-GAL4; UAS-eGFP* upon exposure to 1-octen-3-ol for 1 hr demonstrated stimulation of NO by hemocytes which co-localize with H2 specific antibodies where the insets demonstrate the morphology and size of the co-localized cells in control and exposed brain (c,f). (g) The mean fluorescence pixel intensity (MFPI) of NOS was elevated in hemocytes exposed to 1-octen-3-ol. (n = 100–150 hemocytes; ** = P < 0.01) and represents a significant difference between control and octen-3-ol exposed larvae.
by performing the co-immunostaining with anti-NOS and anti-plasmatocytes (P1) antibodies in the control and 1-octen-3-ol (0.5 ppm for 6 hr) exposed adult brain. Interestingly, few discrete NOS positive plasmatocytes were detected in the control brains while exposed adult brain showed an increased number of NOS positive cells that exhibited rounded to amoeboid cell shape, ranging from 0.5 µm to 2 µm in size that co-localized with plasmatocytes. The quantification of mean fluorescent intensity of NOS and plasmatocytes confirmed the elevated expression level of NOS and hemese in exposed hemocytes as compared to the control hemocytes. Increased expression of NOS positive plasmatocytes in the 1-octen-3-ol exposed adult brain is a provocative finding and reports for the first time presence of increased number of microglial cell-like population in the adult Drosophila capable of expressing NOS and acting possibly as cytotoxic agent due to dysregulated expression of plasmatocytes in response to environmental fungal derived chemical, 1-octen-3-ol.

Several mammalian studies have shown that the overactivation and dysregulation of microglia results in amplification of the neuronal damage induced by foreign stimuli/toxins, thereby enhancing neurotoxicity and neurodegeneration. Our study suggests that exposure to a low dose of 1-octen-3-ol induces similar neuroinflammatory responses in adult Drosophila brain and may provide a mechanistic basis for the reported neurological symptoms associated with exposure to mold.

Multiple epidemiological studies have found a positive association of mold and dampness with multiple allergic and respiratory effects. Few groups have advocated Drosophila models for understanding the genetic components of asthma and other inflammatory types of lung disorders. The Drosophila larval airway system shows striking similarities with that of mammals in terms of its physiology, architecture and reaction towards foreign pathogens along with expression of NOS in larval airway linings. In our study, we detected the up-regulation of NOS in the airway linings of the transgenic strains of 3rd instar btl-GAL4::UAS-GFP larvae exposed to 0.5 ppm of 1-octen-3-ol for 6 hr. Moreover, increased numbers of nuclei were detected in the distal branches of tracheal linings of exposed larvae indicating that exposure to 1-octen-3-ol

Figure 4 | Induction of NOS in adult brain by 1-octen-3-ol. (a) Few NOS positive cells were seen in control adult brains (b) Increased NOS positive cells expressed in adult brain exposed to 1-octen-3-ol for 6 hr which co-localized with adult P1 positive cells. The insets demonstrate the morphology and size of the colocalized cells (c,d). (e) The increase in MFPI for NOS and P1 labeled hemocytes in the exposed adult brains and was expressed as a percentage of that of control adult brains. (f) The Pearson co-relation coefficient for control and exposed adult brains for NOS and adult hemocytes showing the degree of co-localization between NOS positive cells and adult hemocytes (n = 10–12).
possibly led to remodeling of the epithelial lining. Wagner et al.\textsuperscript{60} also reported similar changes in the \textit{Drosophila} tracheal lining challenged with gram negative bacteria. Our study shows that exposure of 1-octen-3-ol induces increase expression of NOS as well as airway remodeling, both of which are common pathological features of asthma and inflammatory lung diseases\textsuperscript{29} and may provide cues for the reported positive association between the mold exposure and respiratory symptoms\textsuperscript{16,61}. In summary, our study demonstrates that a common fungal VOC associated with mold-contaminated damp indoor spaces stimulates a NO mediated inflammatory response in \textit{Drosophila} nervous and respiratory tissues. This work opens a new avenue for gaining a mechanistic understanding of the human health effects by mold-emitted metabolites and also demonstrates the utility of this \textit{in vivo} \textit{Drosophila} model to complement existing cell culture systems for studying VOC toxicity.

**Methods**

\textbf{Drosophila strains.} Unless otherwise stated, all experiments were performed using 48 hr post-eclosion, wild type \textit{y}\textsuperscript{1}, \textit{w}\textsuperscript{1118}, a yellow body and white-eyed strain. The
heterozygous mutant strain for NOS, NOS2431 and y w, repo-GAL4 and UAS-eGFP transgenic lines were obtained from Bloomington Stock Center. All fly stocks were reared on Ward’s Instant Drosophila medium (blue) and all experiments were performed at room temperature. The transgenic lines for hemese, hemocyte-specific transmembrane protein, y w, He-Gal-4; UAS-eGFP (chromosome III), y w, btl-Gal4; UAS-btl::GFP which expresses a Btl::GFP fusion protein in btl-expressing tissues, including trachea were gifts from Dr. Janis D’Oonnell (University of Alabama) and have been described previously13,27.

**Chemicals and exposure conditions.** 1-octen-3-ol (99%), NG-Nitro-D-arginine-methyl ester hydrochloride (D-NAME), NG-Nitro-L-arginine-methyl ester hydrochloride (L-NAME), Minocycline (MC), (Sigma) and incubated for 15 min. Nitrite levels were measured spectrophotometrically at 520 nm with concentrations of standard nitrite calculated against a silver nitrite-derived standard curve. Data were presented as a concentration of nitrite per fly-heads.

**Statistical analysis.** Statistical significance was determined by Student’s one-tailed t-test or one way ANOVA with Dunnett’s post-test using GraphPad Prism software (CA, USA). The relevant details of the statistical analysis are described in the Figure legends.

**Drug treatment.** Drug treatment was performed by giving 0.5 ppm 1-octen-3-ol to wild type flies, the exposed and control (un-exposed) adult flies were dissected in PBS and fixed in acetone for 3 min. The brains were rehydrated with PBST and then blocked with 5% FBS in PBS for 30 min. The overnight incubation at 4°C with primary antibodies: anti-rabbit universal monoclonal NOS (1:1000) (Abcam, Cat. No. ab3142) and anti-mouse P1 antibodies, stimulated with 2% paraformaldehyde (PF) and incubated for 15 min. The supernatants and disease.

**Immunostaining of adult brains with anti-NOS and anti-phospho-tyrosine antibodies.** Immunostaining was performed following the protocol published by Kurucz et al27 with slight modifications. Upon exposure to 0.5 ppm 1-octen-3-ol to wild type flies, the exposed and control (un-exposed) adult flies were dissected in PBS and fixed in acetone for 3 min. The brains were rehydrated with PBST and then blocked with 5% FBS in PBS for 30 min. The overnight incubation at 4°C with primary antibodies: anti-rabbit universal monoclonal NOS (1:1000) (Abcam, Cat. No. ab3142) and anti-mouse P1 antibodies, stimulated with 2% paraformaldehyde (PF) and incubated for 15 min. The supernatants and disease.

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