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Sexual dimorphism in inflammasome-containing extracellular vesicles and the regulation of innate immunity in the brain of reproductive senescent females

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ABSTRACT

A woman's risk for stroke increases exponentially following the onset of menopause; however, the underlying mechanisms responsible for the increased risk remain unknown. The depletion of endogenous estrogen at menopause is known to activate the inflammatory response. Therefore, in this study we have used reproductively senescent (RS) rats to test the hypotheses that (1) inflammasome activation is significantly higher in the brain of RS females (RSF) as compared to their younger counterparts and age-matched senescent male rats, and that (2) RS triggers an innate immune response mediated in part by inflammasome-containing extracellular vesicles (EV) that originate in the female reproductive organs and then spreads to the brain. We tested these hypotheses using male and female Sprague–Dawley rats (Young: 6–7 months and RS: 9–13 months). Hippocampus, gonads and serum were collected. Additionally, cerebrospinal fluid (CSF) of pre- and post-menopausal women (ages 23 to 37 and 52 to 68) was purchased and extracellular vesicles (EV) were isolated from serum and CSF. The Inflammasome proteins caspase-1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and IL-1β were then resolved by immunoblotting. We found that inflammasome protein expression increased significantly in the analyzed tissues in RSF as compared to young females (YF), such difference was not present in age-matched male rat brains. Interestingly, we found that Nik-related kinase (NRK), which is present in female reproductive organs was present in the CSF– and serum-derived EV, suggesting that the source of the EV seen in the brain during RS/menopause originate, in part, in the female reproductive organs. Thus, this study shows for the first time an involvement of the inflammasome originating in the female reproductive system as a contributor to inflammation in the brain that makes the peri-menopausal women's brain more susceptible to neurodegenerative diseases such as stroke.

1. Introduction

One out of five women experience stroke, a condition that disproportionately kills more women than men in the United States. Importantly, observed sex differences in stroke epidemiology have also been reported. Studies from various laboratories have demonstrated that stroke causes a smaller infarct in young adult female rodents as compared to age-matched males (Alkayed et al., 1998; Park et al., 2006). A woman's risk for stroke increases following the onset of menopause (Towfighi et al., 2007). At menopause, production of ovarian hormones such as progesterone and estrogen slowly diminishes (Edwards and Li, 2013). Estrogen has been suggested to function as a potent anti-inflammatory factor (Cushman et al., 1999; Edwards and Li, 2013; Vegeto et al., 2008). Thus, the depletion of estrogen as a result of menopause activates systemic adaptive and innate immune responses (Giannoni et al., 2011). The innate immune response is characterized, in part, by activation of inflammasomes responsible for the activation of caspase-1 and the processing of the pro-inflammatory cytokines IL-1β and IL-18 (de Rivero Vaccari et al., 2008). Studies have shown that the inflammasome is a critical regulator of the inflammatory response in the brain as a result of injury (Abulafia et al., 2009; Adamczak et al., 2012; de Rivero Vaccari et al., 2014; de Rivero Vaccari et al., 2009), disease (de Rivero Vaccari et al., 2016c; Mohamed et al., 2015) and aging (Mawhinney et al., 2011).

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In addition, it has been shown that sex steroids regulate activation of the inflammasome in the brain (Slowik and Beyer, 2015). Recently, we demonstrated that silencing of estrogen receptor beta (ER-β) attenuated the 17β-estradiol-mediated decrease in caspase-1, ASC, and IL-1β (de Rivero Vaccari et al., 2016c), while periodic ER-β agonist treatment significantly decreased inflammasome activation, thus protecting the brain from global ischemic damage in reproductively senescent (RS) female rats (de Rivero Vaccari et al., 2016c). These findings indicate a key role of estrogen in regulation of inflammasome activation in the brain. However, during menopause, the number of ovarian follicles declines, resulting in less production of estrogen. At the same time, the ovaries become less responsive to the pituitary hormones Luteinizing Hormone and Follicle-Stimulating Hormone. These changes in hormonal environment and ovarian senescence set in motion an inflammatory response through extracellular vesicles (EV) that spreads through the body, including the brain.

Over recent years, EV have garnered significant importance due to their ability to carry a cargo that plays an important role in the immune response (Robbins and Morelli, 2014). EV have several roles in cell signaling, including cell-to-cell communication and antigen presentation (Raposo et al., 1996), and they carry tumor antigens (Wolfers et al., 2001) as well as mycobacterial antigens (Giri and Schorey, 2008). We have previously shown that EV carry inflammasome proteins that play a role in the pathology of brain and spinal cord injury (de Rivero Vaccari et al., 2016a) as well as stroke (Kerr et al., 2018a). Here we analyze the role of EV-containing inflammasome proteins as vesicles responsible for carrying inflammasome proteins from the female reproductive organs to the brain as a consequence of RS. Therefore, we hypothesize that RS triggers an innate immune inflammatory response in the female reproductive organs that spreads to the brain, making the brain more susceptible to ischemic damage.

2. Material and methods

2.1. Animals

All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the Animal Care and Use Committee of the University of Miami. Young (6–7 months) or retired breeder (9–13 months) Sprague–Dawley rats of both sexes were purchased from Charles River Laboratories (Wilmington, MA). Prior to any experimentation on female rats, their estrous cycles were monitored by daily examination of vaginal smears. Retired breeder female rats that remain in constant diestrus were considered RS (Raval et al., 2009). Young, RS and age matched male rats were randomly divided mainly in to two cohorts. A cohort of rats was used for the tissue collection. A second cohort of rats was exposed to transient middle cerebral artery occlusion (tMCAO). In the present study we have analyzed the brain and ovaries of 5 young female and 7 reproductive senescent female rats, as well as the brain of 4 young male and 4 old male rats. We also analyzed the brain of 6 young females and 6 reproductive senescent females that underwent tMCAO. For the adoptive transfer experiments we have analyzed the brain of 11 young female rats that received EV from 6 young females and 5 reproductive senescent females.

2.2. Transient middle cerebral artery occlusion

Transient MCAO was adapted from previous publications (Belayev et al., 1996; Lin et al., 2014). In short, animals were anaesthetized with 5% isoflurane and 30:70 percent mixture of O_2 and N_2O followed by rats were intubated endotracheally and immobilized with rocuronium bromide. Transient MCAO was achieved by intraluminal suture. A 30-mm-long 3–0 nylon monofilament suture coated with silicone (Doccol) and was placed 19–20 mm into the internal carotid artery to occlude the ostium of the MCA. The suture was placed in the MCA for 90 min and the drop in cerebral blood pressure was confirmed using Laser Doppler (LDF, Perimed Inc.). The LDF monitoring was done from a single 2 mm burr hole drilled in the zygomatic bone. The LDF signal reading was recorded prior (15–30 min), during (90 min) suture insertion and for 15–30 min during reperfusion. Rats that had less than 50% drop in LDF signal on suture insertion were excluded from the study prior to randomization and allocation to treatment groups. A group of rats were also exposed to sham surgical procedure, for which rats were exposed to anesthesia for a period similar to that of the tMCAO group. Physiological parameters including, pCO_2, pO_2, and pH were maintained within normal limits through the surgery or sham-surgery. Mean arterial blood pressure (MABP) was continuously monitored and head and body temperatures were maintained at 37 °C. Rats exposed to tMCAO or sham surgery, were allowed to survive for 24 h followed by randomly used for blood and brain tissue collection for biochemical assay or infarct volume quantification by 2,3,5-triphenylte- trazolium chloride (TTC) staining described next.

2.3. TTC staining technique

Twenty-four hours after MCAO rats were sacrificed and brains were collected. The brains were sectioned into 1 mm slices beginning from the rostral end into a total of seven slices. The area of infarction was visualized by incubating the sections in 1.5% TTC (2,3,5-triphenylte-trazolium chloride; Sigma Aldrich) in phosphate buffered saline (PBS) for 15 min at 37°C. The sections were then transferred to 10% formalin (Sigma Aldrich). Images of the sections were scanned, and the hemispheres and areas of infarct were measured using ImageJ software as described previously (Koronowski et al., 2015). All outcomes were measured by an investigator blinded to the experimental conditions.

2.4. Isolation of EV from serum

EV were isolated from rat serum using the Total exosome Isolation from serum kit (Invitrogen) as described in (Kerr et al., 2018a). Briefly, 100 µl of each sample were centrifuged at 2000 × *g* for 30 min and the supernatant was then incubated with 20 µl of Total exosome Isolation reagent for 30 min at 4 °C followed by centrifugation at 10,000 × *g* for 10 min at room temperature. Supernatants were then discarded and the pellet was re-suspended in 50 µl of PBS and 100 µl of lysis buffer.

2.5. Isolation of EV from CSF

Cerebrospinal fluid (CSF) from healthy young females (YF, 23–37 years old) and healthy (not suffering from any chronic inflammatory disease) menopausal women (52–68 years old) were purchased from Bioreclamation IVT. To isolate EV from the CSF of women the Total Exosome Isolation From Other Body Fluids kit (Invitrogen) was used according to manufacturer's instructions. Briefly, 700 µl of CSF were centrifuged at 2000 × *g* for 30 min. The supernatant was then further centrifuged at 10,000 × *g* for 30 min. An equal volume of Total Exosome Isolation from Other Body Fluids reagent was then added and samples were incubated at 4 °C for 1 h followed by centrifugation at 10,000 × *g* for 1 h. Supernatants were discarded and the pellet was resuspended in 75 µl of PBS and 100 µl of lysis buffer. Re-suspended EV were then centrifuged at 10,000 × *g* for 5 min and the supernatant was transferred to a new tube until further processing. For immunoblot analyses equal amounts of protein were loaded for all groups (50 µg of protein).

2.6. Protein quantification

To quantify the protein concentration in isolated EV, the Pierce Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scienftific, Inc.) was used according to the manufacturer's instructions. Serum-derived EV were lysed (1:1 dilution) in lysis buffer as described in (de Rivero Vaccari et al., 2016b).

2.7. Adoptive transfer of serum-derived EV from post-menopausal women into young female rats

Serum-derived EV from peri-menopausal women (ages 52 to 68) were injected into YF (6–7 months) rats through the tail vain at a dose of 1.0×10^{10} particles per gram/body weight (Wiklander et al., 2015). Particle count was measured by Nanosight Tracking analysis and samples were diluted accordingly as described in (Kerr et al., 2018a). Animals were sacrificed 24 h after injection of EV and cortex and hippocampus were isolated for immunoblot analysis. Serum samples from healthy females were purchased from Bioreclamation IVT and used as control.

2.8. Immunoblotting

For detection of inflammasome signaling proteins in rat ovaries/reproductive organs, serum-derived EV, hippocampus, cortex, and CSF-derived EV from women, protein lysates were resolved by immunoblotting as described in (de Rivero Vaccari et al., 2015). Briefly, following lysis of the pellet proteins were resolved in 10–20% Criterion TGX Stain-Free precasted gels (Bio-Rad), using antibodies (1:1000 dilution) to NLRC4 (Novus Biologicals), caspase-1 (Novus Biologicals), ASC (Santa Cruz), IL-1β (Cell Signaling), CD81 (Thermo Scientific) and Nik-related Kinase (Thermo Scientific). Quantification of band density was done using the UN-SCAN-IT gel 5.3 Software (Silk Scientific Corporation). Equal amounts of protein (15 μg) were loaded for each experiment. The Chemilluminescence substrate (LumiGlo, Cell Signaling) in PVDF membranes was imaged using the ChemiDoc Touch Imaging System (BioRad).

2.9. Statistical analyses

Data are shown as mean value ± SEM. Investigators blinded to the experimental conditions performed densitometric analysis of immunoblots and infarct volume quantifications of TTC stained brain slices. Statistical comparisons between groups were done using a two-tailed Student's *t*-test. P-values of significance used was $p < 0.05$.

3. Results

3.1. Inflammasome protein expression is increased in the brain of RSF

We have previously shown that inflammasome activation is increased in the aged brain of rats (Mawhinney et al., 2011). However, whether reproductive senescence contributes to the inflammatory innate immune response through the inflammasome in the brain is yet to be determined. Thus, here we tested the hypothesis that inflammasome activation is significantly higher in the brain of RSF (Fig. 1A) as compared to YF and senescent male rats (OM) (Fig. 1F). Accordingly, we measured the protein levels of NLRC4 (Fig. 1B and G), caspase-1 (Fig. 1C and H), ASC (Fig. 1D and I) and IL-1β (Fig. 1E and J) and found that NLRC4 inflammasome protein activation was increased in the brain of RSF when compared to YF (NLRC4 (34% change), caspase-1 (75% change), ASC (54% change) and IL-1β (81% change)), indicating that in the brain of RSF there is a heightened innate immune inflammatory response that is not present in younger females. Moreover, we did not find a difference in the levels of inflammasome proteins in the brain of young and old male rats, suggesting that the inflammatory effects detected in the senescent brain is related to the reproductive senescence specifically in females.

Since, we observed that inflammasome activation is significantly higher in the brain of RSF when compared to YF, next we tested if there is a difference in the degree of post-tMCAO ischemic brain injury between RSF and YF. Our data indicate that 24 h after ischemia, there was a 41% increase in the infarct volume of RSF rats when compared to YF (Fig. 2). Sham animals did not show any infarction (data not shown). Taken together, these findings indicate that the effects of ischemia on the brain are worse in RSF than in YF rats, suggesting that reproductive senescence makes females more susceptible to the effects of ischemia in the brain.

3.2. Inflammasome protein expression is increased in the female organs of RSF

To test our hypothesis that in the RSF brain inflammation spreads from the reproductive organs in EV, we first determined the levels of the inflammasome signaling proteins caspase-1, ASC and IL-1β in the reproductive organs of YF and RSF (Fig. 3A). Accordingly, the protein levels of caspase-1 (Fig. 3B), ASC (Fig. 3C) and IL-1β (Fig. 3D) were elevated in the reproductive organs of RSF when compared to YF. Thus, indicating that there is an increased inflammatory innate immune response present in the reproductive organs of RSF that is mediated by the inflammasome.

3.3. Inflammasome protein expression is increased in serum-derived EV from RSF

EV are known for spreading inflammation across different tissues and organs (Gupta and Pulliam, 2014; Rak and Guha, 2012; Selmaj et al., 2017). To determine if EV contribute to the spread of inflammation in RSF, we isolated EV from the serum of YF and RSF and resolved them by immunoblot for the expression of inflammasome signaling proteins (Fig. 4A). Our findings indicate that the protein expression of caspase-1 (Fig. 4B), ASC (Fig. 4C) and IL-1β (Fig. 4D) is higher in the serum-derived EV from RSF when compared to YF. CD81 (Fig. 4E) was used as a marker of isolated EV and NRK was used as a marker of EV originating in the female reproductive organs (Fig. 4F). Moreover, we immunoblotted the brain from male and female rats as well as the female reproductive organs to test that NRK was only present in the ovaries but not the brain (Fig. 5A). Interestingly, NRK protein expression levels did not differ between YF and RSF (Fig. 5B). We chose NRK based on information from the Human Protein Atlas (http://www.proteinatlas.org/ENSG00000123572-NRK/tissue) that indicates NRK to be present almost exclusively in the ovary and placenta.

3.4. Inflammasome protein expression is increased in CSF-derived EV from menopausal women

If serum-derived, inflammasome-loaded EV originating in the female reproductive organs contribute to brain inflammation, then we would expect to detect higher levels of inflammasome proteins in the CSF of RSF rats. In this study we were unable to obtain enough CSF from rats to isolate EV; therefore, we tested the expression of inflammasome proteins in CSF-derived EV from women over the age of 52 and run those samples along samples from women under 40 (Fig. 6). Interestingly, we found that in the CSF-derived EV of older females the expression of NRK, NRLC4 and caspase-1 was higher than in the CSF-derived EV from YF. The variability observed between groups is probably the common variability that is present in human samples particularly when other comorbidities may be present such as during menopause. However, we did not notice this variability in the isolates from younger females. Unfortunately, we did not have access to information regarding comorbidities that the CSF donors might have had at the time of donation. Taken together, these data suggest that the inflammatory response that reaches the brain through EV carrying inflammasome proteins originates, in part, in the female reproductive organs.

3.5. Transfer of EVs from post-menopausal women activate the inflammasome in the brain of YF rats

To determine if EV from peri-menopausal women contributes to the inflammatory response in the brain, we isolated serum-derived EV from

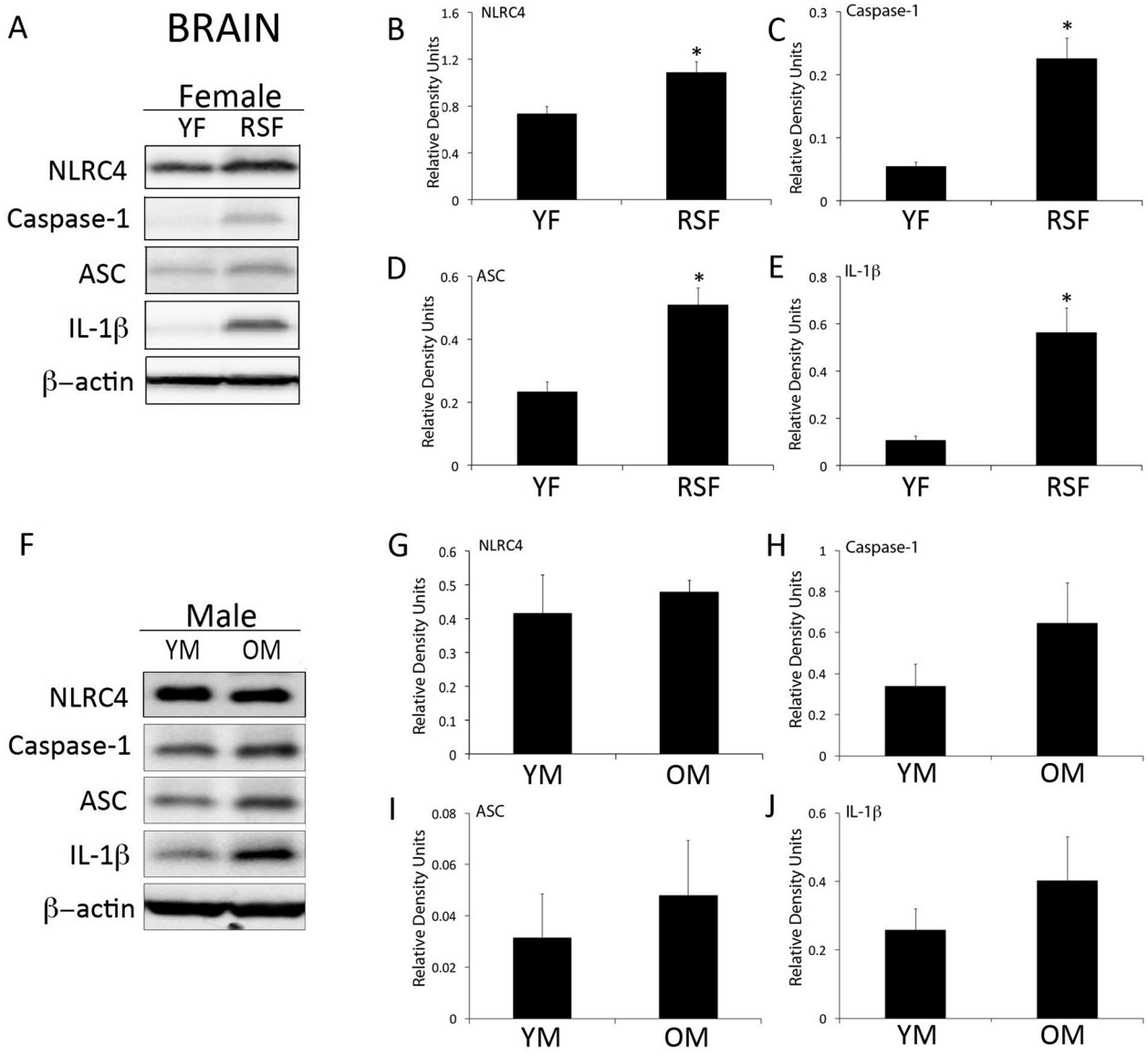

Fig. 1. The brain of RSF expresses higher levels of inflammasome proteins than YF and males. Representative immunoblot of protein lysates that were isolated from the brain of young (YF and YM, 6–7 months) and **A)** reproductive senescent females (RSF, 9–13 months) and **F)** age-matched young (YM) or old (OM) males rats and blotted for **B** and **G)** NLRC4, **C** and **H)** caspase-1, **D and I)** ASC and **E** and **J)** IL-1β. Data were normalized to the β-actin. Data are presented as mean ± SEM. *p < 0.05. N = 5 (YF) and 7 (RSF), N = 4 (YM, Young male), 4 (OM, Old male).

women over the age of 52 and from women under the age of 40 to test the hypothesis that EV from peri-menopausal women induce inflammation in the cortex (Fig. 7A) and hippocampus (Fig. 7F) of YF rats. Accordingly, we found that 24 h after delivery of the EV, capase-1 (Fig. 7C) and ASC (Fig. 7D) were elevated in the cortex of YF rats, whereas we detected no differences in NLRC4 (Fig. 7B) and IL-1β (Fig. 7E). Similarly, in the hippocampus we detected an increase in ASC (Fig. 7I). Thus, these data indicate that EV from RSF containing inflammasome proteins contribute to the inflammatory response that affects the brain in part through the inflammasome.

4. Discussion

In this study, we show for the first time that the inflammasome contributes to the inflammatory response in the brain during menopause, and that this innate immune response arises in part from EV that originate in the female reproductive organs that are carried to the brain in blood and CSF.

Menopause (reproductive senescence) is a natural process of aging in women. The overall process of menopause is slow and lasts for years, and it is mainly divided into peri-menopause and post-menopause. At the time of menopause-transition, the ovaries are depleted of oocytes, and the cyclical production of estradiol and progesterone by the ovaries diminishes and becomes less consistent. Because the life span of women continues significantly beyond the onset of menopause, women are likely to spend one third of their life in the post-menopausal stage, which is also associated with low levels of circulating ovarian hormones, particularly estrogen. Estrogens are known to exert neuroprotective and immunomodulatory effects (Baron, 2006; Cushman et al., 1999; Giefing-Kroll et al., 2015; Vegeto et al., 2008). In menopausal women, the decline in estrogen is associated with increased levels of pro-inflammatory markers such as interleukin (IL)-6, IL-1 and TNF (Pfeilschifter et al., 2002). Additionally, aging also reduces the availability of estrogen receptor subtypes alpha and beta in the brain. In a recent study, we showed that estrogen receptor beta (ER-β) plays a key role in inflammasome protein regulation in the brain of female rats (de Rivero Vaccari et al., 2016c). Since reproductive aging reduces the availability of ER-β in the brain, this could explain why there is

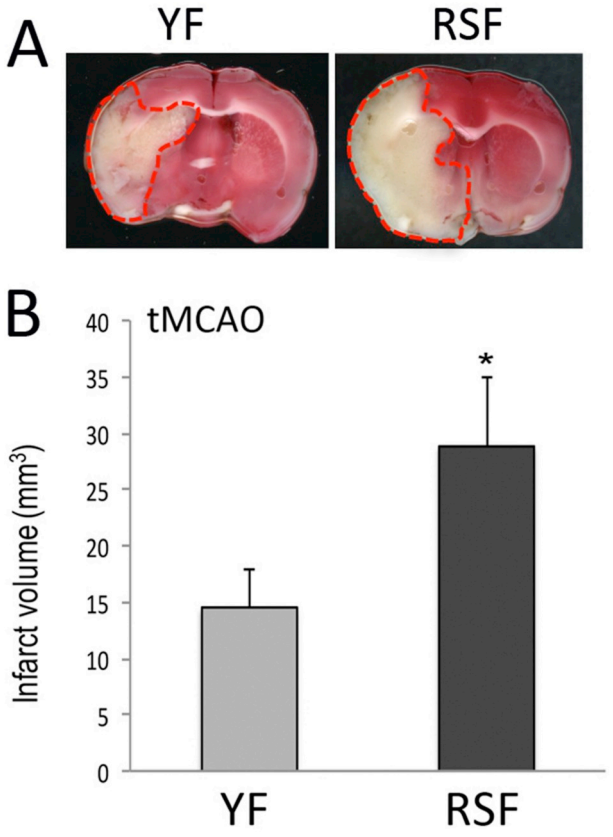

Fig. 2. RSF undergo greater post-tMCAO ischemic damage when compared to YF rats. A) Representative images of TTC stained brain sections after tMCAO. **B)** Infarct volume was measured 24 h following ischemia by analyzing lesion size after rats were subjected to 90 min of tMCAO. Volume was measured in mm^3. Data presented as mean ± SEM. *p < 0.05. N = 6 (YF) and 6 (RSF).

increased post-tMCAO brain damage observed in RSF rats as compared to YF.

Additionally, menopause/reproductive senescence sets in motion a number of systemic structural, biochemical and functional alterations, including in the ovaries and the brain. Here we suggest a novel mechanism by which inflammation takes place in the brain as a result of reproductive senescence in which EV containing inflammasome proteins are secreted from the female reproductive organs into the blood and CSF to then reach the brain, rendering the brain more susceptible to inflammation following a variety of stressors, including stroke.

We have previously shown that inflammasome proteins are present in EV and that inflammasome proteins, as cargo in EV, contribute to the innate immune response in the brain (de Rivero Vaccari et al., 2016b). EV have been shown to contribute to the pathology of diseases (Vella et al., 2008), infections (Izquierdo-Useros et al., 2010), cancer (Luga et al., 2012) as well as in immune signaling (Robbins and Morelli, 2014). In the nervous system, EV are released at synapses upon cell depolarization. Therefore, EV may be involved in the regulation of neuronal excitability (Lachenal et al., 2011) and synaptic plasticity (Goldie et al., 2014). Oligodendrocytes communicate with neurons and astrocytes through EV (Fruhbeis et al., 2013) with a cargo of molecules involved in preventing oxidative stress and a cargo of myelin-related proteins (Kramer-Albers et al., 2007).

In microglia, ATP stimulation of P2X7 results in release of IL-1β in EV (Bianco et al., 2005), which is activated by the inflammasome (Minkiewicz et al., 2013; Silverman et al., 2009). Here we show that EV present in blood and CSF contain inflammasome proteins as well as NRK, a protein that is normally present in female reproductive organs. Thus, we propose that this increase in EV containing inflammasome proteins in RSF may contribute to the exacerbation of brain damage following cerebral ischemia. Moreover, we have recently shown that EV containing inflammasome proteins play a critical role in the pathophysiology of stroke and that these EV containing inflammasome proteins can be potentially used as biomarkers of stroke pathology (Kerr et al., 2018a).

NLRC4 has recently been shown to mediate pyroptosis of microglia following stroke (Poh et al., 2018). Inflammasomes in microglia are major mediators of the innate immune response in a variety of conditions and diseases including trauma (Lee et al., 2018) and neurodegenerative diseases (Gold and El Khoury, 2015). Since microglia play an important role in the regulation of inflammation, and inflammation differs between sexes, then it is imperative to better understand how

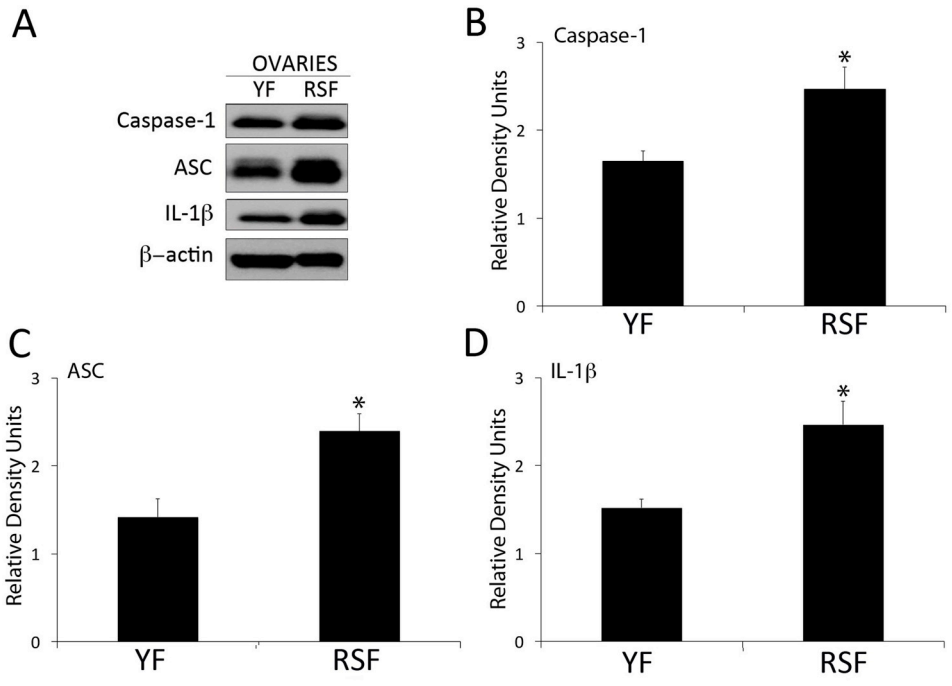

Fig. 3. Ovaries of RSF express high levels of inflammasome proteins. A) Representative immunoblot of protein lysates that were isolated from the ovaries of young (YF, 6–7 months) and reproductive senescent females (RSF, 9–13 month) rats and blotted for **B)** caspase-1, **C)** ASC and **D)** IL-1β. Data were normalized to the β-actin. Data presented as mean ± SEM. *p < 0.05. N = 5 (YF), 7 (RSF).

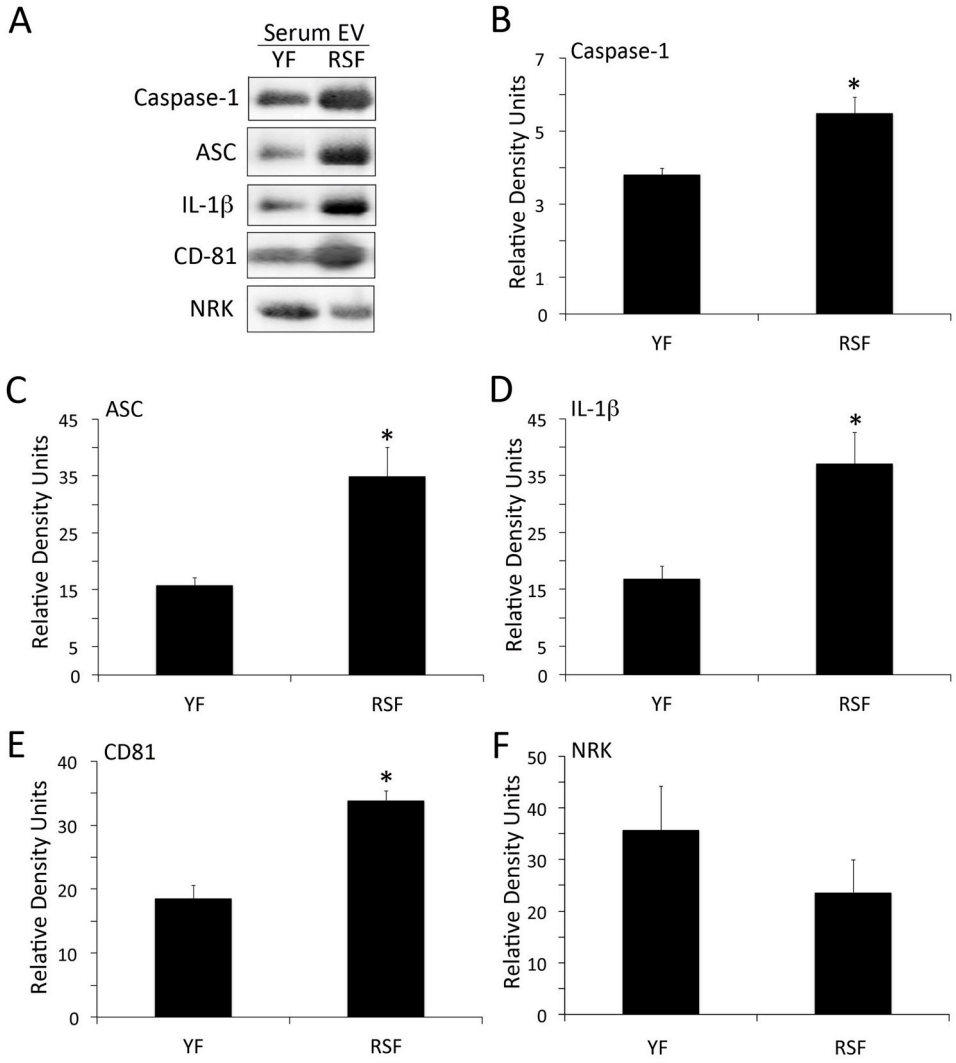

Fig. 4. Inflammasome proteins are elevated in serum-derived EV. EV were isolated from the serum of YF (6–7 months) and RSF (9–13 months) rats. **A)** Representative immunoblot of inflammasome proteins in serum-derived EV. Protein lysates of serum-derived EV were blotted for **B)** caspase-1, **C)** ASC, **D)** IL-1β, **E)** CD81 and **F)** NRK. CD81 was used as a marker for EV and NRK as a marker for an ovary-derived/specific protein. Data presented as mean ± SEM. *$p < 0.05$. N = 4 per group.

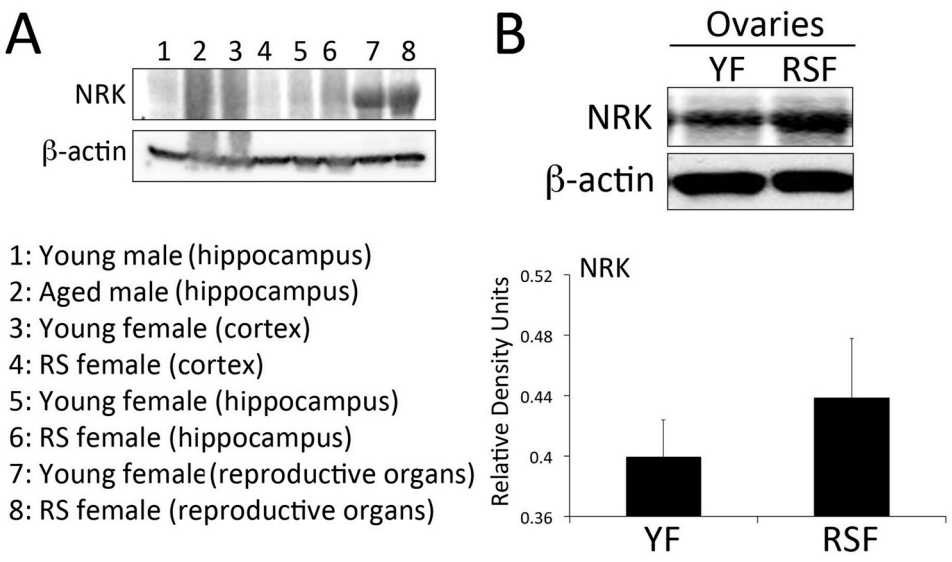

Fig. 5. NRK is expressed in the ovaries but not the brain: A) Immunoblot of protein lysates corresponding to 1) young male (hippocampus), 2) aged male (hippocampus), 3) young female (cortex), 4) RSF (cortex), 5) young female (hippocampus), 6) RSF (hippocampus), 7) YF ovaries and 8) RSF (ovaries). **B)** Representative immunoblot of RSF and YF ovaries blotted for NRK and normalized to β-actin. Data presented as mean ± SEM. N = 5 (YF) and 7 (RSF).

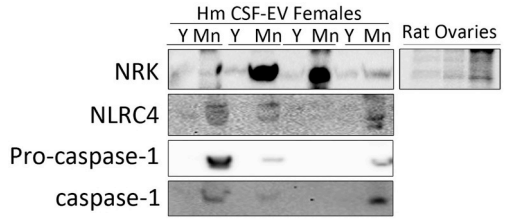

Fig. 6. NRK, caspase-1 and NLRC4 are elevated in CSF-derived EV from menopausal females. EV were isolated from the CSF of YF (23–37 y/o) and RSF (52–68 y/o) and blotted for NRK, NLRC4 and caspase-1. Rat ovaries were loaded as a molecular weight control for NRK. Y: Young, Mn: Menopause.

inflammation affects males and females differently. Understanding this difference will provide important insights regarding why females are more prone to suffer from certain conditions such as AD, depression when compared to men who are more likely to suffer from diseases like Parkinson's disease (Hanamsagar and Bilbo, 2016).

Our findings indicate that the inflammasome proteins NLRC4, caspase-1, ASC and IL-1β significantly increase in the hippocampus, serum, and female reproductive organs of RSF as compared to YF. Similar changes were not observed in the brain of age-matched males. These findings are consistent with higher levels of inflammasome proteins in serum-derived EV. In addition, we were able to detect NRK in these EV. NRK is a protein present in the female reproductive organs (http://www.proteinatlas.org/ENSG00000123572-NRK/tissue). Considering that the origin of EV can be determined by looking at tissue-specific proteins as markers, our findings suggest that the NRK containing EV from serum, in part, originate in the female reproductive organs. Thus, indicating that the female reproductive organs such as the ovaries secrete EV into the bloodstream containing inflammasome proteins. Interestingly, we were able to detect increased inflammasome protein expression in CSF-derived EV from females over the age of 52, when compared to a younger cohort of female samples. This suggests that the

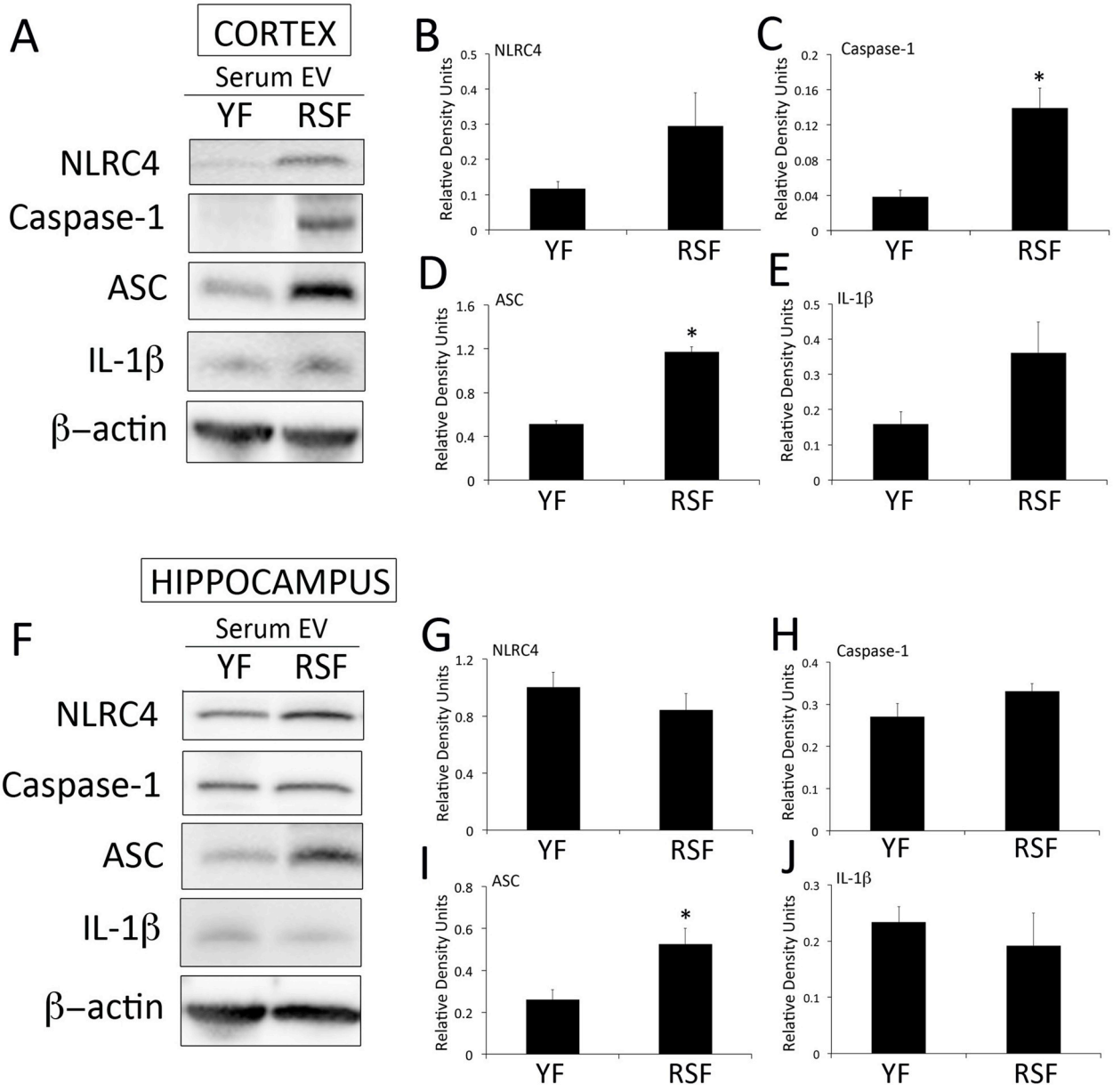

Fig. 7. Serum-derived EV from menopausal females activates the inflammasome in the brain of YF rats. Representative immunoblot of **A)** cortical and **F)** hippocampal lysates from YF rats that were treated with serum-derived EV from young (YF) and menopausal females (RSF). Protein lysates were blotted for **B** and **G)** NLRC4, **C** and **H)** caspase-1, **D** and **I)** ASC and **E** and **J)** IL-1β. Data presented as mean ± SEM. *p < 0.05. N = 6 (YF) and 5 (RSF).

mechanism that we are suggesting in rodents may also extend to humans. Accordingly, since we detected NRK in CSF-derived EV, it is feasible to hypothesize that the origin of the EV containing increased levels of inflammasome proteins originated in the reproductive organs of females.

Importantly, our adoptive transfer experiment in which we isolated EV from the serum of peri-menopausal women and deliver them intravenously to young rats, thus inducing heightened inflammasome activation in the brain, indicates that EV in menopausal women are responsible for the increased inflammatory response present in the brain of YF rats. Similarly, using this approach, we have recently shown a comparable effect of systemic inflammation mediated by EV containing inflammasome proteins in the context of acute lung injury after brain injury (Kerr et al., 2018b).

5. Conclusions

Taken together, we report that inflammasome proteins are elevated in the reproductive organs as well as in the brain of RSF. In addition, this is the first study to show higher levels of inflammasome proteins in serum-derived and CSF-derived EV in RSF. Importantly, we found that NRK, which is mainly present in the reproductive organs of females but absent in the brain, is elevated in CSF-derived EV, which suggest that the EV that are traveling in the body fluids carry inflammasome proteins as a cargo that originates in the reproductive organs and spreads to the brain during menopause, thus producing an exacerbated innate immune response in the brain. Current studies investigating the role of EV in post-ischemic inflammation are underway to understand how modulating EV trafficking can reduce the incidence and impact of cerebral ischemia in peri-menopausal women.

Conflicts of interest

JPdRV is a co-founder and managing member of InflamaCORE, LLC and have patents on inflammasome proteins as biomarkers of injury and disease as well as on targeting inflammasome proteins for therapeutic purposes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.11.018.

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