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Sex differences in the mitochondrial bioenergetics of astrocytes but not microglia at a physiologically relevant brain oxygen tension

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Abstract

Biological sex is thought to influence mitochondrial bioenergetic function. Previous respiration measurements examining brain mitochondrial sex differences were made at atmospheric oxygen using isolated brain mitochondria. Oxygen is 160 mm Hg (21%) in the atmosphere, while the oxygen tension in the brain generally ranges from ~5–45 mm Hg (~1–6% O_2). This study tested the hypothesis that sex and/or brain physiological oxygen tension influence the mitochondrial bioenergetic properties of primary rat cortical astrocytes and microglia. Oxygen consumption was measured with a Seahorse XF24 cell respirometer in an oxygen-controlled environmental chamber. Strikingly, male astrocytes had a higher maximal respiration than female astrocytes when cultured and assayed at 3% O_2. Three percent O_2 yielded a low physiological dissolved O_2 level of ~1.2% (9.1 mm Hg) at the cell monolayer during culture and 1.2–3.0% O_2 during assays. No differences in bioenergetic parameters were observed between male and female astrocytes at 21% O_2 (dissolved O_2 of ~19.7%, 150 mm Hg during culture) or between either of these cell populations and female astrocytes at 3% O_2. In contrast to astrocytes, microglia showed no sex differences in mitochondrial bioenergetic parameters at either oxygen level, regardless of whether they were non-stimulated or activated to a proinflammatory state. There were also no O_2- or sex-dependent differences in proinflammatory TNF-α or IL-1β cytokine secretion measured at 18

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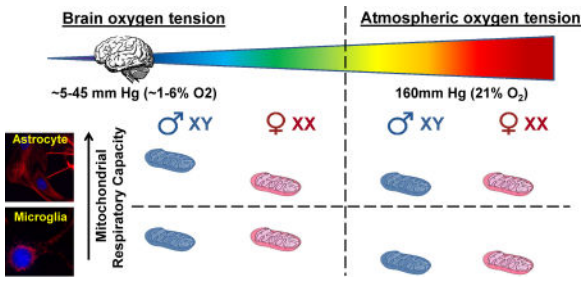
hours activation. Overall, results reveal an intriguing sex variance in astrocytic maximal respiration that requires additional investigation. Findings also demonstrate that sex differences can be masked by conducting experiments at nonphysiological O_2.

Graphical abstract



Keywords

respiration; gender; hyperoxia; hypoxia; lipopolysaccharide; cytokine

1. Introduction

Mitochondria are organelles responsible for ATP production, consuming oxygen in the process. Mitochondrial ATP production is especially important in the brain, as the central nervous system (CNS) consumes ~20% of the total oxygen inspired, while only accounting for ~2% of the total body weight (Silver and Erecinska, 1998). Mitochondrial dysfunction is a nearly ubiquitous occurrence in neurodegenerative diseases (Lin and Beal, 2006, Fiskum et al., 1999) and acute CNS injuries (Fiskum, 2000, Demarest and McCarthy, 2015, Demarest et al., 2016, Robertson et al., 2006). While mitochondrial respiratory impairment is associated with numerous pathologies, evidence for association comes primarily from studies using isolated brain mitochondria, which are derived from both neurons and glia, or from primary neuronal cultures. Roles for mitochondrial respiratory function/dysfunction in neuroglia such as astrocytes and microglia are comparatively understudied, which is surprising since neuroglia are thought to comprise ~50% of the brain, with variation by species (Azevedo et al., 2009, Herculano-Houzel, 2014).

Astrocytes were initially regarded as “glue” or “housekeeping” cells of the brain. However, they are now recognized to have many pivotal functions such as regulation of ion homeostasis (Olsen et al., 2015), neurotransmitter recycling (Schousboe et al., 1993), and control of cerebral blood flow (Cabezas et al., 2014). Astrocytes also carry out important bioenergetic roles in the brain, including regulation of brain glucose uptake, production and storage of brain glycogen, and provision of metabolic and antioxidant support for neurons (Belanger et al., 2011).

Microglia are the resident immune cells of the brain (Kreutzberg, 1996) that play diverse roles in physiology, including surveying the surrounding environment to clear cellular debris, synaptic pruning, and promotion of synaptogenesis (Tremblay et al., 2011, Wu et al., 2015). In response to brain pathology, microglia become “activated” and secrete cytokines,

an initially protective process that becomes maladaptive when failing to resolve in a timely fashion (Brown and Bal-Price, 2003, Block et al., 2007). A shift in cellular bioenergetics from oxidative phosphorylation to glycolysis occurs during microglial activation (Volobouéva et al., 2013, Orihuela et al., 2015).

There is increasing evidence of sex differences in mitochondrial function in both health and disease. A recent study found that isolated brain mitochondria from female mice have higher Complex I-linked respiration than male mitochondria at 3 months, a difference that no longer exists by 20 months or following ovariectomy (Gaignard et al., 2015). Multiple studies demonstrated that the glutathione antioxidant defense system, which protects mitochondrial bioenergetic function, is elevated in females compared to males (Gaignard et al., 2015, Demarest et al., 2016). In adults, this effect is abolished by ovariectomy (Gaignard et al., 2015). While these studies suggest that sex hormones may directly regulate mitochondrial function, fewer studies have examined whether there are intrinsic sex differences in mitochondrial function at the cellular level. A sex difference in respiration was not apparent in brain mitochondria isolated from postnatal day 7 rats (Demarest et al., 2016). However, isolated forebrain mitochondria are derived from multiple cell types which may mask cell type-specific sex differences.

Sexual dimorphism of astrocyte morphology is already evident by the day of birth (Mong and McCarthy, 2002), suggesting that there can be early sex differences in glial cell properties. Dimorphism is thought to occur via an organizational effect of gonadal steroids in males (Mong and McCarthy, 2002). Thus, neonatally prepared glial cells from males will have had hormone exposure *in utero*, allowing for organizational effects (Mong et al., 1996, Mong and McCarthy, 2002), while being devoid of continued gonadal hormone exposure during *in vitro* development. In addition to neonatal astrocytes, sex is also thought to influence neonatal microglial cell properties, including proinflammatory gene expression (Loram et al., 2012). The precedence for intrinsic sex differences in XX vs. XY glial cells makes neonatal primary cultures an excellent system to investigate fundamental mitochondrial sex differences in defined cell populations.

The primary goal of this study was to test the hypothesis that there are sex differences in the mitochondrial respiratory properties of relatively pure populations of rat cortical astrocytes or microglia. An additional goal was to determine whether experimental oxygen tension influences the astrocytic or microglial bioenergetic function of either sex. The vast majority of *in vitro* work on mitochondrial function has been performed at atmospheric oxygen (160 mm Hg, 21% O_2). However, atmospheric O_2 tension is far higher than the pO_2 that reaches cells within the brain (typically 5–45 mm Hg or ~1–6% O_2) (Grote et al., 1996). Oxygen tension regulates many biochemical processes with the potential to impact mitochondrial respiration, including superoxide production (Hoffman et al., 2007), nitric oxide formation (Rengasamy and Johns, 1996) and the stability of oxygen-sensitive transcription factors such as HIF-1α (Semenza, 2012). Therefore, we hypothesized that conducting experiments at a physiologically relevant O_2 level may reveal sex differences in mitochondrial bioenergetics that would not otherwise be observed. The effects of 3% O_2, specifically, were investigated because early studies suggested a reduction in oxidative stress-induced changes in cultured cells at this O_2 level compared to supraphysiological O_2 (Parrinello et al., 2003, Busuttil et

al., 2003). We refer to 3% O_2 as low physiological O_2 because several studies have considered *in vitro* 5% O_2 as brain physiological O_2 (Tiede et al., 2011, Zhu et al., 2012, Sun et al., 2015, Dussmann et al., 2017).

We found that male astrocytes exhibit a higher respiratory capacity than female astrocytes when cultured and assayed at 3% O_2 (dissolved O_2 of ~1.2–3.0%), but not when experiments are conducted at atmospheric 21% O_2. However, we did not find sex differences in bioenergetic parameters or release of two key proinflammatory cytokines in non-stimulated or activated microglia, irrespective of experimental O_2.

2. Materials and Methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

2.2. Preparation of primary rat cortical astrocytes

All procedures were approved by the University of Maryland Institutional Animal Care and Use Committee and were compliant with the NIH Guide for the Care and Use of Laboratory Animals. Primary rat cortical astrocytes were prepared from postnatal day 1 Sprague Dawley rat pups as described (McKenna, 2012). Briefly, rats were sexed visually by comparing the distance between the genitals and anus. Only rats that were easily identifiable as male or female by at least two parties were used in this study. Rat pups were euthanized by decapitation. Cortices were then removed, homogenized by trituration, and vortexed for one minute. Brain homogenate from two pups of the same sex were then passed through a 70 μm filter before plating into two separate tissue culture-treated flasks in Eagle's Minimal Essential Medium (EMEM, Quality Biological, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA, Lonza, Walkerville, MD) and 50 μg/mL gentamicin. One flask was placed in a standard 37°C incubator at 95% air/5% CO_2 (20% O_2, which is 95% of atmospheric 21% O_2). The other flask was placed in a 37°C 92% N_2/5% CO_2/3% O_2 incubator (referred to as 3% O_2). After two days, cell culture medium was changed to gentamycin-free medium and cells were thereafter maintained on medium without gentamicin. At 18 days *in vitro*, cells were trypsinized using TrypLE Express (Thermo Fisher, Waltham, MA), and sub-cultured for at least 24 hours prior to assays. Data represents preparations across 3–4 litters.

2.3. Preparation of primary rat cortical microglia

Primary rat cortical microglia were prepared from cortices of one day old Sprague-Dawley rats as previously described (Wu et al., 2010). Each primary culture preparation combined pups from 2 separate litters, with 3–4 separate preparations being utilized for each study. Briefly, cerebral cortices were dissected, homogenized by serial trituration with progressively narrower serological pipets, and plated in poly-D-lysine-coated culture flasks. Cells were maintained at 20% or 3% O_2, as described above, in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with penicillin (100 IU/mL) plus streptomycin (100 μg/mL) and 10% FBS. Seven days after preparation, flasks

were shaken for one hour at 100 rpm using an Orbi-Shaker™ (Benchmark Scientific, Edison, NJ), after which medium was collected and centrifuged at 1,000*g* for 10 min to isolate microglia. To induce proinflammatory microglial activation, cells were treated with a combination of 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, cat# L2654) and 10 ng/mL recombinant interferon-γ (IFN-γ; R&D Systems, Minneapolis, MN, cat# 485-MI-100) for 18 hours prior to bioenergetic measurements or cytokine quantification.

2.4. Dissolved Oxygen Measurements

Dissolved oxygen at the cell monolayer surface was measured using a commercially available sensor dish reader (SDR; PreSens, Regensburg, Germany) in specialized 24-well O_2-sensing plates (Oxo-Dish OD-24; PreSens). These plates contain an immobilized O_2-sensitive fluorescent patch within the plating surface of each well for measuring dissolved oxygen and come sterilized and calibrated by the manufacturer. Measurements were made at 15 second intervals over a 16 hour period in either a conventional 37°C, 95% air/5% CO_2 humidified incubator or in the 37°C 3% O_2 humidified incubator described above. Astrocytes were plated at 3.6×10^5 cells/well for measurements in the 20% O_2 incubator and at 2.4×10^5 cells/well for measurements at 3% O_2, yielding a comparable cell density at time of analysis (16 hours). A plating volume of 1.2 mL was used for both O_2 levels. Data were acquired using SDR v4.0.0 software (PreSens), and then exported into Microsoft Excel for analysis. Dissolved O_2 at the cell monolayer of microglial cultures was calculated based on the relative O_2 consumption rate of the microglial cultures using Fick's first law:

$$J = D\nabla C - R$$

where J is the flux of O_2 into the system; D is the diffusion coefficient of O_2 into the media; ∇C is the change in O_2 concentration in the x, y, and z directions in the culture media; and R is the cellular O_2 consumption rate (Lewis et al., 2017).

2.5. XF24 microplate-based respirometry

Oxygen consumption rate (OCR) measurements from primary rat cortical astrocytes or microglia were performed using a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) as previously described (Gerencser et al., 2009, Clerc and Polster, 2012). Astrocytes were plated in XF24 V7 plates (Agilent Technologies) at 0.6×10^5 cells per well at 20% O_2 and at 0.4×10^5 cells per well at 3% O_2. Microglia were plated in V7 plates at 1.0×10^5 cells per well. Cells were allowed to attach and culture overnight. Microglia were either untreated or activated for 18 hours prior to assay, as described above. Prior to OCR assays, cells in three of the 24 wells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, pH 8.0). Lysates from these wells were used for protein quantification by the Pierce Micro bicinchoninic acid (BCA) Assay (Thermo Fisher), allowing subsequent normalization of O_2 consumption rates to average protein per well. Artificial cerebrospinal fluid (aCSF) assay medium for respiration measurements consisted of 120 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mM glucose, and 4 mg/mL fatty acid-free bovine serum albumin (BSA), pH 7.4. Cells were incubated in a CO_2-free incubator at 37°C

for 45 minutes prior to assay to allow temperature and pH calibration. XF24 assays consisted of cycles of 3 min mix, 2 min wait, and 2 min measure for astrocytes, and 2 min mix, 1 min wait, and 2 min measure for microglia.

For experiments performed at 3% O_2, the Seahorse XF24 instrument was placed into the workspace of an Xvivo System environmental chamber (Biospherix, Ltd., Parish, NY) that also contained four cell culture incubators with independent gas control. The Xvivo workspace was at room temperature (~25°C) and regulated to 3% O_2 (with no CO_2). A minimum of 4 hours prior to assays, aCSF assay medium and a calibration cartridge containing XF calibrant (Agilent Technologies) were placed into a partitioned 37°C, 3% O_2/97% N_2/0% CO_2 incubator to equilibrate to temperature and O_2. Cells to be assayed at 3% O_2 were cultured within another partitioned incubator set to 3% O_2/92% N_2/5% CO_2 within the Xvivo System. Forty-five minutes prior to assays, cell culture medium was exchanged for 3% O_2-equilibrated aCSF assay medium within the 3% O_2-regulated Xvivo workspace and cells were then transferred to the CO_2-free 3% O_2 partitioned incubator. Injection port drug loading of the cartridge was also conducted at 3% O_2 within the environmental chamber. XF24 assays consisted of the same mix-wait-measurement cycles described above. Importantly, three empty wells of each assay plate (A6, B6, and C6) received four successive injections of 1.0 M sodium sulfite (1:10, 1:11, 1:12, and 1:13 dilutions in the assay wells, respectively) to chemically scavenge oxygen and provide a zero O_2 reference. Sodium sulfite stock (1.0 M) was made fresh immediately prior to each assay by dissolving powder in aCSF assay medium in a glass vial with a tight-fitting lid. For XF assays conducted at 3% O_2, OCRs generated by the XF software were recalculated using the XF Hypoxia Rate Calculator Program (Agilent Technologies). The sodium sulfite zero oxygen reference specifically in wells A6, B6, and C6 is essential for the Hypoxia Rate software to calculate OCR at 3% O_2.

2.6. ELISA analysis of TNF-α and IL-1β

Rat interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were quantified by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols (R&D Systems).

2.7. Statistical analysis

Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc, San Jose, CA). Two-way analysis of variance (ANOVA) was employed to evaluate the statistical significance of oxygen consumption measurements, while three-way ANOVA was used to evaluate the statistical significance of TNF-α and IL-1β measurements. A p value < 0.05 was considered significant. Tukey's post-hoc analysis was used to compare individual groups.

3. Results

3.1. Estimation of dissolved oxygen at the cell monolayer surface

Cells that were assayed at 3% O_2 were also cultured at 3% O_2 and were not exposed to room air prior to measurements. Cells that were analyzed at 21% O_2 were cultured at 20% O_2

(95% of atmospheric 21% O_2) in an incubator containing 95% air and 5% CO_2. Due to cellular O_2 consumption and limitations in cell culture medium O_2 diffusion, dissolved O_2 at the cell monolayer surface is less than the regulated O_2 in air (Abaci et al., 2010). To get a better estimate of the O_2 level at the cell surface for our two O_2 setpoints, we used specialized Oxo-Dish plates containing O_2 sensor patches directly embedded into the plates. For astrocytes cultured in 1.2 mL of media, a volume that produces a similar height of media to that used in Seahorse XF24 assay plates, O_2 at the cell surface during culture was 1.17 ± 0.18% at a set point of 3% O_2 and was 19.71 ± 0.13% O_2 at a set point of 20% O_2 (mean ± SD, n=3).

Next, we used the relative O_2 consumption rates (OCR) of astrocytes and microglia to calculate the cell surface O_2 level for primary microglia cultured at 3% O_2. Because there were no sex differences in basal OCR (see below), cultures from males and females were combined for each cell type. The absolute (non-protein normalized) OCR of astrocytes was 69 ± 23 pmol O_2/min (mean ± SD, n=11) while the absolute OCR of microglia was 49 ± 23 pmol O_2/min (mean ± SD, n=6). Based on the slightly lower O_2 consumption rate of microglia, the dissolved O_2 at the microglial monolayer surface was estimated to be 1.69% during 3% O_2 culture.

During the acquisition of O_2 consumption rates by Seahorse respirometry, cells consume O_2 and frequent media mixing is required. Therefore, during assays, astrocytes likely experience a dynamic O_2 range of ~1.2–3.0% O_2 when measured at 3% O_2 while microglia likely experience a range of ~1.7–3.0% O_2 under the same conditions. Cells measured at 21% O_2 transiently drop below 21% O_2 during 2 min measurements but O_2 level quickly recovers after mixing.

3.2. Astrocyte bioenergetics

The basal OCR of male and female primary rat glial cells was measured at 3% or 21% O_2. Following acquisition of basal OCR, maximal respiration was induced by the addition of the uncoupler FCCP and the cell permeable mitochondrial Complex I substrate pyruvate. Provision of excess substrate was done to preclude any differences due to insufficient substrate supply, enabling us to examine differences in OCR primarily due to differences in electron transport chain function. The Complex III inhibitor antimycin A was added last and any remaining oxygen consumption was regarded as non-mitochondrial respiration.

Primary rat cortical astrocytes showed no significant differences in basal OCR among male and female astrocytes at either 21% or 3% oxygen (Fig. 1A and C–D). There were also no differences among these groups in the amount of basal O_2 consumption used for mitochondrial ATP synthesis, as estimated by the fraction of the basal respiration rate reduced by the ATP synthase inhibitor oligomycin (Fig. 1E and F). However, at 3% O_2 male astrocytes demonstrated a significantly higher maximal respiration rate than female astrocytes (Fig. 1A–D). Male astrocytes at 3% O_2 also had a higher maximal respiration rate than either male or female astrocytes at 21% O_2. The difference in maximal respiration rate remained significant even after respiration was normalized to basal respiration (Fig. 1B), indicating that male astrocytes have greater spare respiratory capacity than female astrocytes at 3% O_2. Spare respiratory capacity, the difference between basal and maximal respiration,

is thought to reflect the capability of cells to respond to increased energy demand with an elevation in oxidative phosphorylation (Nicholls, 2009).

3.3. Microglial bioenergetics

Primary rat cortical microglia were either untreated (CTRL) or stimulated with LPS plus IFN-γ (LPS/IFN-γ) for 18 hours prior to oxygen consumption measurements. There was no difference between male and female cells in basal OCR, maximal OCR, or spare respiratory capacity at either 3% or 21% O_2 (Fig. 2A–D). In both male and female controls, basal OCR was lower at 3% O_2 compared to 21% O_2, an effect that remained consistent following LPS/IFN-γ stimulation (Fig. 2E). There was no oxygen tension-dependent difference in maximal OCR (Fig. 2F), yet spare respiratory capacity of untreated microglia (CTRL) was significantly higher at 3% O_2 compared to 21% O_2 (Fig. 2G). At both 21% O_2 (Fig. 2A and B) and 3% O_2 (Fig. 2C and D), basal and maximal OCR were significantly suppressed by LPS/IFN-γ treatment. There were no sex differences in the extent of inhibition at either oxygen tension. However, both basal and maximal OCR were impaired to a greater degree at 3% O_2 compared to 21% O_2 following LPS/IFN-γ treatment (Fig. 2E and F).

3.4. Microglial release of proinflammatory TNF-α and IL-1β in response to LPS/IFN-γ

A previous study found that neonatal male cortical microglia expressed more IL-1β mRNA than female cells upon 4 hours of LPS stimulation (Loram et al., 2012). Therefore, despite finding no sex differences in the microglial mitochondrial response to LPS/IFN-γ, we decided to test whether sex influences microglial secretion of proinflammatory cytokines. We also evaluated whether physiological oxygen tension, compared to atmospheric O_2, affects cytokine secretion. There were no significant sex or oxygen tension-dependent differences in the levels of TNF-α (Fig. 3A) or IL-1β (Fig. 3B) released by microglia stimulated with LPS/IFN-γ for 18 hours. There were also no differences in basal cytokine release from untreated cells (CTRL, Fig. 3A and B).

4. Discussion

This study demonstrates an intrinsic sex difference in the mitochondrial bioenergetics of rat cortical astrocytes at low physiological 1.2–3.0% O_2 that was not observed at atmospheric O_2. Male astrocytes exhibited a higher maximal respiration rate than female astrocytes, but only when oxygen consumption rate was measured at 3% O_2 following 3% O_2 culture. In contrast to astrocytes, there were no sex differences in the respiratory characteristics of rat cortical microglia at either 3% or 21% O_2, regardless of whether they were non-stimulated or activated by LPS/IFN-γ. Nevertheless, there were some oxygen tension-dependent differences in both nonstimulated and LPS/IFN-γ-stimulated microglia.

Non-stimulated microglia displayed slightly lower basal respiration at 3% O_2 compared to atmospheric O_2. Although maximal OCR was not different, the difference in basal respiration resulted in greater spare respiratory capacity when microglia were at 3% O_2. As expected, LPS/IFN-γ stimulation curbed respiration at both oxygen levels, consistent with the reported metabolic shift from oxidative phosphorylation towards glycolysis upon activation (Volobouéva et al., 2013, Orihuela et al., 2015). However, LPS/IFN-γ induced a

slightly greater suppression of both basal and maximal OCR at 3% O_2 compared to 21% O_2. Activated microglia produce nitric oxide (NO), which contributes to respiratory inhibition (Moss and Bates, 2001). One mechanism by which NO may do so is by competing with molecular oxygen at Complex IV of the electron transport chain (ETC) (Brown and Cooper, 1994, Cleeter et al., 1994). A greater inhibitory effect of NO on the ETC at 1.2–3.0% O_2 compared to 21% O_2 is predicted based on a competition mechanism, potentially accounting for the greater suppression of OCR that was observed when microglia were activated and assayed at 3% O_2.

The Organizational/Activational Hypothesis of hormone action states that gonadallyderived steroid hormones create lasting sex differences in brain circuitry that are then activated by sex-specific hormones in adulthood (McCarthy et al., 2012). Importantly, activation of the testes during a critical window during the late stages of embryonic development leads to a surge in production of the hormone testosterone in males (Gillies and McArthur, 2010, Kight and McCarthy, 2014). This testosterone is aromatized to estradiol. In rodents, it is estradiol that plays a significant role in the masculinization or de-feminization of neural circuitry (referred to as organization) by influencing processes such as synaptogenesis and neurite outgrowth (Wilson and Davies, 2007). Because cultured cells are not exposed to the same levels of sex hormones that circulate in the brain, any sex differences observed *in vitro* would likely be caused by organizational modifications occurring *in utero* or intrinsic differences in gene expression between XX and XY chromosome-containing cells.

One concern involving *in utero* hormone exposure involves the role of alpha-fetoprotein, a plasma glycoprotein produced during fetal life (Andrews et al., 1982). Alpha-fetoprotein is thought to protect the developing female brain against masculinization through the binding of circulating estradiol (Bakker et al., 2006). As the level of alpha-fetoprotein may vary from mother to mother, we used litters from multiple dams to minimize differences due to variability in *in utero* hormone exposure. However, to determine the robustness and generalizability of the astrocyte sex difference identified in Sprague Dawley cortical astrocytes, a future goal is to test whether our findings extend to astrocytes derived from other rat strains, or from other species.

The sex difference in mitochondrial respiration found in astrocytes but not microglia at 3% O_2 is consistent with the possibility of a lasting organizational modification occurring *in utero* specifically in astrocytes. Lasting *in vitro* sex differences have also been found in neurons, for example, in gamma-aminobutryic acid (GABA) responses in hippocampal neuronal cultures (Nunez and McCarthy, 2009). Intrinsic neuronal sex differences in mitochondrial properties have also been reported, including XX-XY differences in mitochondrial biogenesis (Sharma et al., 2014), morphology (Sharma et al., 2014), and recruitment of cell death signaling (Du et al., 2004, Sharma et al., 2014). The observation of mitochondrial sex differences *in vitro* in neurons and astrocytes but not microglia may be explained by the developmental origin of the brain cells. Both neurons and astrocytes are thought to originate from radial glial cells (Malatesta et al., 2003, Malatesta et al., 2000), whereas microglia are thought to arise from precursor cells originating in the yolk sac (Alliot et al., 1999). It may be that lasting sex differences observed in neurons and astrocytes develop from alterations occurring in precursor radial glial cells, adaptations that would not

be found in yolk sac-derived microglia. Nevertheless, literature supports sex differences in microglial cytokine production during early development (Loram et al., 2012, Crain et al., 2013) or following injury (Mirza et al., 2015), indicating that the topic requires further study.

The mechanism underlying the higher respiratory capacity observed in male astrocytes compared to female astrocytes at 3% O_2 remains to be elucidated. It may be that male astrocytes cultured and assayed at 3% O_2 express rate-limiting enzymes involved in aerobic energy metabolism at higher levels than female cells or male cells at 21% O_2. A transcriptome study identified increased expression of the gene encoding Complex I subunit Ndufa5 in astrocytes cultured at 4% O_2 relative to 20% O_2 (Chadwick et al., 2011). However, cells were pooled from both sexes, making the contribution of male astrocytes to this increase unclear. It is also possible that protein degradation/turnover of electron transfer chain subunits is differentially regulated, for example, by damage due to oxidative stress. Such alterations would not necessarily lead to a sex difference in basal respiration, which is regulated by energy demand. However, a relative increase in the levels of ETC proteins is predicted to provide males with a greater respiratory capacity, as indicated by maximal respiration measured in the presence of an uncoupler. Respiration in the presence of uncoupler is independent of energy demand because the protonmotive force required for ATP synthesis is dissipated. Therefore, uncoupled OCR measured with excess substrate is a good measure of ETC capacity.

Perhaps the best-characterized protein that is a candidate for mediating differences in mitochondrial protein expression is hypoxia-inducible factor-1α (HIF-1α), the oxygen-sensitive subunit of the transcription factor HIF-1 (Semenza, 2012). HIF-1α is constitutively degraded at atmospheric O_2, with the prolyl hydroxylase enzymes necessary for this degradation using oxygen as a substrate (Epstein et al., 2001). Because prolyl hydroxylase activity is O_2-dependent, degradation is limited under low-oxygen conditions, including within the physiological oxygen range (Epstein et al., 2001). Stabilization of HIF-1α is necessary for a wide variety of transcriptional processes, including regulation of cellular metabolism (Semenza, 2012) and, specifically, ETC Complex IV subunit composition (Fukuda et al., 2007). Interestingly, sex effects on Complex IV subunit transcription have also been reported (Roemgens et al., 2011). Alternatively, other factors could be responsible for modifying the function of the electron transport chain. For instance, if there is greater basal production of nitric oxide in female astrocytes compared to male cells, the higher NO concentration may result in inhibition of maximal respiration in female cells at 3% O_2. This would lead to a higher respiratory capacity in male cells. The absence of a sex difference at 21% O_2 might then be explained by an inability of NO to effectively compete with O_2 when oxygen is more abundant. Additionally, it is possible that the ETC is modified post-translationally in a sex-dependent manner, such as by S-nitrosylation, phosphorylation, or acetylation. Proteomic studies are needed to help resolve whether there are male-female differences in the composition or post-translational modification of the ETC in cultured astrocytes at 3% O_2.

A limitation of our study is that although the brain experiences a range of oxygen tensions, our experiments were performed at a single physiological oxygen tension. Notably, we were able to estimate O_2 level at the cell monolayer surface at the 3% O_2 set point, showing that

dissolved O_2 is actually ~1.2% during culture of astrocytes and ~1.7% during culture of microglia. These O_2 levels are closer to the bottom of the physiological range rather than the 3.5% midpoint. It is possible that the sex difference in astrocyte mitochondrial bioenergetics observed at 1.2–3.0% O_2 may decrease, increase, or disappear at the upper or lower O_2 limits of the physiological range. Similarly, it is possible that a sex difference in microglial bioenergetics would be revealed elsewhere within the physiological O_2 range.

The most significant contribution of our study is demonstration of the importance of controlling oxygen tension when studying *in vitro* sex differences of glial cells. Additional studies are required to determine the mechanisms behind the dichotomous bioenergetic profiles of male and female astrocytes at low physiological oxygen. It also remains to be seen whether the slight quantitative but not qualitative difference in microglial respiratory impairment at 3% O_2 compared to atmospheric O_2 during proinflammatory activation is functionally significant.

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- Male astrocytes have greater respiratory capacity than female at a physiological O_2.
- No sex differences in astrocyte respiration are seen at atmospheric O_2.
- No sex differences in microglial respiration are observed at either O_2.
- Respiration is more inhibited when microglia are activated at a physiological O_2.

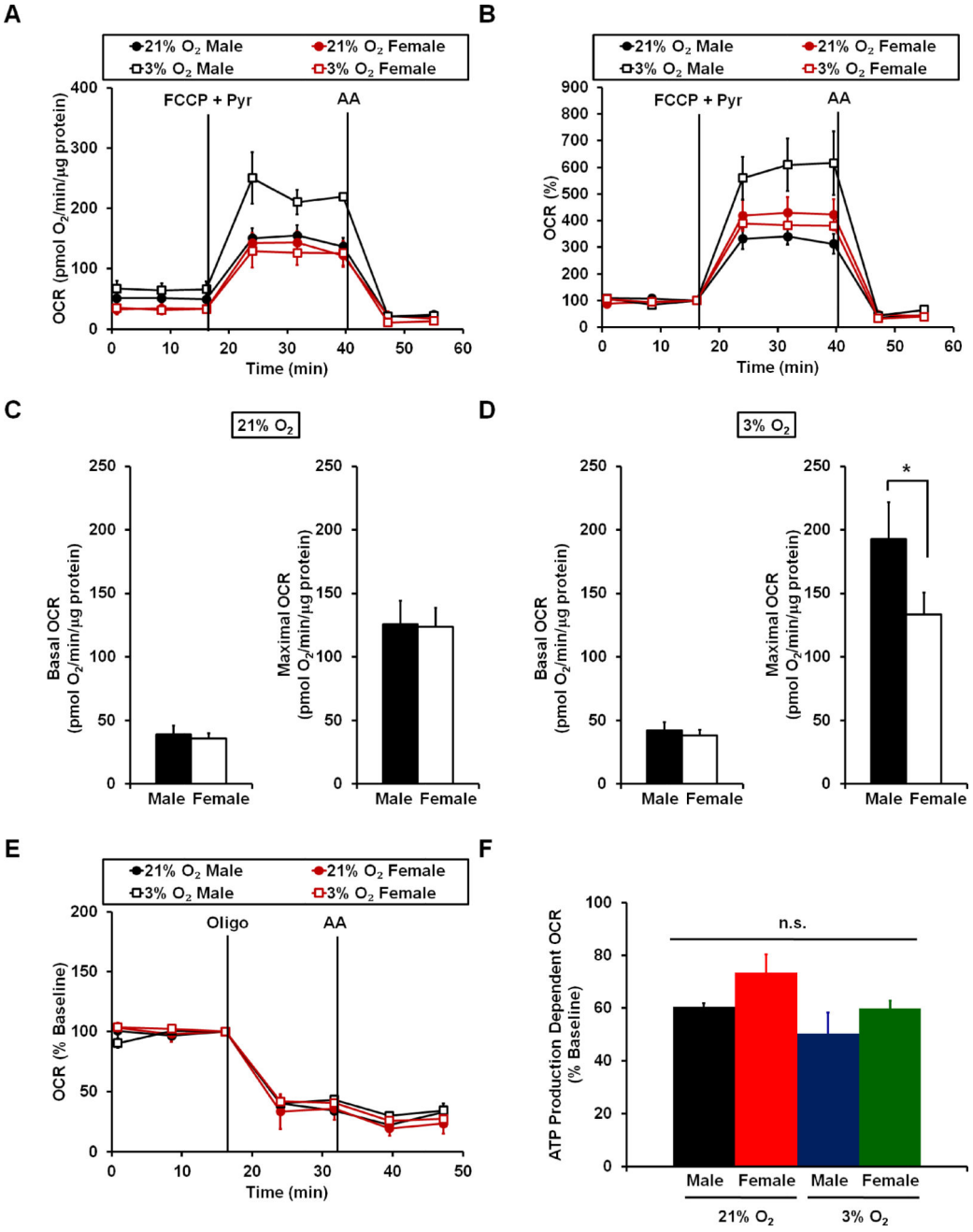

Figure 1. A sex difference in astrocyte respiration is observed at 3% oxygen but not at 21% oxygen

(A) Representative traces of oxygen consumption rate (OCR) measurements from male and female primary rat cortical astrocytes cultured and tested at 21% atmospheric or 3% low physiological oxygen. FCCP (6 µM), pyruvate (Pyr, 10 mM) and antimycin A (AA, 1 µM) were added when indicated. Traces are mean ± standard deviation of three wells of cells from the same animal that were cultured and assayed at the different O_2 values (i.e. one male and one female). (B) Representative traces of the data described in A after normalization to the third basal respiration measurement prior to FCCP + Pyr addition. (C–D) Bar graph representations of basal and maximal OCR at 21% O_2 (C) and at 3% O_2 (D). (E)

Representative traces of normalized OCR measurements in male and female rat cortical astrocytes at 21% and 3% oxygen. Oligomycin (oligo, 0.3 μg/mL), and antimycin A (AA, 1 μM) were added when indicated. (F) Bar graph representation of ATP production-dependent OCR at 21% vs. 3% O_2. Data are presented as mean ± standard error, n=5–8 astrocyte preparations across 3–4 separate litters derived from different dams. *$p<0.05$.

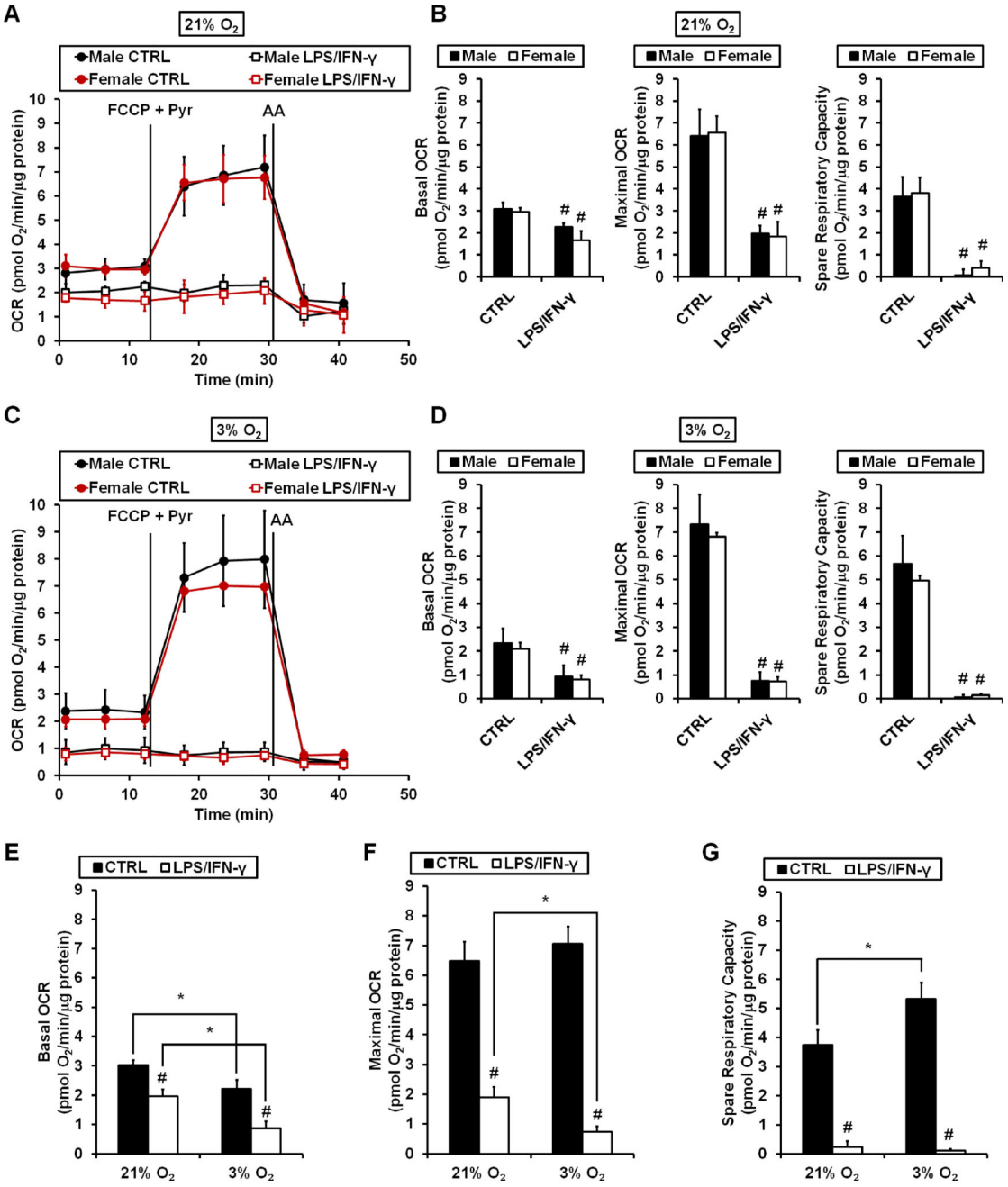

Figure 2. LPS/IFN-γ induces a greater respiratory impairment in microglia at 3% O_2 compared to 21% O_2, regardless of sex

(A) Representative traces of oxygen consumption rate (OCR) measurements from primary rat cortical microglia at 21% O_2 following 18 hours of LPS (100 ng/mL) plus IFN-γ (10 ng/mL) stimulation (LPS/IFN-γ) or control (CTRL) treatment. FCCP (4 μM), pyruvate (Pyr, 10 mM) and antimycin A (AA, 1 μM) were added when indicated. (B) Quantification of basal OCR, maximal OCR, and spare respiratory capacity from the experiments described in (A). (C) Representative traces of OCR measurements from cortical microglia at 3% O_2 following 18 hours of LPS/IFN-γ stimulation or CTRL treatment. Drug additions were as in (A). (D) Quantification of basal OCR, maximal OCR, and spare respiratory capacity from

the experiments described in (C). (E–G) Comparison of basal OCR (E), maximal OCR (F), and spare respiratory capacity (G) from microglia (combined data of male and female) at 21% *vs.* 3% O_2. Traces in (A) and (C) are mean ± standard deviation of three wells of cells from the same microglial preparation and are representative of 3–4 independent experiments using different preparations. Data in (B) and (D) are mean ± standard deviation, n = 3–4 microglial preparations derived from different animals. Data in (E–G) are mean ± standard deviation, n=6–8 microglial preparations derived from different animals. Each individual preparation utilized litters stemming from separate dams. #p <0.05 compared to CTRL of the same sex or the same O_2. *p<0.05 for 21% vs. 3% O_2.

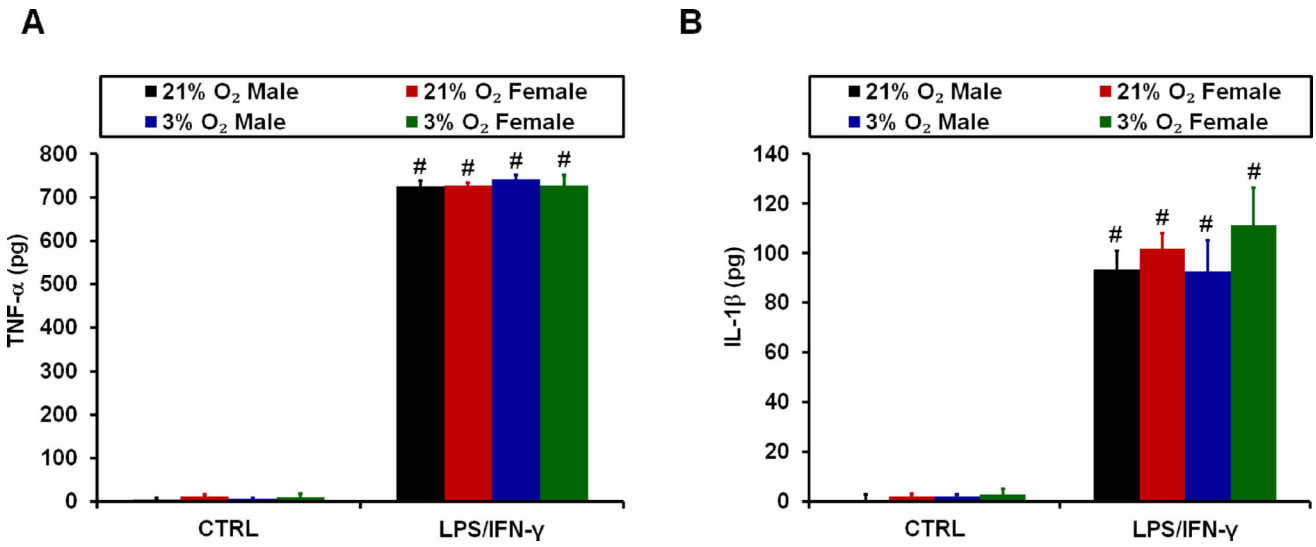

Figure 3. Lack of effect of sex or oxygen tension on microglial release of key proinflammatory cytokines

Primary rat cortical microglia were untreated (CTRL) or treated with 100 ng/mL LPS plus 10 ng/mL IFN-γ (LPS/IFN-γ) for 18 hours, and the release of (A) TNF-α and (B) IL-1β were quantified by ELISA. Data are presented as mean ± standard deviation, n = 3–4 microglial preparations derived from different animals, with each preparation utilizing litters stemming from separate dams. #p <0.05 compared to CTRL.