Cellular and Molecular Neurobiology (2019) 39:321–328
https://doi.org/10.1007/s10571-018-0643-0

BRIEF COMMUNICATION

The Effect of MPP+ on the Viability of Primary Cortical Astrocytes Isolated from Female and Male Wistar Rats of Different Ages

Adriana Alarcón-Aguilar[1] · Armando Luna-López[2] · Norma Edith López-Diazguerrero[1] · Mina Königsberg[1,3]

Received: 3 September 2018 / Accepted: 7 December 2018 / Published online: 11 December 2018

Abstract

Although age is known to be the main risk for developing chronic and neurodegenerative diseases, those illnesses have a different prevalence depending on the sex. It has been questioned whether genetic and hormonal differences are preserved in primary cultures from individuals of different genders. Therefore, here we studied the susceptibility of astrocytes, obtained from female and male Wistar rats of different ages (newborn, 9 and 24 months-old), to the well-known toxin MPP+ after 2 weeks in vitro, at different concentrations and exposure times. Our results showed that there are no variances due to gender, but that there are important differences associated to age in terms of the viability against this toxin.

Keywords Astrocytes · Rats · Sex · Gender · Age · MPP+

Introduction

It is known that many neuropsychiatric and neurodegenerative diseases have different prevalence depending on sex and age (Valentino and Bangasser 2016). It has even been recognized that, although females live longer than males, they have a higher prevalence in diseases and morbidities associated with age (Fischer et al. 2016). Even so, that, for many years, most of the neurobiological studies were conducted on male animal models, due to the variability of the results obtained in females, mainly due to the hormonal effects (Choleris et al. 2018).

As for in vitro research, there are only a few reports studying cells obtained from animals of both genders. Nevertheless, it is known that both neurons and glia have different properties according to sex. In particular, regarding astrocytes, their morphology, number, and cellular responses vary depending on the gender (Pfau et al. 2016; Schwarz and Bilbo 2012).

Recently, many scientific journals, as well as numerous grant programs, are beginning to demand that research should be done on both sexes (Miller et al. 2017). Because of its great importance, we studied the differences between the sexes in terms of their survival response against a toxin well documented and used in neurobiology, such as 1-methyl-4-phenylpyridinium (MPP+). This toxin is known to impair mitochondrial function, and to induce ROS production and cell death in neurons and astrocytes (Przedborski and Vila 2003; Zhang et al. 2010; Alarcón-Aguilar et al. 2014). We also compared the results according to age, since there exist no reports analyzing astrocytes behavior by gender, in adult and old animals.

Materials and Methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co.

✉ Mina Königsberg
mkf@xanum.uam.mx

[1] Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, 09340 Ciudad de México, Mexico

[2] Instituto Nacional de Geriatría, SSA, 10200 Ciudad de México, Mexico

[3] División de Ciencias Biológicas y de la Salud, Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, A.P. 55-535, C.P 09340 Ciudad de México, Mexico

Animals

Primary astrocytes were isolated from the frontal cortex of female and male Wistar rats, divide into: (*Rattus norvegicus*) newborns (3-days old), adults (9-month old), and old (24-month old). The animals were obtained from our institutional closed breeding colony (Universidad Autónoma Metropolitana-Iztapalapa). The animals' treating procedures were always strictly performed according to the Mexican Official Ethics Standard 062-ZOO-1999, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This project was approved by the Universidad Autónoma Metropolitana-Iztapalapa, ethics committee (dictum 1706–2018).

Cortical Astrocytes Isolation and Culture

The astrocytes isolation was performed as reported previously in Lin et al. (2007) and McCarthy and de-Vellis (1980), and cultured using MEM supplemented with 10% FBS, glutamine 0.11%, glucose 0.15% and penicillin/streptomycin 0.1%, as described before in Alarcón-Aguilar et al. (2014).

MPP+ and H_2O_2 Cytotoxic Treatments

After 2 weeks in culture, the astrocytes were reseeded at a cellular density of 2.5×10^5 cells per well on a 24-well multi-chamber. Twenty-four hours after that, 10, 25, 50 or 100 µM MPP+ were added to the culture media for 1, 3 or 5 h at 37 °C and 5% CO_2. In the case of H_2O_2, a single concentration of 100 µM was assessed for 1 or 3 h under the same conditions.

Cellular Viability

The trypan blue assay is used to differentiate dead cells (with membrane disruption) from live cells (with intact membranes). So, the viability was evaluated using this technique as reported elsewhere (Seifrtova et al. 2013). Briefly, cells were trypsinized and a 20 µl aliquot was stained with an equal volume of a 0.4% trypan blue physiological solution. The number of living cells in 10 µl of this suspension was counted using the Vi-Cell-XR 2.03 (Beckman-Coulter).

Statistical Analysis

The data are reported as the mean ± SD of at least three independent experiments in triplicate. A multivariate analysis of variances (MANOVA) was performed to determine the effect of sex and treatments on cell viability, and an ANOVA test was performed, followed by a Tukey–Kramer multiple comparisons test, to determine the differences between the treatments. Values of $p < 0.05$ were considered as statistically significant.

Results

To determine the effect of MPP+ on the viability of the primary astrocytes, we performed a multivariate analysis of variances (MANOVA). The results showed that gender does not affect cellular viability in astrocytes from newborn rats (Fig. 1a: $p = 0.4233$; Fig. 1b: $p = 0.7789$ and Fig. 1c: $p = 0.8590$), adult rats (Fig. 2a: $p = 0.6775$; Fig. 2b: $p = 0.9041$ and Fig. 2c: $p = 0.9805$), or old rats (Fig. 3a: $p = 0.6556$; Fig. 3b: $p = 0.0967$ and Fig. 3c: $p = 0.0912$). On the other hand, the results of the exposure to different MPP+ concentrations during diverse times of exposure showed differences in all the experiments performed. The results presented in Fig. 1a–c show that the astrocytes obtained from newborn rats significantly decreased their viability proportionally to the increase in the concentration of the toxin and the time of exposure ($p < 0.05$, $p < 0.01$). This same susceptibility to concentration and time of exposure was observed in the astrocytes isolated from adults (Fig. 2a–c) and old rats (Fig. 3a–c).

However, the astrocytes from old rats decreased their cell viability by a higher percentage, compared to the adult and newborn astrocytes, in all treatments ($p < 0.05$). The adult cells showed significant differences with respect to the newborns in the concentrations of 25 and 50 µM after the 1 h treatment ($p < 0.05$), as well as with MPP+ 50 and 100 µM for 3 and 5 h ($p < 0.05$).

Additionally, the effect of H_2O_2 100 µM after 1 and 3 h treatments was evaluated and, again, no significant differences were found by gender (Fig. 4a, newborn $p = 0.8169$; Fig. 4b adult $p = 0.9589$, Fig. 4c old $p = 0.7904$). Therefore, the exposure time turned out to be the variable that influenced cell viability ($p < 0.0001$) in all the experimental groups. As in MPP+ treatments, cultures of old astrocytes were more susceptible to the exposure time than adult astrocytes and neonates ($p < 0.05$).

Our results suggest that the increased susceptibility to MPP+ is a function of the age of the organisms from which the astrocytes come, but it is not related to gender.

Discussion

Gender significantly influences the risk of acquiring different illnesses, including cardiovascular, autoimmune and neurodegenerative diseases (Congdon 2018; Ober et al. 2008). Nevertheless, it has been questioned whether the genetic

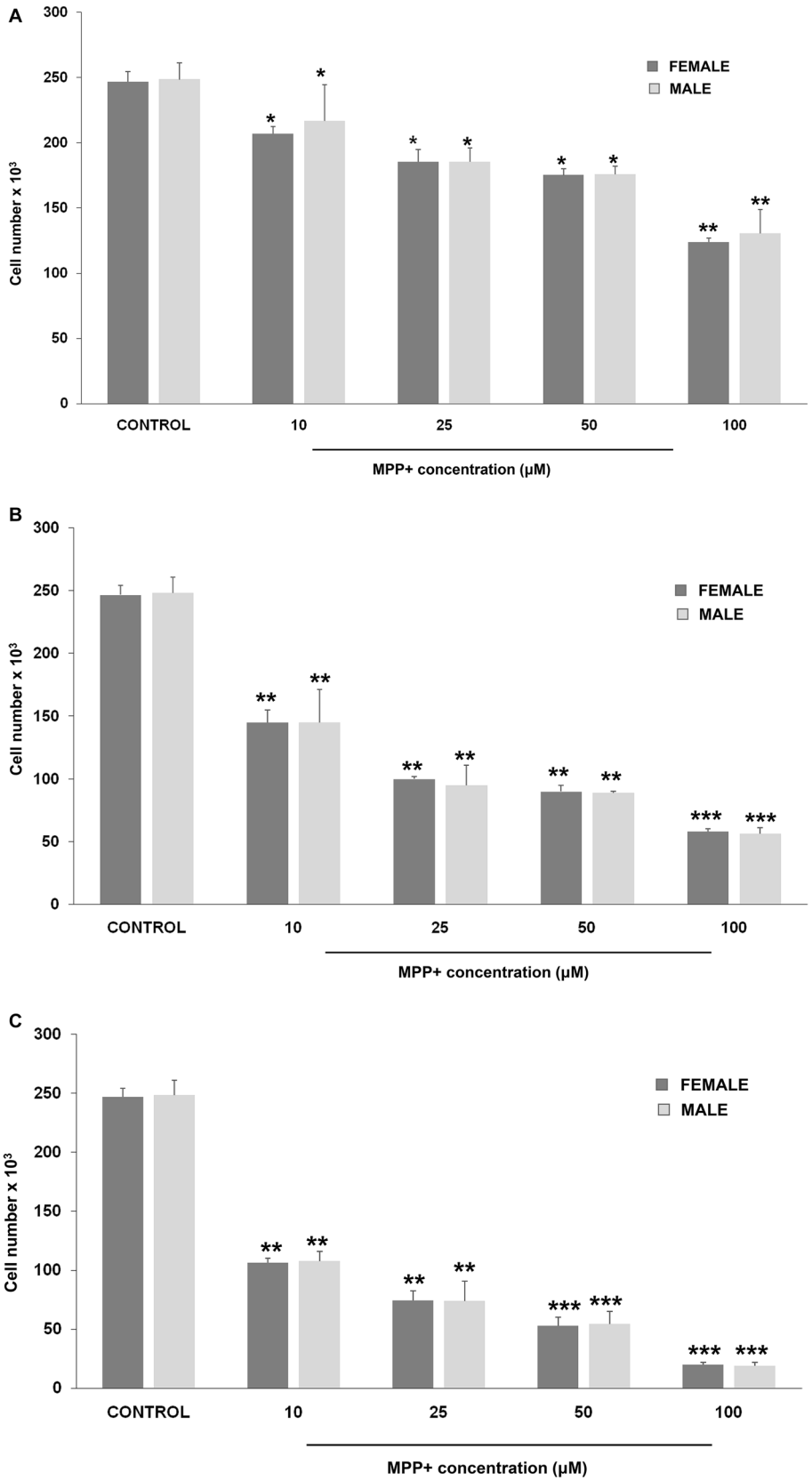

Fig. 1 Cellular viability in neonatal astrocytes. Cortical astrocytes isolated from female (dark gray bars) and male (light gray bars) Wistar newborn rats were exposed to different MPP+ concentrations during different time-points, 1 h (**a**), 3 h (**b**) and 5 h (**c**). The values represent the mean ± SD of three independent experiments. The differences were determined by the ANOVA test followed by a Tukey–Kramer multiple comparisons method. $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to the control

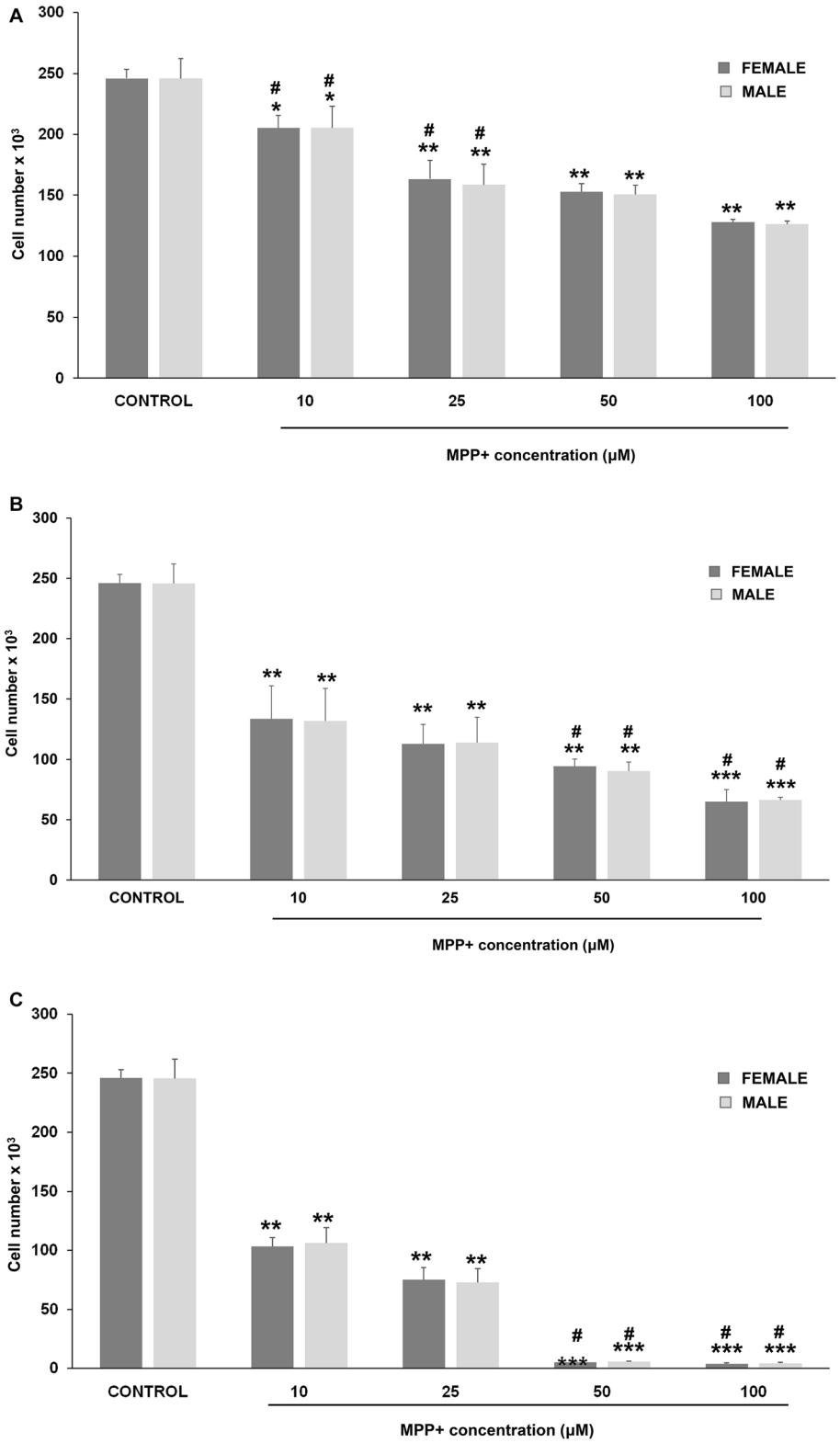

Fig. 2 Cellular viability in adult astrocytes. Cortical astrocytes isolated from female (dark gray bars) and male (light gray bars) 9 months-old Wistar rats were exposed to different MPP+ concentrations during different time-points, 1 h (**a**), 3 h (**b**) and 5 h (**c**). The values represent the mean ± SD of three independent experiments. The differences were determined by the ANOVA test followed by a Tukey–Kramer multiple comparisons method. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with respect to the control

and hormonal differences are preserved in primary cultures from individuals of different genders. From this perspective, we studied the susceptibility of primary astrocytes obtained from male and female Wistar rats of different ages after 2 weeks in culture, in order to avoid the effect of circulating hormones.

Although we found interesting differences in the survival response among the diverse ages studied, both in time and

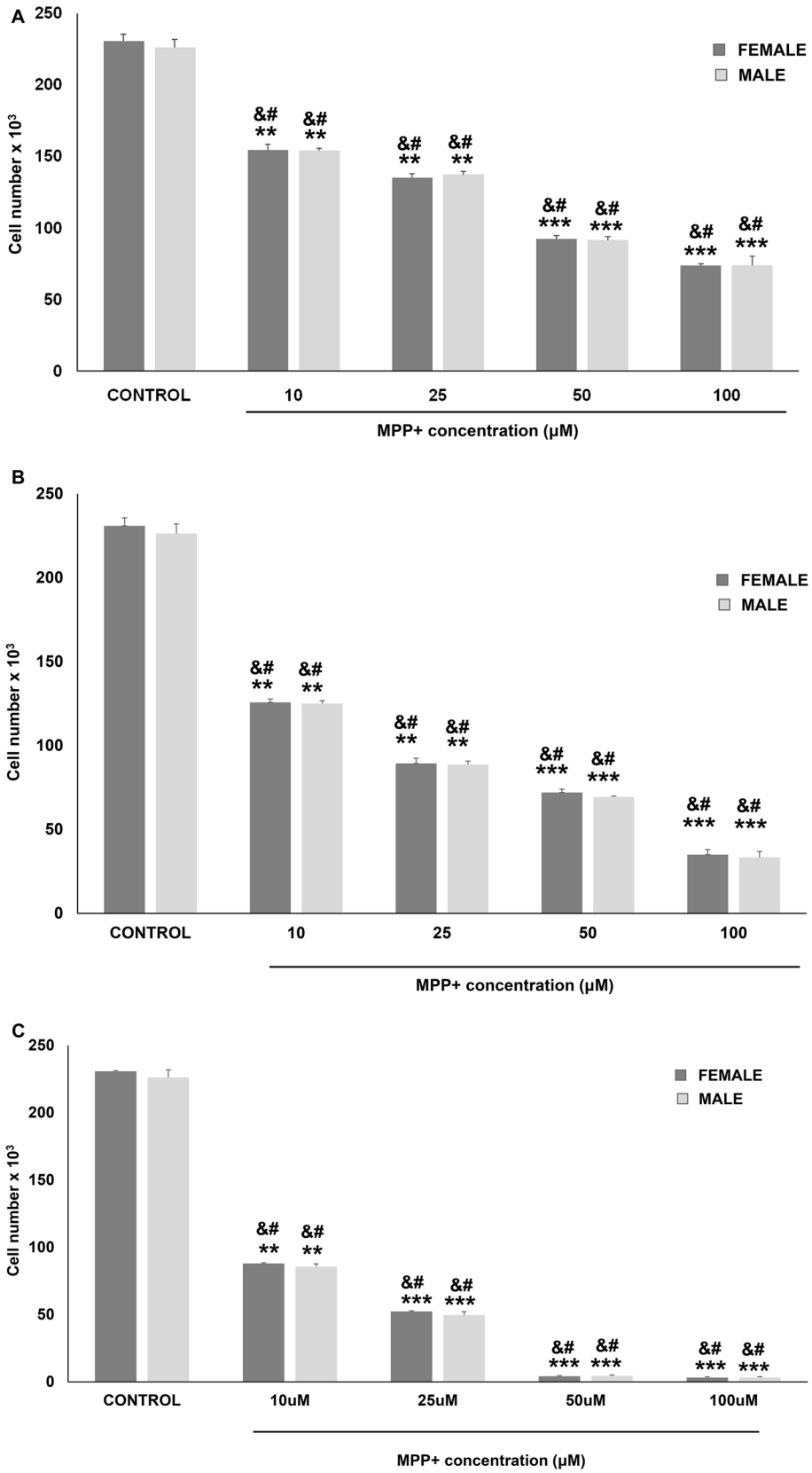

Fig. 3 Cellular viability in old astrocytes. Cortical astrocytes isolated from female (dark gray bars) and male (light gray bars) 24 months-old Wistar rats were exposed to different MPP+ concentrations during different time-points, 1 h (**a**), 3 h (**b**) and 5 h (**c**). The values represent the mean ± SD of three independent experiments. The differences were determined by the ANOVA test followed by a Tukey–Kramer multiple comparisons method. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ with respect to the control, $^{\#}p < 0.05$ with respect to the newborn astrocytes; $^{\&}p < 0.05$ with respect to the adult astrocytes

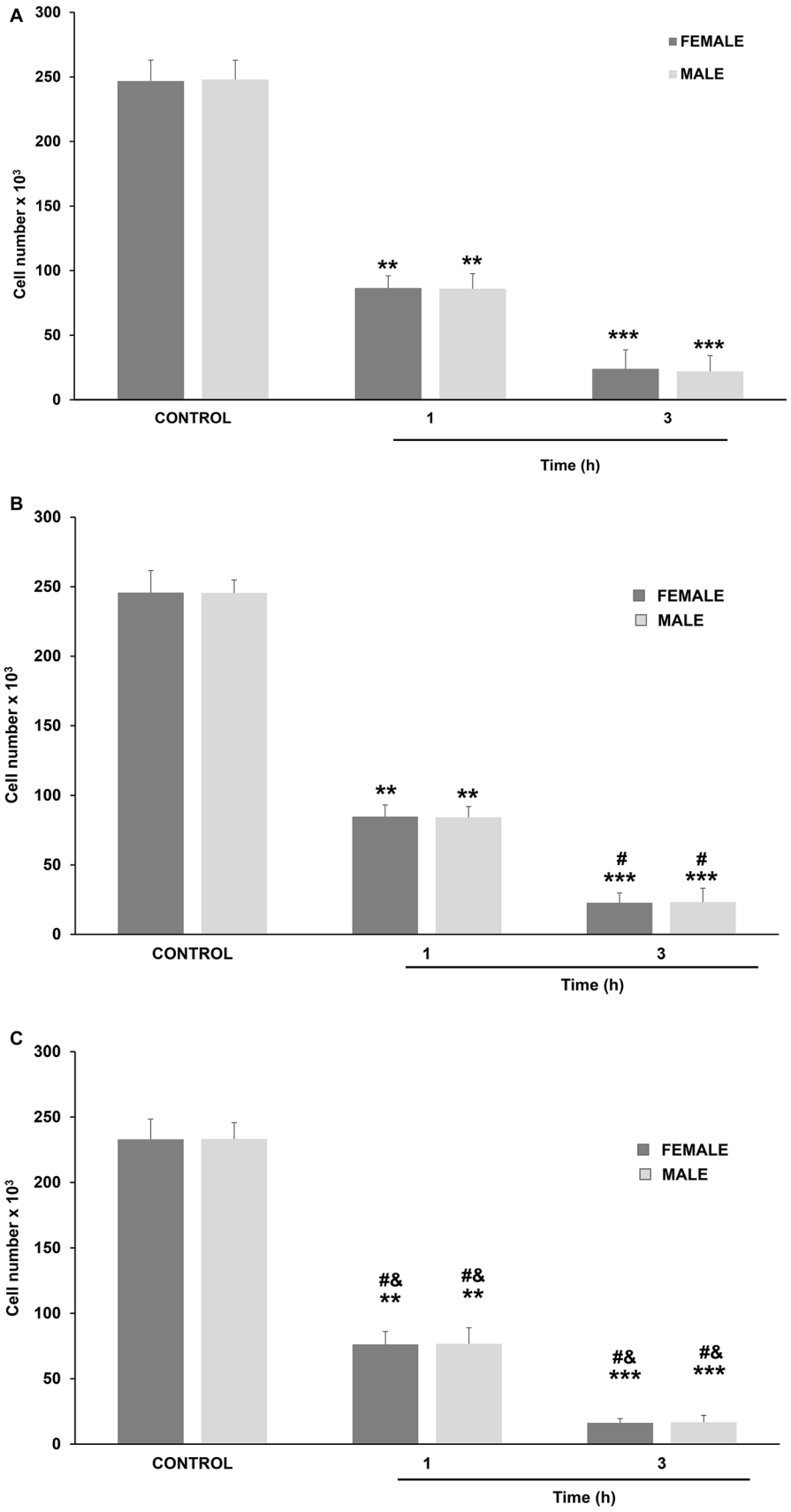

Fig. 4 Cellular viability after H_2O_2 toxic insult. Cortical astrocytes isolated from female (dark gray bars) and male (light gray bars) newborn (**a**), adult (**b**) and old (**c**) rats were exposed to 100 µM H_2O_2 during 1 and 3 h. The values represent the mean ± SD of three independent experiments. The differences were determined by the ANOVA test followed by a Tukey–Kramer multiple comparisons method. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ with respect to the control, $^{\#}p < 0.05$ with respect to the newborn astrocytes; $^{\&}p < 0.05$ with respect to the adult astrocytes

concentration of the toxin exposure, no differences were found between genders. In other species, similar results have been reported. For example in mice, it was shown that when newborn astrocytes of different genders were exposed to the insecticide dimethoate, no changes in cell viability were observed (Astiz et al. 2014). However, in that study the inflammatory profile was higher in males, suggesting that this pro-inflammatory profile might have significant effects at the tissue and organism level. Conversely, another study using microRNA from mice (Mangold et al. 2017) found that females have more pro-inflammatory processes, which is in agreement with the increased activity of astrocytes, as proposed by Chisholm and Sohrabji (2016).

Interestingly, those experiments were performed on astrocytes only 5 days after cultivation, where the hormonal effects in the animal brain might still be present. Liu et al. (2008) reported that newborn female astrocytes were more resistant to oxygen deprivation due to p450 aromatase. However, other studies have not been able to find differences related to sex when the astrocytes were deprived of glucose and oxygen (Johnsen and Murphy 2011). Similarly, Sundar-Boyalla et al. (2011) used cortical and mesencephalon astrocytes of newborn mice exposed to different MPP+ concentrations, and found a more significant death rate in astrocytes from males only in the mesencephalon region, while those from the cortex did not show differences between the sexes. They suggested that the differences could have been related to specific tissue responses related to the physiological and energetic function of each brain region. Again, those experiments were performed treating the astrocytes 4–5 days after isolation, unlike our experiments, where the astrocytes were treated 2 weeks after isolation. One explanation might be that after a long time in vitro, the effect of the circulating hormones might no longer be present, and cells might evade the hormonal-associated effects. However, that idea still needs to be proved.

Regarding the adult and old animals, it is known that the rat's brain presents sexual dimorphism, mainly related to the astrocytes and neurons quantities and morphology in the different brain regions (Pfau et al. 2016). Sexual differences in the organization of the brain are essential for the expression of specific behaviors (Keverne 2008). It has been widely reported that responses to stress in different brain regions of adult organisms are gender-dependent on in vivo models (Lazarus et al. 2015; Morrison and Filosa 2016). However, it is not known whether these differences can be preserved in astrocytes cultures of fully sexually developed individuals, such as adult or aged organisms when they are extracted from their hormonal environment. Most of the work that has been done to demonstrate the different responses to stress that occur in primary cultures of newborn, adults and old animals, have been carried out in males, where the main objective was to demonstrate that the physiological response to stress is age-dependent and not gender-dependent (Longoni et al. 2018; Santos et al. 2018; Souza et al. 2016, 2017).

As far as we know, there are no studies with astrocytes obtained from adult or old rats separated by gender. Interestingly, our results showed no differences in gender when we exposed adult and old astrocytes to H_2O_2 or MPP+ at different concentrations and exposure times. Our results agree with the idea that the response to stress decreases with age. It is still possible that gender-related differences might be preserved in some brain regions or cellular types (other than the cortical astrocytes studied here) characterized by a specific function linked to metabolism or sexual functions that under certain circumstances can be influenced by the hormonal environment (Huxley et al. 2018; Jaber et al. 2018; Santos et al. 2017; Villa et al. 2018). In this sense, it has been reported that the use of hormonal therapies, including estrogen and testosterone, has neuroprotective effects in a variety of contexts (Engler-Chiurazzi et al. 2017; Pike et al. 2009; Rosario et al. 2011).

In summary, our work suggests that the diverse susceptibility to stress in organisms of the same species, given by features such as age and gender, might be studied in vitro; and that, at least in rat primary cortical astrocytes, there are no differences given by gender, but there are significant differences associated to age in terms of cell viability against certain toxins. However, in regards to the inflammatory response resulting from those differences, it would still be necessary to perform more experiments to assess whether or not they depend on gender.

Acknowledgements The authors thank Dr. Wilhelmina Sudarsky for English editing. This work was supported by CONACyT grant FON. INST/298/2016.

Author Contributions Alarcón-Aguilar A and Luna-López A performed the experiments and statistics. López-Diazguerrero NE, Alarcón-Aguilar A and Konigsberg M designed the experiments, analyzed the data, and wrote the paper.

Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to disclose.

References

Alarcón-Aguilar A, Luna-López A, Ventura-Gallegos JL, Lazzarini R, Galván-Arzate S, González-Puertos VY, Morán J, Santamaría A, Konigsberg M (2014) Primary cultured astrocytes from old rats are capable to activate the Nrf2 response against MPP + toxicity after tBHQ pre-treatment. Neurobiol Aging 35(8):1901–1912. https://doi.org/10.1016/j.neurobiolaging.2014.01.143

Astiz M, Acaz-Fonseca E, Garcia-Segura LM (2014) Sex differences and effects of estrogenic compounds on the expression of inflammatory molecules by astrocytes exposed to the insecticide dimethoate.

Neurotox Res 25(3):271–285. https://doi.org/10.1007/s12640-013-9417-0
Austad SN, Fischer KE (2016) Sex differences in lifespan. Cell Metab 23:1022–1033. https://doi.org/10.1016/j.cmet.2016.05.019
Chisholm NC, Sohrabji F (2016) Astrocytic response to cerebral ischemia is influenced by sex differences and impaired by aging. Neurobiol Dis 85:245–253. https://doi.org/10.1016/j.nbd.2015.03.028
Choleris E, Galea LAM, Sohrabji F, Frick KM (2018) Sex differences in the brain: implications for behavioral and biomedical research. Neurosci Biobehav Rev 85:126–145. https://doi.org/10.1016/j.neubiorev.2017.07.005
Congdon EE (2018) Sex Differences in Autophagy Contribute to Female Vulnerability in Alzheimer's Disease. Front Neurosci 12:372. https://doi.org/10.3389/fnins.2018.00372
Engler-Chiurazzi EB, Brown CM, Povroznik JM, Simpkins JW (2017) Estrogens as neuroprotectants: estrogenic actions in the context of cognitive aging and brain injury. Prog Neurobiol 157:188–211. https://doi.org/10.1016/j.pneurobio.2015.12.008
Fischer KE, Hoffman JM, Sloane LB, Gelfond JAL, Soto VY, Richardson AG, Austad SN (2016) A cross-sectional study of male and female C57BL/6Nia mice suggests lifespan and healthspan are not necessarily correlated. Aging 8(10):2370–2391. https://doi.org/10.18632/aging.101059
Huxley VH, Kemp SS, Schramm C, Sieveking S, Bingaman S, Yu Y, Zaniletti I, Stockard K, Wang J (2018) Sex differences influencing micro- and macrovascularendothelial phenotype in vitro. J Physiol 596(17):3929–3949. https://doi.org/10.1113/JP276048
Jaber SM, Bordt EA, Bhatt NM, Lewis DM, Gerecht S, Fiskum G, Polster BM (2018) Sex differences in the mitochondrial bioenergetics of astrocytes but not microglia at a physiologically relevant brain oxygen tension. Neurochem Int 117:82–90. https://doi.org/10.1016/j.neuint.2017.09.003
Johnsen D, Murphy SJ (2011) Isoflurane preconditioning protects astrocytes from oxygen and glucose deprivation independent of innate cell sex. J Neurosurg Anesthesiol 23(4):335–340. https://doi.org/10.1097/ANA.0b013e3182161816
Keverne EB (2008) Visualisation of the vomeronasal pheromone response system. Bioessays 30(9):802–805. https://doi.org/10.1002/bies.20798
Lazarus RC, Buonora JE, Jacobowitz DM, Mueller GP (2015) Protein carbonylation after traumatic brain injury: cell specificity, regional susceptibility, and gender differences. Free Radic Biol Med 78:89–100. https://doi.org/10.1016/j.freeradbiomed.2014.10.507
Lin DT, Wu J, Holstein D, Upadhyay G, Rourk W, Muller E, Lechleiter JD (2007) Ca^{2+} signaling, mitochondria and sensitivity to oxidative stress in aging astrocytes. Neurobiol Aging 28:99–111. https://doi.org/10.1016/j.neurobiolaging.2005.11.004
Liu M, Oyarzabal EA, Yang R, Murphy SJ, Hurn PD (2008) A novel method for assessing sex-specific and genotype-specific response to injury in astrocyte culture. J Neurosci Methods 171(2):214–217. https://doi.org/10.1016/j.jneumeth.2008.03.002
Longoni A, Bellaver B, Bobermin LD, Santos CL, Nonose Y, Kolling J, Dos Santos TM, de Assis AM, Quincozes-Santos A, Wyse ATS (2018) Homocysteine induces glial reactivity in adult rat astrocyte cultures. Mol Neurobiol 55(3):1966–1976. https://doi.org/10.1007/s12035-017-0463-0
López-Diazguerrero NE, López-Araiza H, Conde-Pérezprina JC, Bucio L, Cárdenas MC, Ventura JL, Covarrubias L, Gutiérrez-Ruiz MC, Zentella A, Konigsberg M (2006) Bcl-2 protects against oxidative stress while inducing premature senescence. Free Rad Biol Med 40:1161–1169. https://doi.org/10.1016/j.freeradbiomed.2005.11.002
Mangold CA, Wronowski B, Du M, Masser DR, Hadad N, Bixler GV, Brucklacher RM, Ford MM, Sonntag WE, Freeman WM (2017) Sexually divergent induction of microglialassociated neuroinflammation with hippocampal aging. J Neuroinflamm 14(1):141. https://doi.org/10.1186/s12974-017-0920-8
McCarthy KD, de Vellis (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85(3):890–902. https://doi.org/10.1083/jcb.85.3.890
Miller LR, Marks C, Becker JB, Hurn PD, Chen WJ, Woodruff T, McCarthy MM, Sohrabji F, Schiebinger L, Wetherington CL, Makris S, Arnold AP, Einstein G, Miller VM, Sandberg K, Maier S, Cornelison TL, Clayton JA (2017) Considering sex as a biological variable in preclinical research. FASEB J 31(1):29–34. https://doi.org/10.1096/fj.201600781R
Morrison HW, Filosa JA (2016) Sex differences in astrocyte and microglia responses immediately following middle cerebral artery occlusion in adult mice. Neuroscience 339:85–99. https://doi.org/10.1016/j.neuroscience.2016.09.047
Ober C, Loisel DA, Gilad Y (2008) Sex-specific genetic architecture of human disease. Nat Rev Genet 9(12):911–922. https://doi.org/10.1038/nrg2415
Pfau DR, Hobbs NJ, Breedlove SM, Jordan CL (2016) Sex and laterality differences in medial amygdala neurons and astrocytes of adult mice. J Comp Neurol 524(12):2492–2502. https://doi.org/10.1002/cne.23964
Pike CJ, Carroll JC, Rosario ER, Barron AM (2009) Protective actions of sex steroid hormones in Alzheimer's disease. Front Neuroendocrinol 30(2):239–258. https://doi.org/10.1016/j.yfrne.2009.04.015
Przedborski S, Vila M (2003) The 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. Ann N Y Acad Sci 991:189–198
Rosario ER, Chang L, Head EH, Stanczyk FZ, Pike CJ (2011) Brain levels of sex steroid hormones in men and women during normal aging and in Alzheimer's disease. Neurobiol Aging 32(4):604–613. https://doi.org/10.1016/j.neurobiolaging.2009.04.008
Santos RS, de Fatima LA, Frank AP, Carneiro EM, Clegg DJ (2017) The effects of 17 alpha-estradiol to inhibit inflammation in vitro. Biol Sex Differ 8(1):30. https://doi.org/10.1186/s13293-017-0151-9
Santos CL, Roppa PHA, Truccolo P, Fontella FU, Souza DO, Bobermin LD, Quincozes-Santos A (2018) Age-dependent neurochemical remodeling of hypothalamic astrocytes. Mol Neurobiol 55(7):5565–5579. https://doi.org/10.1007/s12035-017-0786-x
Schwarz JM, Bilbo SD (2012) Sex, glia, and development: interactions in health and disease. Horm Behav 62:243–253. https://doi.org/10.1016/j.yhbeh.2012.02.018
Seifrtova M, Havelek R, Soukup T, Filipova A, Mokry J, Rezacova M (2013) Mitoxantrone ability to induce premature senescence in human dental pulp stem cells and human dermal fibroblasts. J Physiol Pharmacol 64(2):255–266
Souza DG, Bellaver B, Bobermin LD, Souza DO, Quincozes-Santos A (2016) Anti-aging effects of guanosine in glial cells. Purinergic Signal 12(4):697–706. https://doi.org/10.1007/s11302-016-9533-4
Souza DG, Bellaver B, Terra SR, Guma FCR, Souza DO, Quincozes-Santos A (2017) In vitro adult astrocytes are derived from mature cells and reproduce *in vivo* redox profile. J Cell Biochem 118(10):3111–3118. https://doi.org/10.1002/jcb.26028
Sundar-Boyalla S, Barbara Victor M, Roemgens A, Beyer C, Arnold S (2011) Sex- and brain region-specific role of cytochrome c oxidase in 1-methyl-4-phenylpyridinium-mediated astrocyte vulnerability. J Neurosci Res 89(12):2068–2082. https://doi.org/10.1002/jnr.22669
Valentino RJ, Bangasser DA (2016) Sex-biased cellular signaling: molecular basis for sex differences in neuropsychiatric diseases. Dialog Clin Neurosci 18(4):385–393
Villa A, Gelosa P, Castiglioni L, Cimino M, Rizzi N, Pepe G, Lolli F, Marcello E, Sironi L, Vegeto E, Maggi A (2018) Sex-specific features of microglia from adult mice. Cell Rep 23(12):3501–3511. https://doi.org/10.1016/j.celrep.2018.05.048
Zhang D, Hu X, Qian L, O'Callaghan JP, Hong JS (2010) Astrogliosis in CNS is there a role for microglia? Mol Neurobiol 41:232–241