ADULT MICE EXPOSED TO AEROSOLIZED *ALTERNARIA* EXHIBIT NEUROINFLAMMATION IN THE BRAINSTEM BUT NOT REST OF BRAIN

By

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Abstract

Environmental exposure to inhaled man made pollutants can cause an inflammatory response in both the pulmonary system and central nervous system (CNS). This environmentally triggered neuroinflammation correlates with increased susceptibility to neurodevelopmental and neurodegenerative disorders. As yet, few studies have defined whether exposure to common fungal antigens is able to trigger systemic allergic inflammation and induce an inflammatory response in the CNS. In Southern California, a common fungal trigger for allergies is *Alternaria*. Therefore, we chose to test whether weeklong exposure to aerosolized *Alternaria* antigens would trigger neuroinflammation in the brain as a whole, or in brain regions associated with lung function. We used qPCR to quantify regulation of microglial activation (P2Y12), proinflammatory responses (iNOS), and tissue repair/anti-inflammatory responses (Arginase 1 and TGFβ). Currently our studies indicate that while there was no overt change in inflammatory markers in the brain as a whole, inhaled exposure to *Alternaria* antigens did trigger neuroinflammation in the brain stem. This inflammation was characterized by increased microglial activation and decreased anti-inflammatory gene expression. In aggregate, our data demonstrate that exposure to common environmental allergic triggers not only induce systemic inflammatory responses, but also responses in specific brain regions. The observed reduction in anti-inflammatory molecules suggests that these brain regions would have increased susceptibility to injury or pathogen associated brain insults.

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Introduction

Environmental exposure to inhaled man made toxicants has been known to not only cause systemic inflammation in both the pulmonary system and central nervous system, but also correlate with increased susceptibility to neurodevelopmental and neurodegenerative diseases. Previously, our lab has shown that systemic inflammation, such as those that derive from intraperitoneal injections of lipopolysaccharides, can alter the immunological profile of the brain. However, few studies have attempted to define whether exposure to common fungal antigens can trigger allergic inflammation and induce an inflammatory response in the CNS with similar effects. We therefore looked to determine whether inhalation of an aerosolized, naturally occurring irritant can lead to neuroinflammation in the brain, and if so, identify whether that neuroinflammation affects the whole brain or is region-specific.

To do this, we utilized *Alternaria alternata* as our environmental irritant. *Alternaria* is a common fungus of Southern California that can generally be found in soil and plants. As a major aeroallergen in many parts of the world, *Alternaria* has been known to cause extensive inflammation in the lungs, and lead to active asthma symptoms following chronic exposure (Salo *et al.* 2006). Sensitivity to this fungus has also been increasingly recognized as a major determinant for the development and persistence of asthma, the severity of the asthma, and asthma exacerbations (Bush and Prochnau 2004). It is because of its prevalence in the environment and its ability to cause systemic inflammation that *Alternaira* serves as a good candidate as our environmental trigger.

Now among one of the earliest signs of CNS inflammation is through the activation of microglia, or the resident immune cells of the brain (Carson *et al.* 2004). As

the tissue macrophage of the central nervous system, microglia play a critical role in the maintenance, defense, and repair processes of the CNS, and thus continuously monitor neuronal function. In response to injury or any form of neuronal damage, microglia typically migrate to the damaged site, secrete cytokines or neurotrophic factors, and phagocytose cellular debris (Linnartz *et al.* 2010). However, when microglia convert from an immunologically silent state to an activated state, they can exhibit either one of two types of behavior—neurotoxicity or neurotrophy. Microglia can exhibit neurotoxic behavior by producing pro-inflammatory mediators such as cytokines and reactive oxygen species, resulting in microglial phagocytosis during neuroinflammatory processes (Linnartz *et al.* 2010). On the other hand, microglia can also display neurotrophic behavior by triggering both anti-inflammatory and tissue-repair molecules, resulting in wound healing and the resolution of inflammation (Linnartz *et al.* 2010). Regardless of the activated state, microglia are known to play a vital role in maintaining neuronal homeostasis, and it is through their immunoreceptors that a wide variety of functions can be determined.

To assess for neuroinflammation in response to environmental triggers, we targeted four biological markers—P2Y, G-protein coupled 12 receptor (P2Y12), Transforming Growth Factor-Beta (TGFβ), Arginase 1 (ARG 1), and Inducible Nitric Oxide Synthase (iNOS). P2Y12 is a G-protein coupled receptor that is only expressed on microglia and is able to detect nucleotides upon injury (Moore *et al.* 2015). Because it is ADP-responsive, increased levels of P2Y12 expression is not only indicative of microglia activation, but may suggest cellular damage in the CNS. TGFβ, on the other hand, plays a critical role in immunoregulation as an anti-inflammatory biomarker (Suzumura *et al.*

1993). Although typically expressed at low levels by neural cells, TGFβ becomes upregulated in response to brain insults. By analyzing the relative mRNA expression of TGFβ following *Alternaria* exposure, we can determine whether or not a brain insult did occur, and if anti-inflammatory processes took place. The last two biomarkers we targeted were ARG 1 and iNOS. Both ARG 1 and iNOS are competitive receptors for arginine; however, the resulting mechanism for each is antagonistic to each other. Arginase 1 converts arginine into polyamines and ornithines, resulting in tissue-repair processes that focus on wound healing and matrix deposition (Cherry *et al.* 2014). On the other hand, iNOS converts arginine into nitric oxide and other reactive oxygen species, resulting in pro-inflammatory processes that typically involve microglia phagocytosis. Although both pathways are mechanistically different, the increased level of one or the other is indicative of an inflammatory response, and thus we can analyze their expression, as well as the other biomarkers, to determine whether or not *Alternaria* is sufficient to induce systemic inflammation in the CNS.

Methodology

Animals:

Wild type (C57BL/6) mice were purchased from Jackson laboratories and housed in our vivarium. All mice were bred and housed at the University of California, Riverside animal facility with a 12/12-h light/dark cycle (lights on at 06.00 h) under specific pathogen free conditions. Both male and female mice were used in the experiment, with no preference given to any one sex. The Institutional Animal Care and Use Committee at UCR approved all of the animal studies.

Alternaria exposure:

Mice were exposed to aerosolized *Alteranria* continuously for 5 days in a containment chamber designed and built at UC Riverside as part of the BREATHE project (Bridging Regional Ecology and Aerosolized Toxins to understand Health Effects). Once the mice were placed in, the chamber was closed and not opened until the day of analysis. The dosing concentration and particle size were kept constant throughout the 5 days of treatment. The naive animals were kept outside the chamber in the same room. Following treatment, the mice were sacrificed, and their brains removed and processed for RNA isolation and qPCR analysis.

RNA isolation:

Animals were anesthetized and transcardially perfused with saline. The brains were immediately harvested and the right and left hemispheres where mechanically separated. The RNA was isolated from either a single hemisphere or both. The brain tissue was

then homogenized as described by a previous experiment (Puntambekar *et al.* 2011), and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). A phase separation with chloroform followed (Fisher Scientific, Fair lawn, NJ, USA), and finally RNA was precipitated using Isopropanol (Fisher Scientific, Fair lawn, NJ, USA).

qPCR:

The extracted RNA was converted into cDNA using the first strand cDNA Kit (GE healthcare, Pittsburgh, PA, USA) following the manufacturer's protocol using a CFX96 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The relative number of gene transcripts was determined using the calibration standards method for each of the tested genes. The regular PCR products performed with the same gene primers were used to generate the standards for qPCR. The resulting standards were diluted to obtain a standard curve of 50pg, 5pg, 0.5pg, 0.05pg, 0.005pg and 0.0005 pg for the qPCR analysis. Care was taken to make sure that the copy number of HPRT transcripts was in the same order of magnitude in all samples tested because each gene tested was normalized to HPRT expression.

Statistical Analysis

All data were graphed using Prism6 (GraphPad Software, San Diego, CA, USA) and analyzed using a one-way ANOVA. All data are presented as the mean ± SEM. Statistical significance was assigned at $p < 0.05$, with one asterisk designating $p < 0.05$.

Analytical Discussion

Chamber animals showed robust changes in mRNA expression within the brainstem, but little to no change in mRNA expression within the rest of brain.

Groups of mice were either exposed to room air (naïve) or aerosolized *Alternaria* extract (chamber) continuously for 5 days. Following treatment, quantification of mRNA levels demonstrated that only chamber mice exhibited robust changes in neuroimmune biomarker expression. More specifically, however, those changes were seen only within the brainstem but not the rest of the brain **(Fig. 1)**. As seen in **Figure 1**, there is a significant increase in P2Y12 expression in the brainstem of chamber mice (p-value < 0.05). As mentioned previously, P2Y12 is an ADP-responsive G-protein coupled receptor that is solely expressed on microglia and is able to detect nucleotides upon injury (Moore *et al.* 2015). An increased level of P2Y12 expression is typically indicative of microglial activation. The activation of these tissue macrophages essentially signifies a disruption in the neuronal homeostasis of the CNS, indicating possible cellular damage and systemic inflammation.

We further observed a significant decrease in mRNA levels for both TGFβ and Arginase 1 in the brainstem of chamber mice (p-value < 0.05). TGFβ, or transforming growth factor-beta, has been known for specifically inhibiting inflammation caused by microglia (Suzumura *et al.* 1993). Due to its role in immunoregulation, this reduction in TGFβ expression not only suggests a limited response in anti-inflammatory processes, but perhaps indicates an imbalance affecting neuronal homeostasis. The decreased levels of Arginase 1 further implicates this finding. ARG 1 plays a critical role in tissue-repair functions such as wound healing and matrix deposition via the conversion of arginine

into polyamine and ornithines (Cherry *et al.* 2014), and so the observed reduction in both anti-inflammatory and tissue-repair molecules suggests that this brain region would have increased susceptibility to injury or pathogen associated brain insults.

Interestingly, however, no significant differences in iNOS expression and in the ARG 1/iNOS ratio were seen in the brainstem of chamber mice. iNOS is a pro-inflammatory biomarker that produces large amounts of nitric oxide and other reactive oxygen species in the brain (Sierra *et al.* 2014). Given its role in pro-inflammatory processes, we suspected a significant increase in iNOS expression following *Alternaria* exposure, but instead we observed a reduced expression, though not statistically significant. We further analyzed the ARG 1/iNOS ratio to determine if the difference in ARG 1 levels had caused a polarization in the brain's immune response. We found, however, that no significant shift was determined via statistical analysis. Nevertheless, we believe that the increase in P2Y12 expression as well as the significant decreases in both protective (ARG 1) and anti-inflammatory (TGFβ) molecules are fairly indicative of a perturbed CNS following *Alternaria* inhalation.

This same phenomenon, however, is not seen in the rest of the brain. As seen in **Figure 2**, the graphs for TGFβ, ARG1, and iNOS appear to resemble a similar pattern relative to that seen in the brainstem. However, upon statistical analysis, there appears to be no significant difference in P2Y12, TGFβ, ARG 1, iNOS, and ARG 1/iNOS expression between the naïve and chamber in the rest of brain homogenates **(Fig. 2)**. This supports the idea that *Alternaria* can perhaps trigger neuroinflammation in the brain, but only to specific parts of the brain such as those associated with respiratory function.

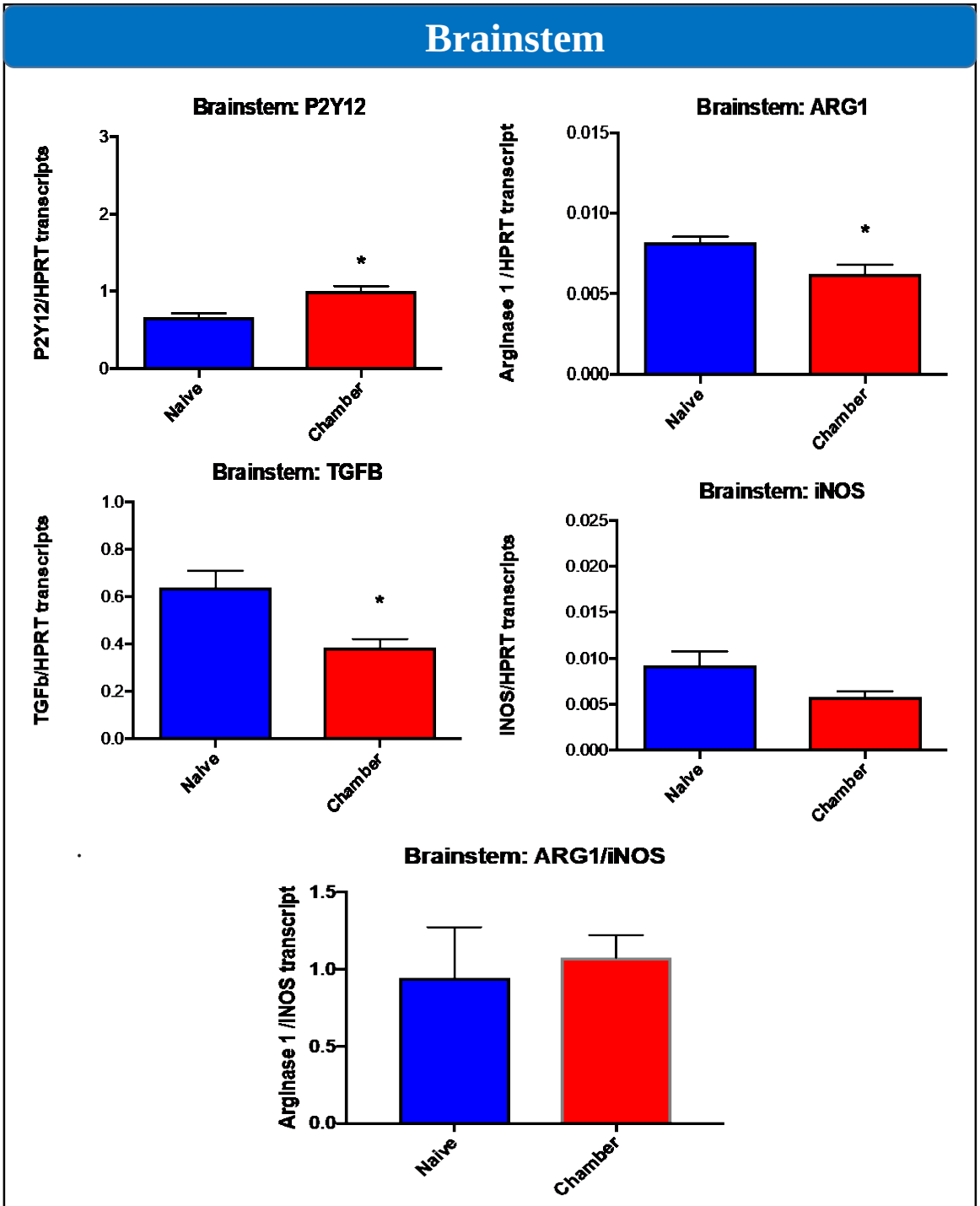

Figure 1. Quantification of neuroimmune biomarker mRNA isolated from brainstem tissue using SYBR-green qPCR. Groups were either exposed to room air (naïve) or aerosolized ALT extract (chamber) continuously for 5 days. Values for gene expression were normalized to HPRT for each gene of interest. Data is represented as means ± SEM; n = 3 for naïve and chamber. Statistical differences were calculated by one-way ANOVA. *$p < 0.05$

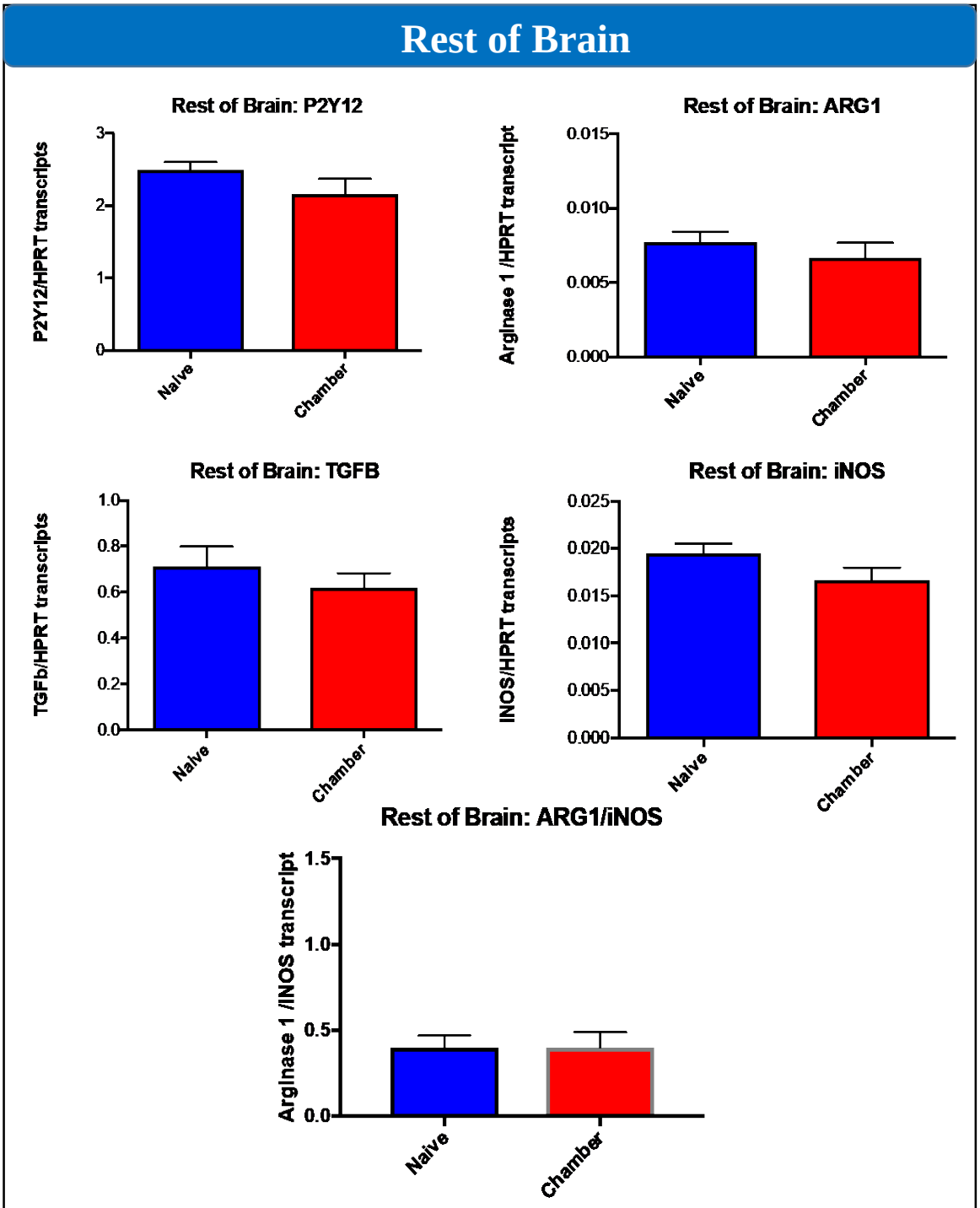

Figure 2. Quantification of neuroimmune biomarker mRNA isolated from the rest of brain tissue using SYBR-green qPCR. Groups were either exposed to room air (naïve) or aerosolized ALT extract (chamber) continuously for 5 days. Values for gene expression were normalized to HPRT for each gene of interest. Data is represented as means ± SEM; n = 3 for naïve and chamber. Statistical differences were calculated by one-way ANOVA. *$p < 0.05$

Conclusion

In this study, we sought to define whether exposure to common fungal antigens is able to trigger systemic allergic inflammation and induce an inflammatory response in the CNS. Currently, our study indicates that while there was no overt change in inflammatory markers in the brain as a whole, inhaled exposure to *Alternaria* antigens did trigger neuroinflammation in the brainstem. Characterized by increased microglial activation (P2Y12) and decreased anti-inflammatory (TGFβ) and tissue-repair (ARG 1) gene expression, our data demonstrate that exposure to common environmental allergic irritants induces systemic inflammatory responses, but in a region-specific manner. The reduced expression of anti-inflammatory molecules not only further indicates a perturbed CNS following *Alternaria* inhalation, but also suggests that this brain region could have increased susceptibility to injury or pathogen associated brain insults.

Despite our limited sample size, our findings herein serve as preliminary data regarding the effects of environmental exposure on CNS inflammation. We found that aerosolized *Alternaria* alone was sufficient to cause changes in the mRNA expression of neuroimmune biomarkers. However, given the increasing concern of environmental pollutants on our health, we aim to develop further studies that examine the relationship between environmentally triggered neuroinflammation and increased susceptibility to neurodevelopmental and neurodegenerative diseases in the future.

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