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# Microglial P2 Purinergic Receptor and Immunomodulatory Gene Transcripts Vary By Region, Sex, and Age in the Healthy Mouse CNS

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## Abstract

Inflammatory damage in many neurodegenerative diseases is restricted to certain regions of the CNS, and while microglia have long been implicated in the pathology of many of these disorders, information comparing their gene expression in different CNS regions is lacking. Here we tested the hypothesis that the expression of purinergic receptors, estrogen receptors and other neuroprotective and pro-inflammatory genes differed among CNS regions in healthy mice. Because neurodegenerative diseases vary in incidence by sex and age, we also examined the regional distribution of these genes in male and female mice of four different ages between 21 days and 12 months. We postulated that pro-inflammatory gene expression would be higher in older animals, and lower in young adult females. We found that microglial gene expression differed across the CNS. Estrogen receptor alpha (*Esr1*) mRNA levels were often lower in microglia from the brainstem/spinal cord than from the cortex, whereas tumor necrosis factor alpha (*Tnfα*) expression was several times higher. In addition, the regional pattern of gene expression often changed with animal age; for example, no regional differences in P2X7 mRNA levels were detected in 21 day-old animals, but at 7 weeks and older, expression was highest in cerebellar microglia. Lastly, the expression of some genes was sexually dimorphic. In microglia from 12 month-old animals, mRNA levels of inducible nitric oxide synthase, but not *Tnfα*, were higher in females than males. These data suggest that microglial gene expression is not uniformly more pro-inflammatory in males or older animals. Moreover, microglia from CNS regions in which neuronal damage predominates in neurodegenerative disease do not generally express more pro-inflammatory genes than microglia from regions less frequently affected. This study provides an in-depth assessment of regional-, sex- and age-dependent differences in key microglial transcripts from the healthy mouse CNS.

**Keywords:** Purinergic; P2X receptors; P2Y receptors: Tumor necrosis factor alpha; Estrogen receptor; Inducible nitric oxide synthase; Inflammation

## Introduction

As the primary resident immune cell population in the central nervous system (CNS), microglia react to signals of injury or infection by producing proinflammatory cytokines, chemokines, and reactive oxygen species [1,2]. Microglia also produce anti-inflammatory cytokines and perform neuronal support functions through their production of neurotrophins and growth factors [3]. An imbalance in these functions, resulting in uncontrolled microglial inflammation, is thought to contribute to the pathology of many neurodegenerative disorders. Neurodegenerative pathologies result in neuronal damage that often predominates in particular regions of the CNS: the hippocampus early in Alzheimer's disease (AD), the brain stem and spinal cord in multiple sclerosis (MS), and the substantia nigra in Parkinson's Disease (PD). Inflammation, often attributed to microglial activation, is common to all neurodegenerative disorders. However, despite inflammation in multiple CNS regions, neuronal death in many of these conditions seems only to occur in very discrete regions. For example, in PD, activated microglia are found in many CNS regions including the putamen, hippocampus, and temporal cortex, but neuronal death is largely limited to the substantia nigra [4]. Although the reasons for this remain poorly understood, it may be that characteristics of specific local CNS environments, to which microglia contribute, favor pathogenesis in certain regions. In addition, since neuronal populations are heterogeneous in the CNS, differences in the local environments they create could lead to regional differences in microglial characteristics that reciprocally influence neuron function. In support of this notion, overall microglial density varies by brain region [5,6], although most of these studies were done in the context of activating stimuli *in vivo* [7-10]. *In vitro*, microglial activity and gene expression also varies depending upon the brain region from which they originated [11,12], but little is known about regional differences in microglial gene expression in unactivated microglia from healthy animals. Many key inflammatory and neurotrophic microglial genes that have been implicated in both the beneficial and toxic activities of microglia are regulated by purinergic receptor (P2R) signaling, cell surface receptors for extracellular nucleotides that regulate many important microglial activities (reviewed in ref. [13,14]). These include tumor necrosis factor alpha (TNFα), inducible nitric oxide synthase (iNOS), interleukin-10 (IL-10), and brain derived neurotrophic factor (BDNF) [15-20]. The expression of many of these genes, and indeed many neurodegenerative diseases themselves, display sexual dimorphisms and change with animal age [21,22]. Additionally, a number of these are influenced by estrogen receptors (ER) in neurons, and ER activation can be anti-inflammatory and neuroprotective [23]. Therefore, in the present study, we tested the hypotheses that: 1) the expression of purinergic receptors, estrogen receptors and other neuroprotective and pro-inflammatory genes in freshly-isolated microglia differ among CNS regions in healthy untreated mice; 2) pro-inflammatory gene expression is higher in microglia from older animals than young; and 3) microglial gene expression is sexually

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dimorphic in adult animals. To test these hypotheses, we evaluated the basal expression of neuroprotective and pro-inflammatory genes in microglia freshly-isolated from the CNS of healthy mice. Five different CNS regions were evaluated: cerebral cortex, hippocampus, cerebellum, brain stem and spinal cord, and midbrain, in male and female mice of four different ages, ranging from 21 days old to 12 months. We found that microglia are regionally heterogeneous, with expression of many genes varying as much as 17-fold between different CNS regions. The pattern of gene expression across the CNS often changed as the animal aged, and in some cases, was also sexually dimorphic. This study provides the first in-depth assessment of the temporal and regional purinergic, inflammatory and neurotrophic gene expression profiles in freshly-isolated microglia from healthy mice of both sexes over age. This information will aid in better understanding the role of microglia in the normal CNS and help to identify their contributions to neurodegenerative disease processes.

## Materials and Methods

### Animals

C57Bl/6 mice were housed under standard conditions in an AAALAC-accredited animal facility according to protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. All animals were housed with *ad libitum* food and water, on a 12 hour light/dark cycle. Experiments were performed using animals aged 21 days (21 d), 7 weeks-old (7 wk), 4 months-old (4 mo), and 12 months-old (12 mo). All animals were acclimated to housing facilities for one week prior to use in the experiments. Adult males were housed individually, and mature females were housed together. The 12 mo mice were retired breeders, and the females had not born a litter for at least two months prior to sacrifice; all other animals were virgins. All efforts were made to minimize the number of animals used while allowing the formation of statistically reliable conclusions.

### CD11b$^+$ cell isolation

Mice were euthanized and then perfused with cold phosphate buffered saline (PBS) to remove the majority of circulating immune cells from the CNS vasculature. CNS tissues were removed, and the brains cleaned of meninges and dissected into five portions: cerebral cortex (abbreviated throughout as "cortex"), hippocampus, cerebellum, brainstem/spinal cord ("BS/SC"), and the remaining brain tissue, which will be referred to "midbrain." Due to the small amount of tissue within some regions, the dissected tissues of 3-7 mice of the same age and sex were combined for each region prior to isolation of the CD11b$^+$ cells, with an average of five mice per pool. CD11b$^+$ cells were isolated as previously described [21-24] using magnetic bead assisted cell sorting. The average purity of isolated cells having the characteristics of microglia was 97% as determined by CD11b/CD45 staining and FSC/SSC by flow cytometry. The CD11b$^+$ cells will be referred to as "microglia." The purity of the isolated cell populations were not significantly different among the CNS regions examined (data not shown).

### RNA extraction/reverse transcription

TriReagent (Sigma-Aldrich, St. Louis, MO) was used to extract RNA from freshly-isolated microglia as we have previously described [21-24]. cDNA was synthesized from 1 µg of total RNA using MMLV Reverse Transcriptase (Invitrogen) as previously described [25].

### Quantitative PCR

Real-time PCR using Power SYBR Green (Applied Biosystems) was performed on the cDNA using the ABI 7300 system as previously described [21-24]. The primer sequences are shown in Table 1 and were designed to span introns whenever possible to discount any product from genomic DNA. NCBI BLAST analysis was used to assess primer specificity prior to use. The dissociation curves for each sample had a single peak and an observed Tm that was consistent with the amplicon length for each gene. Serial dilutions were used to test primer efficiency. Relative gene expression levels were determined using the $\Delta\Delta C_T$ method from averaged duplicate $C_T$ measurements. Based on our previous studies using freshly-isolated microglia [21], we normalized the expression of each gene to the levels of *β-actin (ActB)* detected in the same sample using the following primer sets: forward-ACCCTAAGGCCAACCGTGAA, reverse-AGAGCATAGCCCTCGTAGATGG; or forward-CACAGCTTCTTTGCAGCTCCTT, reverse- ACGACCAGCGCAGCGATAT. In adult males, genes with the highest expression relative to each other were (in descending order): *P2rX7*, *P2rX4*, *P2rY12*, *P2rY13*, and *P2rY6*; those with moderate expression: *Tnfα*, *Esr1*, *Bdnf*, and *P2rX1*; and those with low expression: *P2rY14*, *P2rY2*, *Il-10*, *P2rX6*, *Ifnβ*, *P2rY1*, *iNos*, *P2rY4*, and *P2rX3*. The average $C_T$ for *β-actin* was 18; the average $C_T$ for the other genes varied by brain region, but their overall expression relative to each other was consistent with our previous studies [21-24]. Forty-five cycles of PCR were run and all detected genes had $C_T$ values below 35 in a majority of the samples examined.

### Statistical analysis

Statistical analyses were performed on $\Delta\Delta C_T$ data using a one-way ANOVA followed by the Tukey-Kramer Multiple Comparisons, or unpaired t-tests as appropriate, using Sigma Stat 3.1 software. Statistical significance was set at the 95% confidence limit ($p<0.05$). # represents $0.05<p \leq 0.10$, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$. Quantitative data are expressed as the mean ± SEM, of n=3 independent experiments, with tissue originating from 3-7 mice per experiment. For clarity, due to the large quantity of data collected from this study, data are shown only for those CNS regions among which mRNA expression varied for a given gene. Expression levels for regions not shown are

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *Bdnf* | GGACGGTCACAGTCCTAGAGAAA | CATTGCGAGTTCCAGTGCC |
| *Esr1* | TGCGCAAGTGTTACGAAGTGG | TCATGTCTCCTGAAGCACCCA |
| *Esr2* | GCTGGCTGACAAGGAACTGGT | CGAGGTCTGGAGCAAAGATGA |
| *Ifnβ* | ATGGTGGTCCGAGCAGAGATC | GCCTGCAACCACCACTCATT |
| *Il-10* | GCCTTATCGGAAATGATCCA | TCTCACCCAGGGAATTCAAA |
| *iNos* | TGACGCTCGGAACTGTAGCAC | TGATGGCCGACCTGATGTT |
| *Tnfα* | TGTAGCCCACGTCGTAGCAA | AGGTACAACCCATCGGCTGG |
| *P2rX1* | CAGAAAGGAAAGCCCAAGGTATT | CACGTCTTCACAGTGCCATTG |
| *P2rX2* | GCTGCTCATTCTGCTTTACTTCG | TCCCACACTTTGTGTTCCGA |
| *P2rX3* | AAGGCTTCGGACGCTATGC | GATGACAAAGACAGAAGTGCCCT |
| *P2rX4* | AGACGGACCAGTGATGCCTAAC | TGGAGTGGAGACCGAGTGAGA |
| *P2rX5* | GATGTGGCAGACTTTGTCATTCC | CCTTCACGCTCAGCACAGATG |
| *P2rX6* | ACGTGTTCTTCCTGGTAACCAACT | TGGACATCTGCCCTGGACTT |
| *P2rX7* | ACAATGTGGAAAAGCGGACG | TCAATGCACACAGTGGCCA |
| *P2rY1* | AGCAGAATGGAGACACGAGTTTG | GGGATGTCTTGTGACCATGTTACA |
| *P2rY2* | GAAGAACTGGAGCAGGCGCT | CCATTGCCCTGGACCTGATC |
| *P2rY4* | CTGCAAGTTCGTCCGCTTTC | GTATTGCCCGCAGTGGATG |
| *P2rY6* | TGAAAACAACGAGGAACACCAA | CAGCCTTTCCTATGCTCGGA |
| *P2rY12* | CACAGAGGGCTTTGGGAACTTA | TGGTCCTGCTTCTGCTGAATC |
| *P2rY13* | CAGCTGAGTCTCTTCCAAAACAAA | TGCATCCCAGTGGTGTTGAT |
| *P2rY14* | CCACCACAGACCCTCCAAAC | CAACACGGGAATGATCTGCTTT |

**Table 1:** Primer sequences.

not significantly different from those in cortex, unless otherwise stated. Data are graphed (in arbitrary units) comparing gene expression among different brain regions.

## Results

### ERα mRNA levels are higher in microglia isolated from cortex than brainstem/spinal cord (BS/SC)

Estrogen has been shown to produce many anti-inflammatory effects in microglia [26]. Here we found that expression of *ERα* (*Esr1*) mRNA was significantly higher in microglia isolated from the cortex than the BS/SC in male mice at all four of the examined ages, though to varying levels of significance (Figure 1). In females, *ERα* mRNA levels were also higher in cortical microglia than in microglia from BS/SC, but this difference was only statistically significant at 12 mo. *ERα* expression in microglia from other CNS regions were similar to those in the cortex (data not shown). *ERβ* (*Esr2*) was not detected in microglia from any CNS region, in male or female mice of any age, consistent with previous reports [21,27].

### mRNA levels of Bdnf, Ifnβ, and Il-10 did not vary significantly among CNS regions

Microglial production of neurotrophins such as BDNF, and anti-inflammatory cytokines including IFNβ and IL-10 function together to control (inactivate) the pro-inflammatory immune response and exert protective/supportive effects on neurons. Contrary to our hypothesis, we found no statistically significant differences in the expression of *Bdnf*, *Ifnβ* or *Il-10* in microglia isolated from different CNS regions of mice at any age; there were also no sex differences detected (data not shown).

### Tnfα mRNA expression is higher in microglia from BS/SC than other CNS regions

Because TNFα and iNOS are canonical pro-inflammatory genes that are both often upregulated in activated microglia, we also evaluated their expression. *Tnfα* mRNA levels were four to 17-times higher in microglia from the BS/SC than the cortex, depending on animal age (Figure 2). At 21 d and 7 wk, BS/SC microglia from both males and females displayed higher *Tnfα* expression than microglia from any

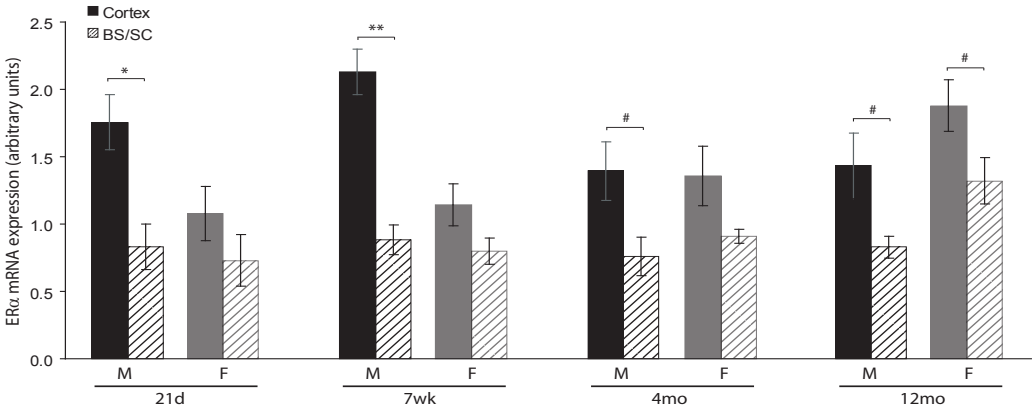


**Figure 1:** ERα mRNA levels are higher in microglia isolated from cortex than brainstem/spinal cord. ERα mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β−actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # 0.05 < p ≤ 0.10; * p ≤ 0.05; ** p ≤ 0.01.

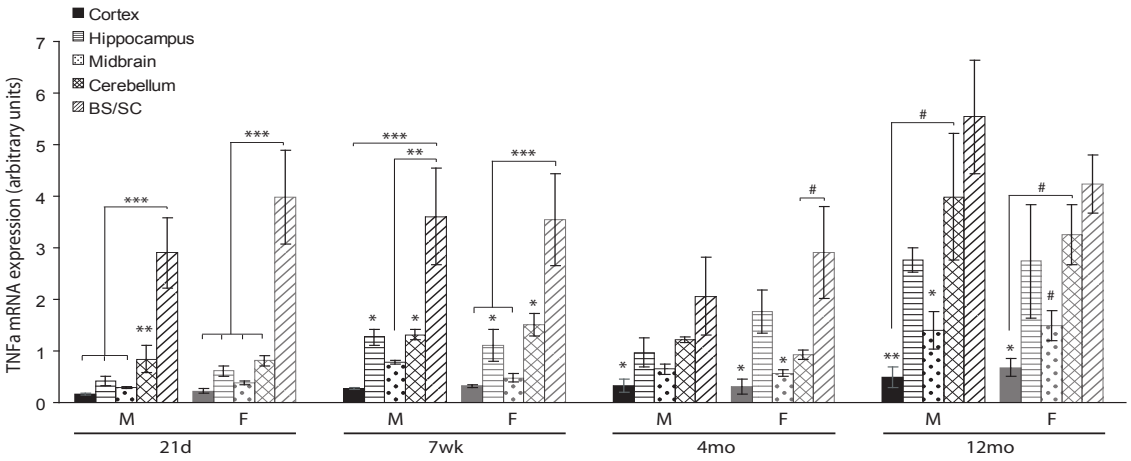


**Figure 2:** TNFα mRNA levels are higher in microglia isolated from the brainstem/spinal cord than other CNS regions. TNFα mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β−actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # 0.05 < p ≤ 0.10; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.005. All comparisons without brackets are relative to the spinal cord.

other CNS region examined. In cerebellar microglia from 12 mo mice, *Tnfα* mRNA levels were also significantly higher than those in the cortex. Interestingly, with age, *Tnfα* mRNA levels appear to increase in microglia in all brain regions, although cortical microglia showed the smallest increase. No sex differences in *Tnfα* gene expression were noted at any age in any region evaluated.

## iNos mRNA expression in BS/SC microglia is sexually dimorphic

*iNos* expression differed between microglia derived from BS/SC and cortex at 21 d and 12 mo (Figure 3). These differences are sexually dimorphic: microglial expression at 21 d in male BS/SC was higher than in cortex, whereas in females, levels in BS/SC and cortex were almost identical. At 12 mo, *iNos* mRNA levels in female cortical microglia were higher than in BS/SC, whereas in males, *iNos* levels were extremely low or undetectable (consistent with our previous observations in whole brain microglia [28]). *iNos* mRNA levels in microglia from other brain regions examined were not significantly different from expression in cortical microglia at any age.

## The expression of several microglial P2X receptors differs by region, age and sex

Many pro-inflammatory microglial factors, including iNOS and TNFα, are regulated by P2 purinergic receptors [15-17,29,30], so we next examined the expression of all 14 known rodent P2Rs in these samples. Microglial *P2X1* (*P2rX1)* expression was higher (with varying levels of statistical significance) in microglia from the cortex than BS/SC in both male and female mice after 21 d (Figure 4). *P2X1* mRNA levels in microglia from other CNS regions were similar to those in cortical microglia at most of the ages examined. *P2X4 (P2rX4)* mRNA levels were higher in male cerebellar microglia than in microglia from other CNS regions, and they were significantly higher than in BS/SC

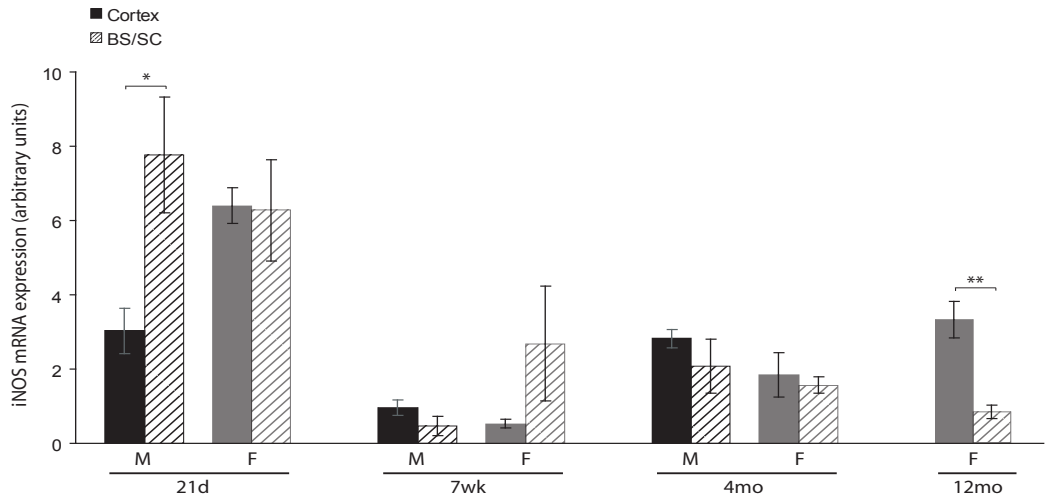


**Figure 3:** iNos expression in the brainstem/spinal cord microglia is sexually dimorphic. iNos mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β−actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). * $p \leq 0.05$; ** $p \leq 0.01$.

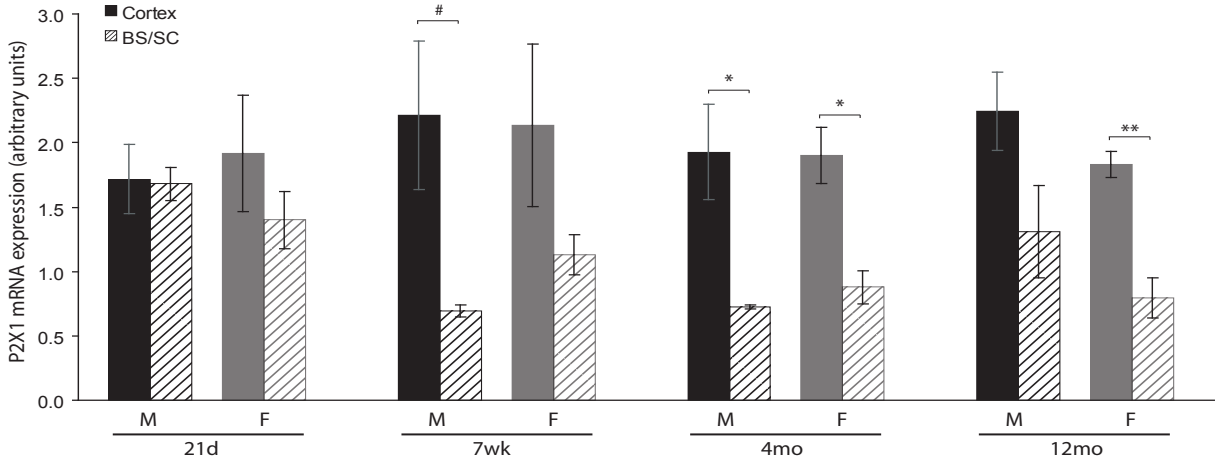


**Figure 4:** P2X1 mRNA levels are lowest in microglia isolated from the brainstem/spinal cord. P2X1 mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β-actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # $0.05 < p \leq 0.10$; * $p \leq 0.05$; ** $p \leq 0.01$.

microglia at all four ages examined (Figure 5). In 7wk animals of both sexes, *P2X4* expression in cerebellar microglia was also significantly higher than in cortical microglia; this remained true in 4mo males. In 21 d females, *P2X4* mRNA levels in BS/SC microglia were higher than in brain, in contrast to males where expression in BS/SC microglia was lower. *P2X7 (P2rX7)* mRNA levels in cerebellar microglia were also significantly higher than those in microglia isolated from other brain regions in males after 21 d (Figure 5). In females, *P2X7* expression in cerebellar microglia was significantly higher than in BS/SC at 7 wk and 12 mo. In addition, whereas *P2X7* expression in male cortical microglia appeared to decrease with age, it remained relatively steady in female cortical microglia. By 12 mo, *P2X7* mRNA levels in cortical microglia from females was significantly higher than BS/SC microglia. *P2X2 (P2rX2)* was not detected in microglia isolated from any CNS region in healthy male or female mice, at any of the ages examined, consistent with our previous results in whole mouse brain [21]. We previously found *P2X5* (*P2rX5)* expression only detectable in whole brain microglia from 3 day-old mice [21]; here we also find that *P2X5* is not detectable in microglia at any of the older ages examined in this study, in any region, suggesting that its role in microglia may be restricted to early CNS development. No notable differences were found in the expression of *P2X3* (*P2rX3)* or P2X6 (*P2rX6)* (data not shown).

## P2Y receptor mRNA levels are differentially expressed in cortical microglia and vary by CNS region and age

In 21 d males, *P2Y2* (*P2rY2)* mRNA expression was significantly lower in cortical microglia than in microglia from hippocampus, cerebellum, and BS/SC (Figure 6). At 7 wk, *P2Y2* expression in BS/SC microglia was significantly higher than in hippocampal and cerebellar microglia. In female 21 d mice, cerebellar microglial *P2Y2* expression was higher than in hippocampal or cortical microglia. At 7 wk, BS/SC microglia became the population with the highest *P2Y2* expression in both males and females. There were no significant differences in expression among other brain regions at 4 mo or 12 mo in either sex, and levels were not significantly different than those at 7 wk. *P2Y2* expression in midbrain microglia was not significantly different from cortical microglia in any of the ages examined. *P2Y6 (P2rY6)* mRNA levels in cortical microglia were significantly higher than in BS/SC microglia in 7 wk females, and in both sexes at 12 mo (Figure 7); expression in microglia isolated from other brain regions was similar to that in cortical microglia at all ages examined. *P2Y12 (P2rY12)* mRNA levels were similar in cortical, midbrain, and hippocampal microglia in all groups. Expression was two to three-times higher in microglia from cortex than those from BS/SC in both sexes and at all ages examined (Figure 8), with the exception of 21 d females. *P2Y12* expression in

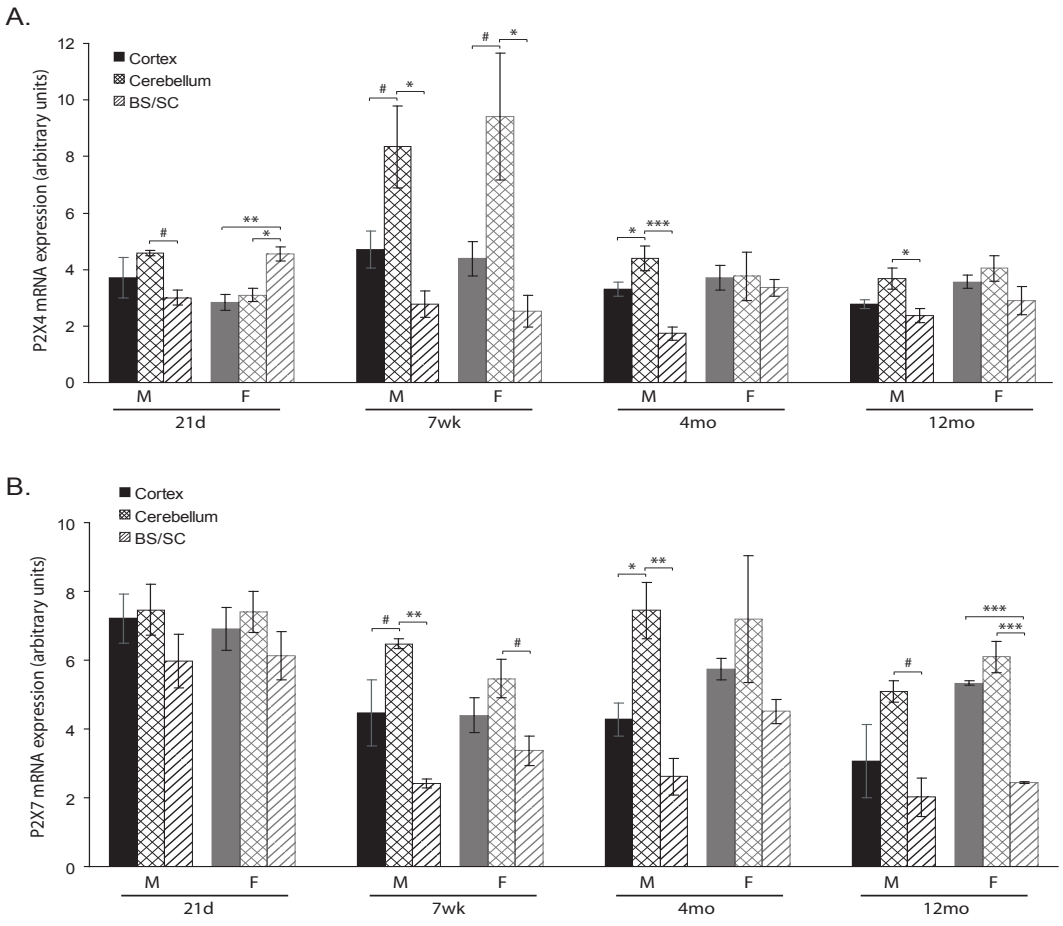


**Figure 5:** P2X4 and P2X7 mRNA levels are higher in male microglia isolated from the cerebellum than other CNS regions. P2X4 (A) and P2X7 (B) mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β-actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # 0.05 < p ≤ 0.10; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.005.

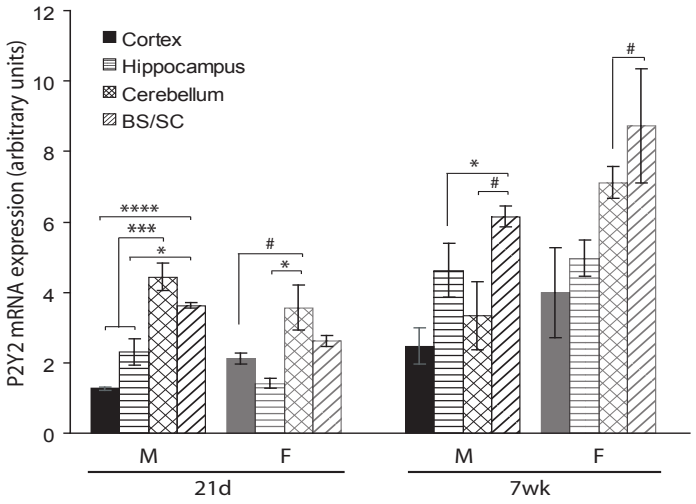


**Figure 6:** P2Y2 mRNA levels are highest in cerebellar microglia at 21d and highest in brainstem/spinal cord microglia at 7wk. P2Y2 mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β−actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # 0.05 < p ≤ 0.10; * p ≤ 0.05; *** p ≤ 0.005.

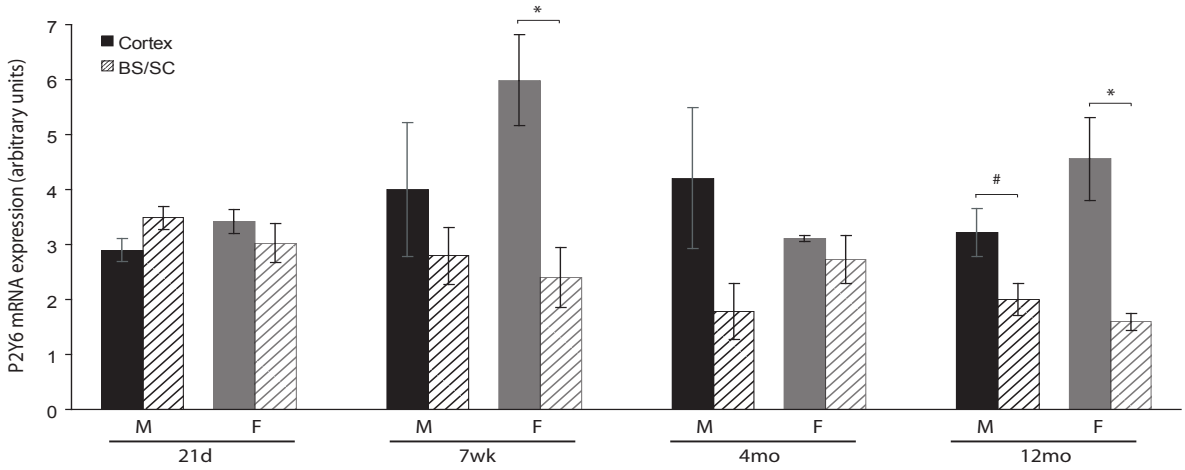


**Figure 7:** P2Y6 mRNA levels are higher in microglia isolated from cortex than brainstem/spinal cord. P2Y6 mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β−actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # 0.05 < p ≤ 0.10; * p ≤ 0.05.

cerebellar microglia was intermediate, being significantly lower than cortical microglial levels and significantly higher than BS/SC microglia, in both males and females at 7 wk and older. Similarly, microglial *P2Y13 (P2rY13)* mRNA levels were higher in cortical microglia than in BS/SC microglia, and cerebellar microglial levels were intermediate, although unlike in males and other ages, these differences did not reach statistical significance at 21 d in either sex, or in 4 mo females (Figure 8). In addition, *P2Y13* expression in midbrain and hippocampal microglia was similar to that in cortical microglia, except at 12 mo when mRNA levels in female hippocampal microglia were significantly lower. No notable differences were detected in the expression of *P2Y1 (P2rY1)*, *P2Y4 (P2rY4)*, or *P2Y14 (P2rY14)* in microglia from any of the regions examined (data not shown).

## Discussion

In the present study we investigated the expression of purinergic receptors and associated immunomodulatory genes in freshly-isolated (uncultured) microglia from five different CNS regions of healthy male and female mice ranging in age from 21 days to 12 months. We previously demonstrated that expression of many of these genes is sexually dimorphic and varies by age in microglia derived from whole brain [21,28], but information on regional expression was lacking. To our knowledge, this is the first study to comprehensively examine the regional transcript levels of these genes in microglia *in vivo*. Estrogen has many anti-inflammatory effects in microglia [26]. While estrogen function in microglia has been studied for over two decades, the regional distribution of *ERα* expression in freshly-isolated microglia from different CNS regions has not previously been addressed. We find that microglial *ERα* expression is lowest in microglia from the BS/SC. This is particularly interesting due to the strong effect of estrogens in MS, where spinal cord lesions are frequent, and symptom severity and relapse rates increase when estrogen levels are low [26,31]. It is tempting to speculate that low microglial *ERα* expression in the spinal cord may render them less able to counteract disease-induced pro-inflammatory

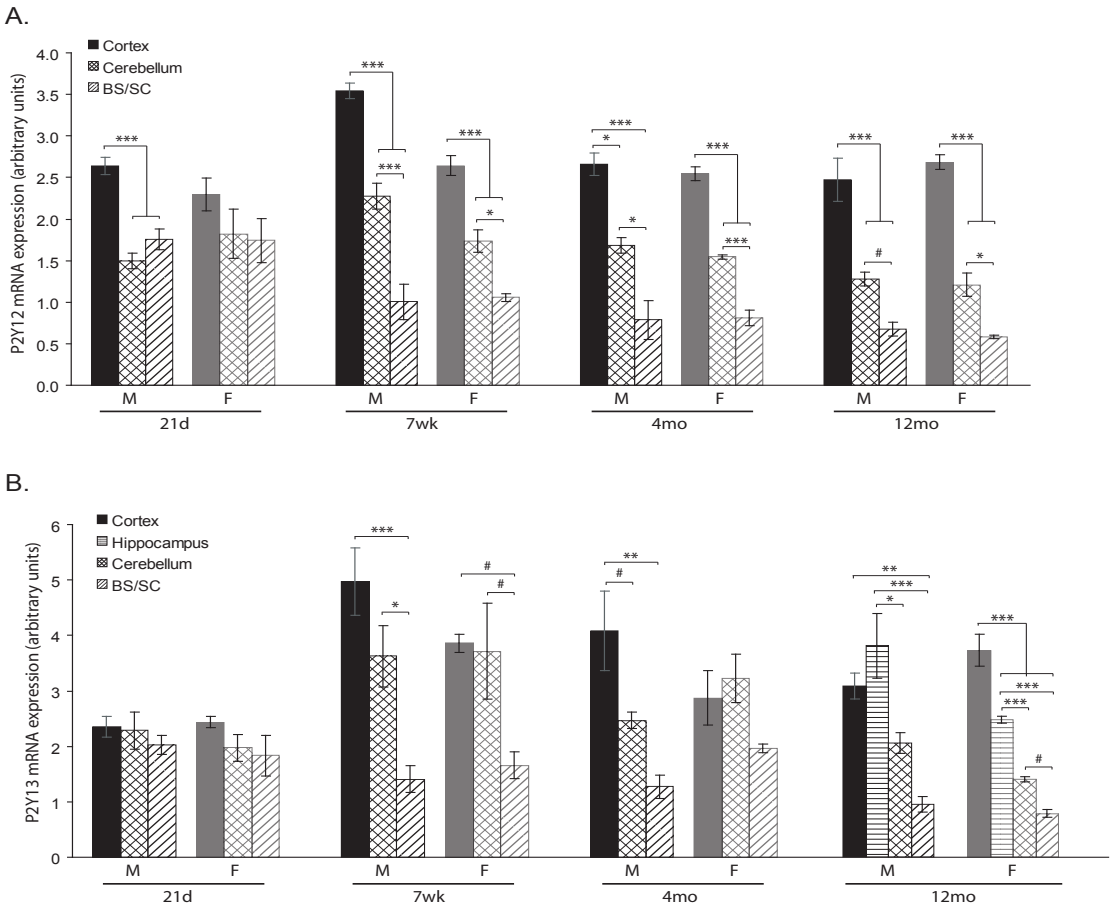


**Figure 8:** P2Y12 and P2Y13 mRNA levels are lowest in microglia isolated from brainstem/spinal cord. P2Y12 (A) and P2Y13 (B) mRNA levels in freshly-isolated microglia from CNS regions of male and female mice of different ages were determined using qRT-PCR. Expression was normalized to β−actin and relative levels (± SEM) are graphed in arbitrary units. M – male (black colored bars), F – female (grey colored bars). # 0.05 < p ≤ 0.10.

signals in the context of decreased estrogen. That *ERβ* mRNA was not detected in microglia from any CNS region, in either sex at any age, supports the idea that this receptor is absent in microglia from healthy C57Bl/6 mice [21,27], and makes the potential influence of *ERα* expression more significant, as it would be the only known route for classical, genomic estrogen effects in microglia. Although differences in microglial *Bdnf* mRNA levels have been observed in microglia derived from different regions *in vitro* [32], *in situ* hybridization and immunohistochemistry in brain and spinal cord did not show differences between CNS regions [33]. These results are consistent with our observations here that no age-, region- or sex-dependent differences in microglial *Bdnf* mRNA levels were detected. As expected in the healthy, uninjured CNS, microglial expression of cytokines and pro-inflammatory molecules in general tended to be low. We found microglial *Il-10* and *Ifnβ* mRNA levels to be low, and like *Bdnf*, there were no statistically-significant differences among the CNS regions examined, though they were lowest in cortical microglia. Whether the expression levels of these genes change during CNS injury or disease, or if their activities contribute to pathogenesis is not yet known; however, the answer will likely be CNS condition-and region-specific. Although the pro-inflammatory mediators TNFα and iNOS are both frequently upregulated in activated microglia, we found significant differences in their expression in microglia from the uninjured CNS. *Tnfα* mRNA levels were higher than the other cytokines evaluated, and consistent with previous observations in culture [11], seemed to be higher in microglia from the hippocampus than from the cortex, although this difference was not consistently statistical significant. *Tnfα* expression in cerebellar microglia was higher still than in hippocampus, and BS/SC microglia showed the greatest expression at all ages. While the regional expression pattern of *iNos* was different than that of *Tnfα*, the simultaneous increase of both *Tnfα* and *iNos* in the cortex between 7 wk and 12 mo in females may reflect a gradual increase in the inflammatory environment in the female cortex with age. However, it is important to note that the actions of TNFα are not necessarily detrimental, as it is also involved in neurogenesis, reducing neuronal injury and promoting neuronal plasticity [34-36], and microglial TNFα production following ischemic injury can be neuroprotective [36]. Thus, TNFα production by microglia in the non-injured CNS may also be similarly beneficial. Future studies will be needed to determine the nature of these effects, which are also likely to be region- or situation-specific. Previous studies demonstrate that P2X1 expression in microglia *in vivo* changes with animal age [21,37,38]. In the present study, we found *P2X1* mRNA levels to be significantly higher in cortical microglia than in BS/SC microglia in mice ages 7 wk and older. Although little is currently known about the function of P2X1 signaling in microglia, P2X1 can heterotrimerize with P2X2, P2X4, and P2X5 [39,40]. Since we find that *P2X2* and *P2X5* mRNAs are undetectable in microglia at these ages, P2X1/P2X4 is likely to be the major P2X1

heteromer expressed in microglia. Our transcript data therefore suggest that there is less potential for P2X1/P2X4 heteromers in BS/SC microglia than in cortical microglia. The differences in signaling pathways and microglial functions initiated by P2X1 homomers versus P2X1/P2X4 heteromers in these CNS regions, and the molecular mechanisms underlying differential *P2X1* gene regulation in microglia from the cortex and BS/SC are not yet known. Previous studies of P2X4 [41-45] and P2X7 distribution throughout the CNS have produced conflicting results [46,47]. While prior studies did not distinguish cell type, other studies have noted P2X4 expression in microglia in the healthy brain [21,37,38]. In the present study, we find that *P2X4* expression is highest in microglia from the cerebellum with a distribution most closely matching that reported by Collo and Seguela et al., [43,44]. We found a similar distribution of *P2X7* mRNA; *P2X7* levels are highest in microglia from the cerebellum of mice 7 wk and older. This similarity is noteworthy because these genes are located on the same chromosome, and heterotrimers of P2X4/P2X7 have been reported in macrophages [48]. The impact of increased microglial P2X4 and P2X7 levels in the cerebellum is not yet known; stimulation of P2X7 receptors in microglia increases production of pro-inflammatory cytokines including TNFα, IL-6, and IL-1β (reviewed in [49]), while P2X4 receptor stimulation increases microglial BDNF production [18,19]. Since the cerebellum continues to develop into early adulthood, P2X receptor signaling may play a supporting role in the maturation of neuronal circuitry/connections during this critical developmental period. P2Y receptor expression also varied by age, sex and region. While previous P2Y receptor studies in the CNS showed expression in the brain or spinal cord microglia [21,50,51], these regions were studied separately, prohibiting comparisons of relative expression. Here we found that *P2Y2* mRNA levels were lower in male cortical microglia at 21 d, but not after 7 weeks, or in females. In contrast, *P2Y6* expression in cortical microglia was significantly higher than in BS/SC microglia in 7wk females and in 12 mo animals of both sexes. Due to the prevalence of synaptic remodeling in the cortex, perhaps the phagocytic activities of microglia associated with *P2Y6* are more necessary in that CNS region. *P2Y12* consistently showed higher expression in microglia from the cortex compared to BS/SC; cerebellar levels were intermediate at ages 7wks and older. Likewise, *P2Y13* expression in cortical microglia was significantly higher than in BS/SC and cerebellum at these ages. P2Y12 has many similarities to the less-studied P2Y13: the genes are located on the same chromosome and the receptors respond to the same agonists and many of the same antagonists [40]. Thus, receptor agonist binding studies using ADP and [$^{35}$S]GTPγS [52] may have also included observations of P2Y13. Additionally, in microglia derived from whole mouse brain, we previously found that both P2Y12 and P2Y13 expression increased with animal age [21]. Although each of these P2Y receptors play roles in important microglial activities: P2Y2 in microglial recruitment and activation [53,54], P2Y6 in phagocytosis [55], P2Y12 in microglial migration and chemotaxis [56-58], and P2Y12 and P2Y13 in promoting neuropathic pain [59,60], their function in adult cortical microglia, where they appear to be highly expressed in adulthood, are not yet clear. Sexual dimorphisms in *ERα, iNos, P2Y2* and *P2Y6* were also noted in this study. These genes are involved in multiple microglial functions including phagocytosis, inflammation, and neuroprotection. Sex differences in the expression of these genes, coupled with their changes with age, may contribute to neurodegenerative diseases that also vary in incidence by sex and age. Although the sexual dimorphisms observed differ by gene, most are evident at adult ages, suggesting that gonadal hormones likely play a role; additional studies are needed to test these ideas directly.

## Summary

In this study we found that microglial gene expression is regionally heterogeneous; differences were identified in half of the genes examined here. Based on the gene expression profiles we evaluated, microglia from the BS/SC are the most different from microglia derived from other CNS regions. In general, gene expression in BS/SC microglia tended to be lower than in microglia from other regions, with the striking exception being *Tnfα*, which was many times higher in BS/SC microglia. While microglia clearly have regional specialization, the significance of differences in the expression of individual genes is not yet clear. At the ages evaluated, microglia from CNS regions frequently associated with neuronal degeneration did not generally express more pro-inflammatory genes than those less frequently affected. Moreover, the regional microglial gene expression profiles often changed with age, suggesting that microglia are not uniformly more pro-inflammatory in older animals, or in one sex. Although some sexual dimorphisms in gene expression within particular CNS regions were identified, in general, sex differences were not as great as regional differences. The new information presented in this study will lead to a better understanding of the role of microglia in aging and pathology, and will set the stage for future studies targeting region-specific, therapeutic manipulation of microglia.

## Competing Interests

The authors declare no conflict of interest.

## Authors' Contributions

JC carried out all animal experiments, RNA analyses, generated the figures and performed statistical analyses. JW conceived of the study, helped with statistical analyses and obtained funding. JC and JW drafted the manuscript.

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