

## RESEARCH

# Chronic estrogen affects TIDA neurons through IL-1 $\beta$ and NO: effects of aging

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## Abstract

Women are chronically exposed to estrogens through oral contraceptives, hormone replacement therapy or environmental estrogens. We hypothesized that chronic exposure to low levels of estradiol-17 $\beta$  (E2) can induce inflammatory and degenerative changes in the tuberoinfundibular dopaminergic (TIDA) system leading to reduced dopamine synthesis and hyperprolactinemia. Young (Y; 3–4 months) and middle-aged (MA; 10–12 months) Sprague–Dawley rats that were intact or ovariectomized (OVX) were either sham-implanted or implanted with a slow-release E2 pellet (20 ng E2/day for 90 days). To get mechanistic insight, adult 3- to 4-month-old WT, inducible nitric oxide synthase (iNOS) and IL-1 receptor (IL-1R) knockout (KO) mice were subjected to a similar treatment. Hypothalamic areas corresponding to the TIDA system were analyzed. E2 treatment increased IL-1 $\beta$  protein and nitrate levels in the arcuate nucleus of intact animals (Y and MA). Nitration of tyrosine hydroxylase in the median eminence increased with E2 treatment in both intact and OVX animals. There was no additional effect of age. This was accompanied by a reduction in dopamine levels and an increase in prolactin in intact animals. E2 treatment increased nitrate and reduced dopamine levels in the hypothalamus and increased serum prolactin in WT mice. In contrast, the effect of E2 on nitrate levels was blocked in IL-1R-KO mice and the effect on dopamine and prolactin were blocked in iNOS KO animals. Taken together, these results show that chronic exposure to low levels of E2 decreases TIDA activity through a cytokine-nitric oxide-mediated pathway leading to hyperprolactinemia and that aging could promote these degenerative changes.

## Key Words

- ▶ estradiol
- ▶ dopamine
- ▶ tyrosine hydroxylase
- ▶ TIDA neurons
- ▶ nitration
- ▶ iNOS
- ▶ IL-1 $\beta$

*Journal of Endocrinology*  
(2019) **240**, 157–167

## Background

Chronic exposure to low levels of estrogen is known to accelerate reproductive aging in female rats (Kasturi *et al.* 2009). In fact, during the normal aging process, female rats go through a period of constant estrous when they reach middle age. During this time, serum estradiol levels are moderately elevated for a period of 6 months.

This results in a gradual decline in hypothalamic dopamine levels with a concurrent increase in serum prolactin levels. This chronic hyperprolactinemia predisposes older rats to mammary and pituitary tumors (Meites 1991). Although the exact mechanisms by which hypothalamic dopamine levels decrease is not clear, it is reasonable to assume that

it is associated with a reduction in tuberoinfundibular dopaminergic (TIDA) neuronal function since dopamine produced by these neurons is the primary inhibitor of prolactin secretion in rats (Meites 1991).

TIDA neurons have estrogen receptors and are susceptible to the influences of estrogens (Mitchell *et al.* 2003, Ribeiro *et al.* 2015). Previous studies have demonstrated that a single dose of estradiol valerate can induce an inflammatory reaction in the arcuate nucleus that contains TIDA neuronal cell bodies (Brawer *et al.* 1978). A similar reaction can be induced after chronic exposures to low doses of estradiol-17 $\beta$  (E2) as well. This effect is evident when slow-release E2 pellets are implanted in young animals to mimic the estradiol levels seen in aging rats. We have shown that such an E2 exposure can increase interleukin-1 $\beta$  (IL-1 $\beta$ ) and nitric oxide-related free radicals in the arcuate nucleus, causing a decrease in TIDA function. This results in reduced dopamine release from TIDA terminals that is followed by an increase in serum prolactin levels (Gonzalez *et al.* 2004). Although these animals were only 6 months old at the end of E2 exposure, their TIDA neurons were functionally similar to 18-month-old rats. This poses several lines of enquiry. Does chronic low-dose E2 exposure produce an even more conspicuous effect on TIDA neurons in aging animals? What is the exact cascade of events that lead to TIDA neuronal dysfunction?

There is some evidence to suggest that aging can independently contribute to increased IL-1 $\beta$  (Tha *et al.* 2000) and free-radical generation in the brain (de Oliveira *et al.* 2012). Elevations in nitric-oxide-related free radicals can cause nitration of certain proteins that are rich in tyrosine moieties resulting in altered function. Tyrosine hydroxylase that is crucial for the biosynthesis of dopamine is one such enzyme that is prone to nitration (Ara *et al.* 1998). Nitration of this enzyme results in a loss of enzyme function leading to reduced dopamine synthesis. However, it is not clear where IL-1 $\beta$  and nitric-oxide-related free radicals fit in the cascade. Therefore, it is important to use mechanistic approaches to study the exact sequence of events leading to hyperprolactinemia. It is also important to understand if aging per se could contribute to this phenomenon.

In the present study, we hypothesized that aging would exacerbate the effects of chronic E2 exposure on TIDA neurons and endogenous estradiol contributes to the degenerative changes. In order to test this, we used both young and aging rats and both intact and ovariectomized (OVX) animals in both the age groups. In addition, we used specific knockout (KO) animal models

to understand the inflammatory cascade sequence in greater detail. Inflammatory changes in the TIDA neurons were compared to another dopaminergic system, viz the nigrostriatal dopaminergic system that does not influence prolactin secretion.

## Methods

### Ethical treatment

Animals were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

### Animals

Young (3–4 months) and middle-aged (10–12 months) female Sprague–Dawley rats were obtained from Harlan Inc. (Indianapolis, IN). Breeding pairs of young (6–8 weeks old) female and male WT (C57BL/10J; Stock No. 000664) and interleukin-1 receptor KO (IL-1R $^{-/-}$ ; B6.129S7-Il1r1<sup>tm1mx</sup>/J; Stock No. 003245) and inducible nitric oxide synthase (iNOS) KO (iNOS $^{-/-}$ ; B6.129P2-Nos2<sup>tm1Lau</sup>/J; Stock No. 002609) mice were purchased from Jackson Laboratories. Housing was light-controlled (lights on from 07:00 to 19:00h) and air-conditioned (23 $\pm$ 2°C) and the animals were fed chow and water *ad libitum*. Mice were bred and female offspring were genotyped and used in the study when they were 3–4 months old.

### Treatment

A large cohort of young and middle-aged animals ( $n=250$ ) was used and animals were each randomly assigned to one of four treatment groups: young and middle-aged, intact, sham-implanted (YIS and MIS respectively) and young and middle-aged, intact, implanted with a slow-release E2 pellet (YIE and MIE respectively). The E2 pellet was capable of releasing 20ng of E2/day (Innovative Research America, Sarasota, FL, USA) for 90 days. Similarly, there were four groups of OVX animals: young and middle-aged OVX sham (YOS and MOS respectively) or OVX animals implanted with an E2 pellet (YOE and MOE—young and middle-aged animals respectively). The E2 pellets produced serum estradiol levels of ~45pg/mL at 30 days, ~70pg/mL at 60 days and ~100pg/mL at 90 days of exposure (Kasturi *et al.* 2009). All E2-treated intact animals were in estrus at the end of treatment and were killed by

rapid decapitation at noon. Control animals were killed when they were in estrus and OVX animals were also killed at noon. Brains and serum were collected and stored at  $-80^{\circ}\text{C}$  until further analysis. Sets of animals ( $n=6-8$ ) were used for different measures. For example, serum hormones, dopamine in the median eminence and caudate putamen and gene expression studies in the arcuate nucleus and substantia nigra were done in one set of animals. Protein expression was measured in a separate set of animals. WT, IL-1R-KO and iNOS-KO animals were raised in our colony. When they were 3 months old, they were either sham-implanted or implanted with slow-release E2 pellets ( $2\mu\text{g}/\text{day}$ ; Innovative Research America) for 90 days. All animals were killed by rapid decapitation at the end of treatment on the day of estrus at noon. Brains and serum were collected and stored at  $-80^{\circ}\text{C}$  until further analysis.

### Estradiol and prolactin analysis

Serum samples were analyzed for E2 and prolactin levels by double-antibody radioimmunoassay. The analysis was performed using reference preparations and primary antibody for prolactin developed at the NHPP, NIDDK. For prolactin, the reference preparation was rPrl-RP3 and the primary antibody was rabbit anti-rat Prl S9. A commercial coat-a-count RIA kit (Siemens Medical Solutions Diagnostics) was used to measure serum estradiol levels. Intra-assay variability was 4.2% for prolactin and 3.5% for estradiol.

### Brain microdissection

Brains were sectioned at  $300\mu\text{m}$  intervals in a cryostat and mounted on glass slides. The slides were transferred to a cold stage maintained at  $-10^{\circ}\text{C}$  and the substantia nigra, and caudate putamen were dissected with a  $500\mu\text{m}$  micropunch, using Palkovits' microdissection technique (Paxinos & Watson 1986) with the stereotaxic atlas as a reference (Paxinos & Watson 1998). Care was taken to include all subdivisions of these nuclei. The median eminence was dissected out by placing the punch partially on the ventral aspect of the hypothalamus, on sections obtained from 2.12 to 3.3 mm posterior to the bregma. After removing the median eminence, the punch was placed on the mid-ventral line to dissect the arcuate nucleus, 2.12–3.6 mm posterior to the bregma.

### IL-1 $\beta$ and iNOS RT and qRT-PCR

mRNA was isolated from the arcuate nucleus and substantia nigra samples using the MELT Total Nucleic Acid Isolation

System (Ambion) according to the manufacturer's protocol. The concentration of the isolated mRNA ( $\text{ng}/\mu\text{L}$ ) was determined using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). The RT<sup>2</sup> First Strand Kit (SABiosciences, Frederick, MD, USA) was used to generate cDNA from 400 ng of mRNA, which was then used to perform quantitative real-time PCR (qRT-PCR) as described before (Sirivelu *et al.* 2012). RT<sup>2</sup> Real-Time PCR SYBR Green/ROX Master Mix and custom primer pairs for rat IL-1 $\beta$ , rat iNOS and rat  $\beta$ -actin were obtained from SABiosciences. PCR master mix, 12.5  $\mu\text{L}$  was mixed with 1  $\mu\text{L}$  of the primer pair, 1  $\mu\text{L}$  of cDNA and 10.5  $\mu\text{L}$  of RNase free water, for a total volume of 25  $\mu\text{L}$  per well. PCR quantification was performed with the 7500 Real-Time PCR machine (Applied Biosystems). The following cycling program was used: 1 cycle at  $95^{\circ}\text{C}$  for 10 min and 40 cycles at  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 1 min. The mRNA for IL-1 $\beta$  and iNOS were normalized to  $\beta$ -actin, the housekeeping gene. The values in the intact and OVX groups were expressed relative to the YIS and YOS groups respectively.

### IL-1 $\beta$ and nitrate analysis

Arcuate nucleus and substantia nigra samples were homogenized in ice-cold PBS (4.73 g monobasic sodium phosphate; 9.74 g dibasic sodium phosphate; 9 g sodium chloride; autoclaved nanopure water to 1000 mL), pH 7.5. An aliquot of the homogenate was saved for protein analysis. The remaining homogenate was used in duplicate to measure IL-1 $\beta$  and nitrate levels using a rat IL-1 $\beta$  enzyme immunometric assay kit (Assay Designs, Ann Arbor, MI, USA) and a total nitric oxide kit (Assay Designs) respectively. The sensitivity of the nitric oxide assay was  $3\mu\text{M}$  and the intra-assay variability was 3.27%. The sensitivity of the IL-1 $\beta$  assay was 25 pg and the intra-assay variability was 15.24%.

### Dopamine HPLC-EC analysis

Median eminence and caudate putamen samples were homogenized in 0.5 M  $\text{HClO}_4$ . After saving an aliquot for protein assay, the remaining homogenate was centrifuged at 14,500 g for 5 min at  $4^{\circ}\text{C}$ . From each sample, 60  $\mu\text{L}$  of supernatant along with the internal standard (0.05 M dihydroxybenzylamine) was injected into a high performance liquid chromatography electrochemical detection (HPLC-EC; Shimadzu, Columbia, MD, USA) system as described before (MohanKumar *et al.* 2011). The sensitivity of the system is  $<1\text{ pg}$ .

## Immunoprecipitation of tyrosine hydroxylase

Tyrosine hydroxylase (TH) in the ME samples was immunoprecipitated as described before (Kasturi *et al.* 2009, MohanKumar *et al.* 2011). Briefly, median eminence and caudate putamen homogenates containing 50 µg of protein were used for immunoprecipitation with protein A agarose (KPL, Gaithersburg, MD, USA) and anti-TH antibody (EMD Millipore). After overnight incubation in binding buffer, TH was eluted using 40 µL of Elution buffer (KPL). The supernatant contained the immunoprecipitated TH and was divided into two equal fractions for Western blot analysis.

## Tyrosine hydroxylase and nitrotyrosine Western blot analysis

TH that was immunoprecipitated from median eminence and caudate putamen samples was subjected to Western blotting according to standard protocols that have been described earlier (Kasturi *et al.* 2009, MohanKumar *et al.* 2011). The proteins were transferred to nitrocellulose membranes using semi-dry blotting. The membranes were probed with either TH or nitrated tyrosine (1:1000 and 1:500 respectively; Abcam) primary antibody in TBST. The membranes were incubated with fluorescent secondary antibody (anti-rabbit IgG IRDye800 conjugated fluorescent secondary antibody; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) at a dilution of 1:10,000. The membranes were scanned with an Odyssey Infrared imaging system (Li-COR Biosciences, Lincoln, NE, USA). The blot image pixels were quantified using the ImageJ software version 1.38 (NIH).

## Protein analysis

Protein concentrations in the microdissected tissue homogenates were determined using a micro bicinchoninic acid assay (Pierce). Concentrations of dopamine and other factors were expressed as per µg protein.

## Statistical analysis

Differences in various parameters between the treatment groups were analyzed using two-way ANOVA with treatment and age as factors followed by Tukey's multiple comparison *post hoc* test. The effects of treatment, age and their interaction are presented.  $P < 0.05$  was considered significant.

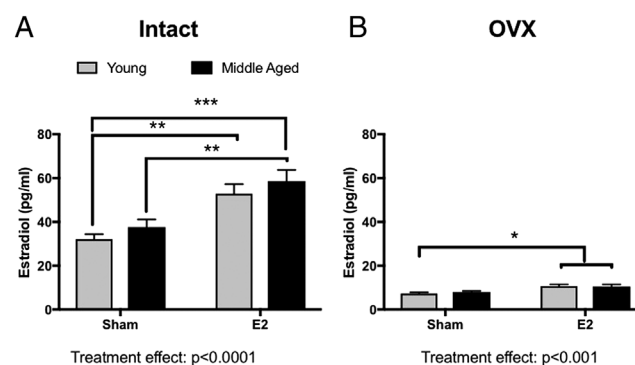
## Results

### Serum estradiol

E2 treatment increased serum E2 levels (pg/mL; mean  $\pm$  s.e.) both in the young ( $52.8 \pm 4.4$ ) and middle-aged animals ( $58.6 \pm 5.1$ ) when compared to the corresponding sham-implanted animals ( $32.13 \pm 2.2$  and  $37.6 \pm 3.4$  in YIS and MIS groups respectively;  $P < 0.01$ ; Fig. 1A). ANOVA revealed a significant treatment effect ( $P < 0.0001$ ). In OVX animals (Fig. 1B), E2 treatment produced a very modest increase in serum E2 levels in both YOE ( $10.7 \pm 0.8$ ) and MOE ( $10.4 \pm 1$ ) groups compared to sham-implanted rats ( $7.3 \pm 0.5$  and  $8 \pm 0.5$  in YOS and MOS groups respectively;  $P < 0.05$ ). ANOVA revealed a significant treatment effect in OVX animals as well ( $P < 0.001$ ).

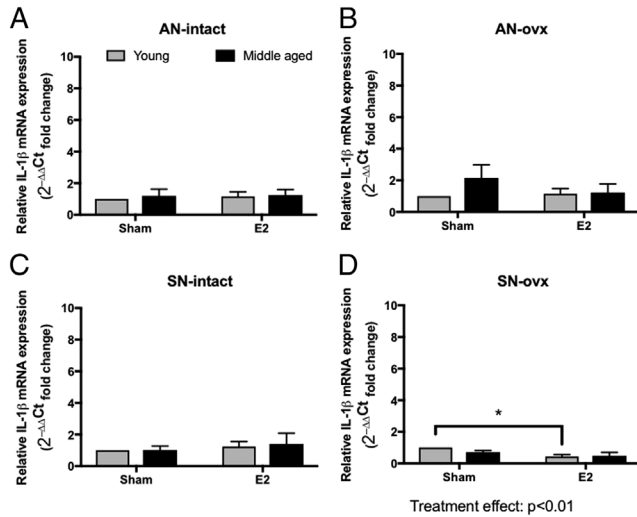
### IL-1 $\beta$ mRNA and protein expression in the arcuate nucleus and substantia nigra

IL-1 $\beta$  gene expression was unchanged in the arcuate nucleus in both intact and OVX animals (Fig. 2A and B). However, in the substantia nigra, E2 treatment produced a significant decrease in IL-1 $\beta$  gene expression in OVX rats ( $P < 0.05$ ; Fig. 2D). E2 treatment increased IL-1 $\beta$  protein levels (mean  $\pm$  s.e.; pg/µg protein) in the arcuate nucleus of middle-aged rats ( $47 \pm 10.8$ ) when compared to sham-implanted rats ( $9.8 \pm 2.6$  and  $14.6 \pm 4.4$  in YIS and MIS rats respectively;  $P < 0.01$ ) (Fig. 3A). It also increased IL-1 $\beta$  levels in young animals ( $33.9 \pm 4.7$ ) compared to their



**Figure 1**

(A) Serum E2 levels (mean  $\pm$  s.e.; pg/mL) in reproductively intact young (3–4 months) and middle-aged (10–12 months) sham (YIS and MIS, respectively;  $n = 9$  and  $8$  respectively) rats, rats treated with slow-release E2 pellets for 90 days (YIE and MIE;  $n = 9$  per group). \*\* indicates significant difference ( $P < 0.01$ ) and \*\*\* indicates  $P < 0.001$ . (B) Serum E2 levels in ovariectomized young (3–4 months) and middle-aged (10–12 months) sham (YOS and MOS, respectively;  $n = 9$  per group) rats and rats implanted with slow-release E2 pellets for 90 days (YOE and MOE;  $n = 9$  per group). \* indicates significant difference from YOS and MOS ( $P < 0.05$ ).



**Figure 2**

(A) IL-1 $\beta$  mRNA gene expression (fold change) in the arcuate nucleus (AN) of MIS, YIE and MIE rats were normalized to gene expression in YIS rats with a  $\Delta\Delta$ Ct value of 1.0. (B) IL-1 $\beta$  mRNA gene expression (fold change) in the arcuate nucleus of MOS, YOE and MOE rats normalized to YOS rats with a  $\Delta\Delta$ Ct value of 1.0. (C) IL-1 $\beta$  mRNA gene expression (fold change) in the substantia nigra (SN) of intact young and middle-aged rats (sham and E2 implanted) normalized to young intact sham (YIS) rats with a  $\Delta\Delta$ Ct value of 1.0. (D) IL-1 $\beta$  mRNA gene expression fold change in the substantia nigra (SN) of ovariectomized young and middle-aged rats (sham and E2 implanted) normalized to young ovariectomized sham (YOS) rats with a  $\Delta\Delta$ Ct value of 1.0. \* indicates significant difference from YOS ( $P < 0.05$ ),  $n = 6$  per group.

sham-implanted counterparts ( $P < 0.05$ ). ANOVA revealed a significant treatment effect ( $P < 0.001$ ). No changes in IL-1 $\beta$  protein levels were observed in the substantia nigra (Fig. 3C and D).

### iNOS gene expression and nitrate levels in the arcuate nucleus and substantia nigra

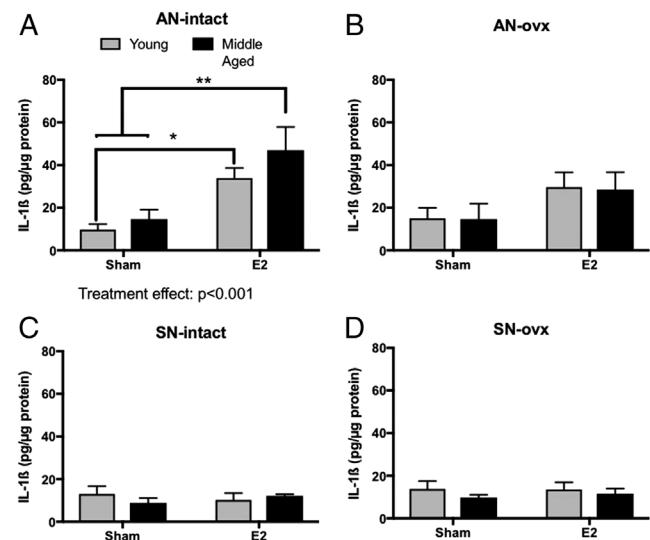
E2 treatment produced a significant upregulation in iNOS mRNA expression in the arcuate nucleus of MIE animals compared to young rats (both sham and E2 treated) (Fig. 4A,  $P < 0.01$ ). iNOS gene expression was also elevated in MIS rats compared to YIS rats; however, no change was seen in the OVX groups (Fig. 4B). There was a significant effect of treatment ( $P < 0.05$ ) and age ( $P < 0.01$ ). In contrast, iNOS gene expression was increased in the substantia nigra of YOE animals compared to the MOS group ( $P < 0.05$ ; Fig. 4D). There was a significant age effect ( $P < 0.01$ ).

E2 treatment increased nitrate levels (mean  $\pm$  s.e.;  $\mu$ M/ $\mu$ g protein) in the arcuate nucleus of both young ( $4.7 \pm 0.7$ ) middle-aged intact animals ( $4.9 \pm 1.2$ ) compared to their sham-implanted counterparts ( $1.5 \pm 0.4$  and

$1.58 \pm 0.2$  respectively) ( $P < 0.05$ ; Fig. 5A). ANOVA revealed a significant effect of treatment ( $P < 0.01$ ). However, there were no differences in the levels of nitrate in the arcuate nucleus of OVX animals or in the substantia nigra.

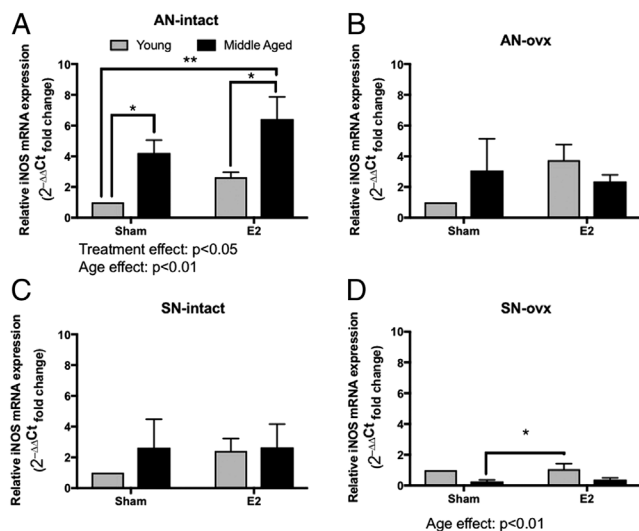
### Ratio of nitrated tyrosine hydroxylase: tyrosine hydroxylase in the median eminence and caudate putamen

The ratio of nitrated tyrosine hydroxylase to total tyrosine hydroxylase in the median eminence increased in an E2 exposure-dependent manner in young, intact animals (Fig. 6A;  $P < 0.05$ ). Although the levels of nitrated TH appeared to be higher in MIS rats, they were not different from YIS rats. In OVX animals, nitrated TH increased significantly in both young and aging animals with E2 treatment (Fig. 6B). There was a significant treatment ( $P < 0.05$ ) and age effect ( $P < 0.05$ ) in intact rats and a significant treatment effect ( $P < 0.01$ ) in OVX rats. A sample Western blot from the arcuate nucleus is shown in Fig. 6C. There was no appreciable change in nitrotyrosine visualized on the immunoblots of samples from the caudate putamen in any of the treatment groups (data not shown).



**Figure 3**

(A) Interleukin 1-beta (IL-1 $\beta$ ) concentrations (pg/ $\mu$ g protein; mean  $\pm$  s.e.) in the arcuate nucleus of sham-implanted YIS and MIS rats, and rats treated implanted with E2 pellets (YIE and MIE). \* indicates significant difference from YIS ( $P < 0.05$ ); \*\* indicates significant difference from both YIS and MIS ( $P < 0.01$ ).  $n = 6$  per group for YIS, MIS and MIE. YIE had  $n = 8$ . (B) IL-1 $\beta$  concentrations in the arcuate nucleus of ovariectomized young and middle-aged sham (YOS and MOS, respectively) rats, and rats implanted with E2 pellets (YOE and MOE).  $n = 6$  per group. (C) IL-1 $\beta$  concentrations in the substantia nigra of intact sham (YIS and MIS) rats, and young and aging rats implanted with E2 pellets (YIE and MIE). (D) IL-1 $\beta$  concentrations in the substantia nigra of ovariectomized sham (YOS and MOS) and E2 implanted animals (YOE and MOE).  $n = 6$  per group.

**Figure 4**

(A) iNOS mRNA gene expression (fold change) in the arcuate nucleus (AN) of MIS, YIE and MIE rats were normalized to YIS rats with a  $\Delta\Delta Ct$  value of 1.0. \* indicates significant difference between MIS and YIS and between MIE and YIE ( $P < 0.05$ ). \*\* indicates significant difference between MIE and YIS ( $P < 0.01$ ).  $n = 6$  for YIS and MIE;  $n = 8$  for MIS and  $n = 7$  for YIE. (B) iNOS mRNA gene expression (fold change) in the arcuate nucleus of MOS, YOE and MOE rats normalized to YOS rats with a  $\Delta\Delta Ct$  value of 1.0. (C) iNOS mRNA gene expression (fold change) in the substantia nigra (SN) of intact young and middle-aged rats (sham and E2 implanted) normalized to young intact sham (YIS) rats with a  $\Delta\Delta Ct$  value of 1.0. (D) iNOS mRNA gene expression fold change in the substantia nigra (SN) of ovariectomized young and middle-aged rats (sham and E2 implanted) normalized to young ovariectomized sham (YOS) rats with a  $\Delta\Delta Ct$  value of 1.0. \* indicates significant difference from YOS ( $P < 0.05$ ).  $n = 6$  per group for graphs B–D.

#### DA concentrations in the median eminence and caudate putamen of young and middle-aged animals

DA in the median eminence (mean  $\pm$  s.e.; pg/ $\mu$ g protein) of YIE ( $26.34 \pm 9.14$ ) and MIE rats ( $75.3 \pm 14.8$ ) was significantly lower compared to MIS animals ( $222.56 \pm 70.7$ ;  $P < 0.05$ ; Fig. 7). DA levels in the YOE group was unaffected compared to YOS rats, but it was markedly reduced in MOE rats ( $22.5 \pm 2.1$ ) compared to the YOE group ( $201.3 \pm 44.2$ ;  $P < 0.05$ ; Fig. 7B). While there was a significant treatment effect ( $P < 0.001$ ) and interaction ( $P < 0.05$ ) in intact animals, there was a significant age effect ( $P < 0.05$ ) in OVX animals. In the caudate putamen, there was no significant difference in DA levels between the treatment groups (Fig. 7C and D).

#### Serum prolactin

Serum prolactin levels (mean  $\pm$  s.e.; ng/mL; Fig. 8A and B) increased in MIE ( $33.7 \pm 3.3$ ) animals compared to sham-implanted rats ( $9.8 \pm 2.6$  (YIS) and  $12 \pm 3.9$  (MIS)) ( $P < 0.001$ ).

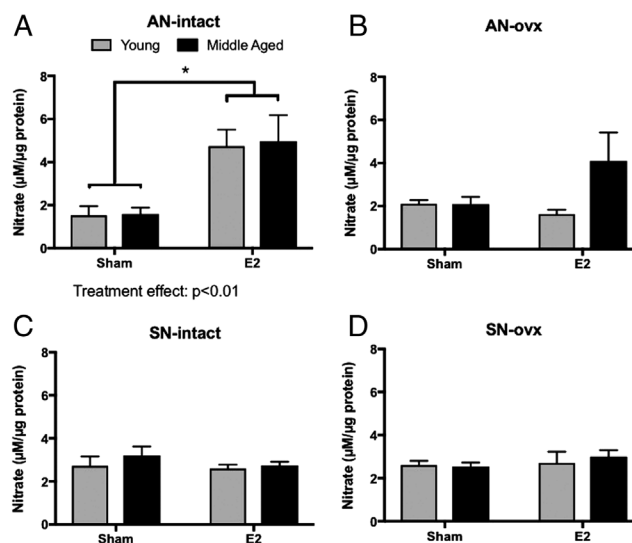
There was a significant effect of E2 treatment ( $P < 0.0001$ ). There were modest increases in prolactin levels in older OVX animals that was not significant; however, ANOVA revealed a significant effect of age ( $P < 0.05$ ).

#### Nitrate in the hypothalamus of WT and IL-1R (–/–) mice

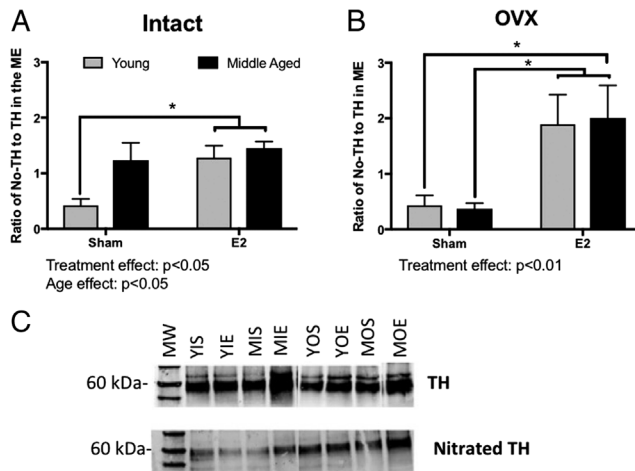
E2 exposure significantly increased nitrate levels ( $\mu$ M/ $\mu$ g protein) in WT animals ( $4.8 \pm 0.6$ ) compared to sham-implanted animals ( $2.4 \pm 0.1$ ;  $P < 0.05$ ). In contrast, E2 treatment did not affect nitrate levels in IL-1R $^{-/-}$  mice indicating that E2-induced increase in nitrate is probably mediated through IL-1 (Fig. 9). There was a significant effect of genotype ( $P < 0.01$ ), treatment ( $P < 0.05$ ) and interaction ( $P < 0.05$ ).

#### Effects of E2 on DA and prolactin in iNOS (–/–) mice

E2 exposure significantly decreased DA in the median eminence (mean  $\pm$  s.e.; pg/ $\mu$ g protein) in WT mice ( $5.5 \pm 1.2$ )

**Figure 5**

(A) Nitrate concentrations ( $\mu$ M/ $\mu$ g protein; mean  $\pm$  s.e.) in the arcuate nucleus (AN) of intact young (3–4 months) and middle-aged (10–12 months) sham (YIS and MIS, respectively) rats, and rats treated with slow-release E2 pellets for 90 days (YIE and MIE). \* indicates significant difference between E2 treated and sham-implanted rats ( $P < 0.05$ ).  $n = 7$  for YIS;  $n = 8$  for YIE;  $n = 6$  for MIS and MIE groups. (B) Nitrate concentrations in the arcuate nucleus of ovariectomized young and aging animals that were sham-implanted or implanted with E2 pellets.  $n = 6$  per group. (C) Nitrate concentrations in the substantia nigra (SN) of intact young and middle-aged sham (YIS and MIS, respectively) rats, and rats treated with slow-release E2 pellets (YIE and MIE).  $n = 6$  per group. (D) Nitrate concentrations in the substantia nigra of ovariectomized young and aging animals that were sham-implanted or implanted with E2 pellets.  $n = 6$  per group.

**Figure 6**

(A) The ratio of nitrotyrosine (NO-TH) to tyrosine hydroxylase (TH) in the median eminence (ME) of intact young (3–4 months) and middle-aged (10–12 months) sham (YIS and MIS, respectively) rats, rats treated with slow-release E2 pellets (YIE and MIE). \* indicates significant difference from YIS ( $P < 0.05$ ).  $n = 6$  per group. (B) The ratio of nitrotyrosine to tyrosine hydroxylase in the median eminence of ovariectomized young and middle-aged sham (YOS and MOS, respectively) rats, and rats treated with slow-release E2 pellets (YOE and MOE). \* indicates significant difference from YOS and MOS groups ( $P < 0.05$ ).  $n = 6$  per group. (C) Representative Western blot depicting differences in the protein expression of tyrosine hydroxylase and nitrated tyrosine hydroxylase in the median eminence from different treatment groups.

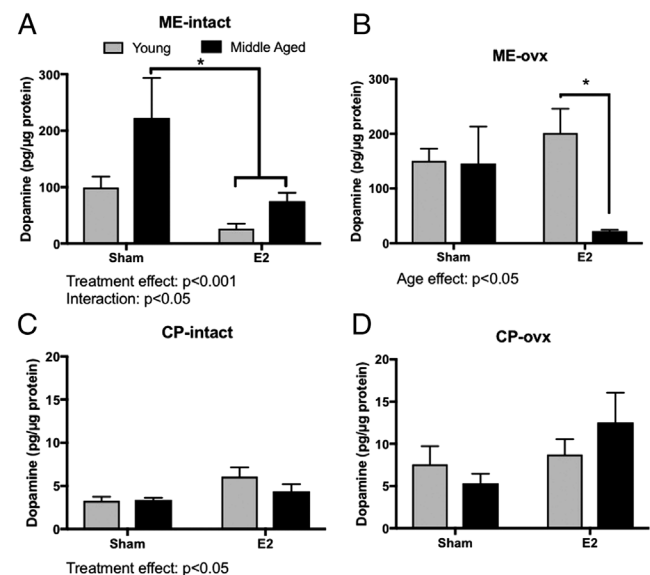
compared to sham-treated animals ( $28.8 \pm 2$ ;  $P < 0.05$ ). In contrast, E2 treatment did not alter DA levels in iNOS $^{-/-}$  animals (Fig. 10A). There was a significant treatment effect ( $P < 0.05$ ). E2 treatment significantly increased serum prolactin (mean  $\pm$  s.e., ng/mL) in WT mice ( $39.8 \pm 1$ ) compared to sham-implanted animals ( $21.8 \pm 2.2$ ;  $P < 0.05$ ). In contrast, E2 exposure did not affect serum prolactin in iNOS $^{-/-}$  animals (Fig. 10B). There was a significant treatment effect ( $P < 0.0001$ ) and a significant genotype effect ( $P < 0.01$ ).

## Discussion

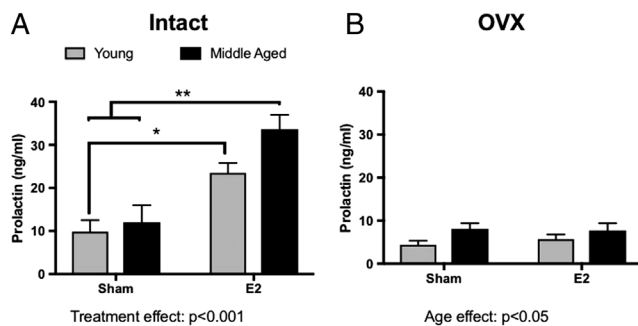
Estrogens are considered to be neuroprotective, beneficial for learning, memory and vital for neuroregeneration (Brann & Mahesh 2014). However, it is important to consider the type of estrogen, dose and the duration of exposure because all these play a crucial role in the outcome. The present study demonstrates how chronic exposures to low levels of E2 can affect a specific dopaminergic system in the brain to cause hyperprolactinemia.

We have previously demonstrated that chronic low-dose exposure to E2 can cause cessation of estrous cycles and induce reproductive senescence (Kasturi *et al.* 2009).

This effect was mediated through a cytokine-nitric oxide-mediated neurodegenerative mechanism in the brainstem that reduced the synthesis of norepinephrine leading to cessation of reproductive cycles (Kasturi *et al.* 2013). Since norepinephrine and dopamine have the same biosynthetic pathway, we predicted that a similar cascade would be in operation in another aging phenomenon viz hyperprolactinemia that is induced during the constant estrous phase of aging in rats. Prolactin secretion is under the inhibitory control of dopamine that is produced by TIDA neurons in the hypothalamus (Meites 1991). Other studies have shown that chronic exposure to E2 does indeed affect these neurons (Demarest *et al.* 1984, Morel *et al.* 2009). We have observed this phenomenon in young, intact animals where low-dose E2 exposure produced glial activation – characterized by an increase in the astrocyte-specific marker glial acidic fibrillary protein (GFAP), increased production of the proinflammatory cytokine IL-1 $\beta$  and increased production of nitric oxide in the arcuate nucleus (MohanKumar *et al.* 2011).

**Figure 7**

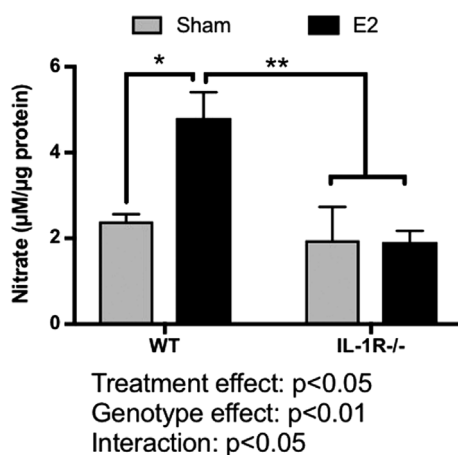
(A) Dopamine levels (mean  $\pm$  s.e.; pg/ $\mu$ g protein) in the median eminence (ME) of reproductively intact young (3–4 months) and middle-aged (10–12 months) sham (YIS and MIS, respectively) rats and rats treated with slow-release E2 pellets (YIE and MIE). \* indicates significant difference between MIE and YIE groups ( $P < 0.05$ ); \*\* indicates  $P < 0.01$  between MIS and YIE and \*\*\* indicates  $P < 0.001$  between YIS and YIE. YIS:  $n = 6$ ; YIE and MIE:  $n = 7$  per group and MIS:  $n = 6$ . (B) Dopamine levels in the median eminence of ovariectomized rats. \* indicates significant difference from YOE ( $P < 0.05$ ). (C) Dopamine levels in the caudate putamen (CP) of intact young and middle-aged sham (YIS and MIS, respectively) rats and rats treated with slow-release E2 pellets for 90 days (YIE and MIE). (D) Dopamine levels in the caudate putamen (CP) of ovariectomized rats that were sham-implanted or treated with slow-release E2 pellets for 90 days (YOE and MOE).  $n = 6$  per group for graphs B–D.

**Figure 8**

(A) Serum prolactin levels (mean  $\pm$  s.e.; ng/mL) in young intact (YIS:  $n = 9$ ) and middle-aged (MIS:  $n = 8$ ) sham rats and rats treated with slow-release E2 pellets for 90 days (YIE and MIE:  $n = 10$  and  $9$  respectively). \* indicates significant difference from YIS ( $P < 0.05$ ) and \*\* indicates  $P < 0.01$  from sham-implanted rats. (B) Serum prolactin levels in OVX young (YOS) and middle-aged (MOS) sham rats and rats implanted with slow-release E2 pellets for 90 days (YOE and MOE).  $n = 9$  per group.

In the present study, we wanted to explore if endogenous estrogens and aging contribute to the neurodegenerative changes observed in TIDA neurons after chronic low-dose E2 exposure.

Estrogen is known to inhibit TIDA neuronal function (Simpkins *et al.* 1982, Demarest *et al.* 1984, Sarkar *et al.* 1984, Shaw-Bruha *et al.* 1996, DeMaria *et al.* 2000, Ribeiro *et al.* 2015) and induce hyperprolactinemia and pituitary prolactinomas in rodents (Koch *et al.* 1980, Smythe & Brandstater 1980, Sarkar *et al.* 1984). There is also an association between hyperprolactinemia in women and the use of estrogenic oral contraceptives

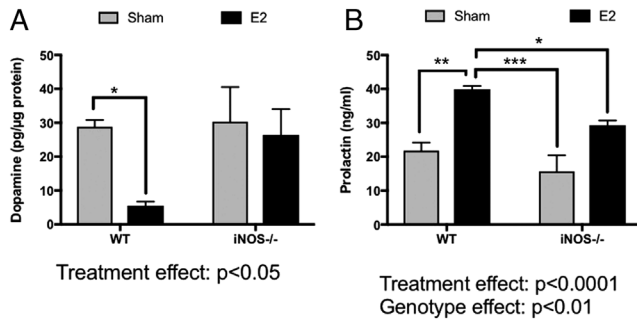
**Figure 9**

Nitrate concentrations ( $\mu\text{M}/\mu\text{g}$  protein, mean  $\pm$  s.e.) in the hypothalamic arcuate nucleus of reproductively intact young (3–4 months) wild type and IL-1R<sup>-/-</sup> KO mice that were sham-implanted or implanted with slow-release E2 pellets for 90 days. \* indicates significant difference from WT sham group ( $P < 0.05$ ) and \*\* indicates  $P < 0.01$  from IL-1R<sup>-/-</sup> groups. WT sham:  $n = 7$ ; WT-E2:  $n = 8$ ; IL-1R<sup>-/-</sup>  $n = 6$  per group.

(Alvarez-Tutor *et al.* 1999, Krysiak *et al.* 2015). In fact, acute treatment with E2 failed to stimulate prolactin secretion in animals subjected to pituitary stalk sectioning (Murai & Ben-Jonathan 1990). In the same study, a tyrosine hydroxylase inhibitor was able to stimulate prolactin secretion robustly from anterior pituitary lactotrophs (Murai & Ben-Jonathan 1990) suggesting that disruption of TIDA activity is a prime regulator of prolactin secretion. However, the underlying mechanisms are not clear. In young, intact, Sprague–Dawley rats, we have previously demonstrated that a low-dose chronic E2 exposure decreased dopamine content in the median eminence and induced hyperprolactinemia. In the present study, we proposed that there would be an age-associated decline in the function of TIDA neurons and that this age-associated neurodegenerative effect would be exacerbated with E2 exposure. We found that an aging effect was apparent in iNOS mRNA levels in the arcuate nucleus and nitration of tyrosine hydroxylase in the median eminence where these parameters were increased in MIS animals and not in YIS animals. Although IL-1 $\beta$  protein, nitrate and nitrated tyrosine hydroxylase were modestly increased in YIE animals, it was overshadowed by the increases observed in MIE animals which were significantly different compared to younger rats. However, dopamine levels were markedly lower in younger animals compared to older rats. These results indicate that the effects of E2 exposure are masked by aging. It is likely that older animals have probably been exposed to more number of endogenous E2 surges than younger rats and have lower TIDA function as a result (Sanchez *et al.* 2003). Moreover, aging is known to affect estrogen receptor- $\alpha$  expression in the arcuate nucleus, but the findings are not consistent (Funabashi *et al.* 2000, Wilson *et al.* 2002). Reduced expression of these receptors in the arcuate nucleus could affect how the TIDA neurons of older rats respond to exogenous E2 administration.

In order to determine if endogenous E2 contributed to this phenomenon, we used OVX rats in the experiment. In these animals, E2 pellet implantation produced a modest but significant increase in E2 levels compared to sham-implanted rats with no change in serum prolactin levels, IL-1 $\beta$  mRNA, iNOS mRNA and nitrate levels in the arcuate nucleus. IL-1 $\beta$  protein levels were elevated in the arcuate nucleus of YOE animals only, while nitrated tyrosine hydroxylase levels were increased in both YOE and MOE. The MOE group had higher levels of IL-1 $\beta$ , and nitrate levels in the arcuate nucleus, which led to the increase in nitrated tyrosine hydroxylase contributing to the reduced dopamine in this group. However, there was no corresponding increase in prolactin in this group.



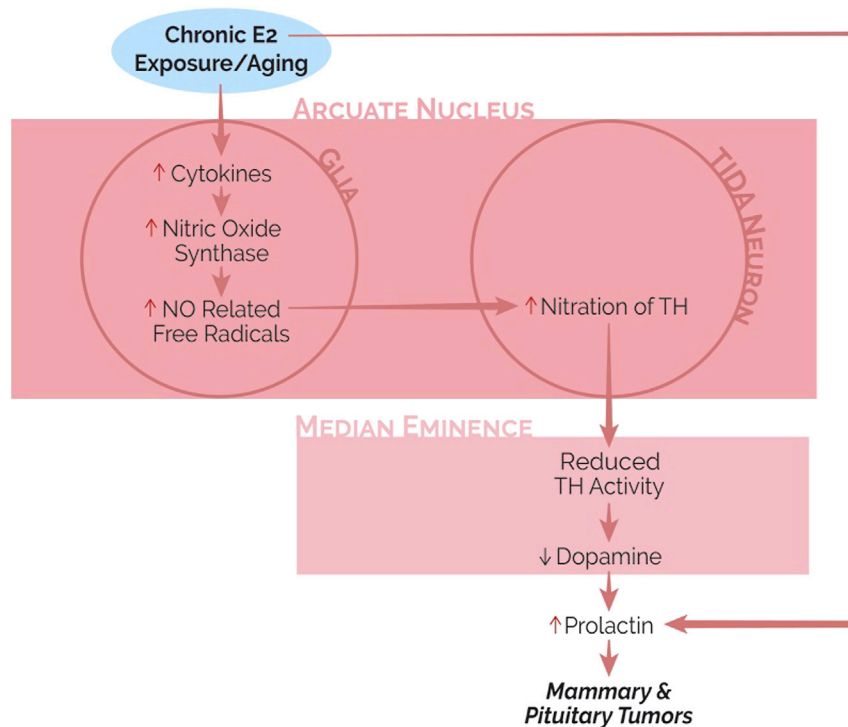


**Figure 10**  
(Panel A) DA concentrations (pg/μg protein, mean ± s.e.) in the hypothalamus and (panel B) serum PRL (ng/mL ± s.e.) in wild type and iNOS(−/−) KO mice that were either sham-implanted or implanted with slow-release E2 pellets for 90 days. \* indicates significant difference ( $P < 0.05$ ); \*\* indicates  $P < 0.01$  and \*\*\* indicates  $P < 0.001$ . WT:  $n = 8$  per group; iNOS<sup>−/−</sup>:  $n = 7$  per group.

The reason for this effect is not clear and needs further investigation.

Results from this study indicate the possible involvement of cytokines and nitric oxide-related free radicals in E2-induced TIDA neuronal degeneration. To understand the exact sequence of events, both IL-1R-KO mice and iNOS-KO mice were used. E2 treatment reduced nitrate production in the arcuate nucleus of IL-1R-KO mice indicating that IL-1β is essential for nitric oxide production. Moreover, the reduction in dopamine was blocked in the iNOS KO mice. This suggests that cytokine

production is followed by nitric-oxide-related free-radical generation in this model. Cytokines and nitrates are known to play a role in neurodegenerative processes (Giasson *et al.* 2000, McCann *et al.* 2000, Reynolds *et al.* 2007). Increase in IL-1β immunoreactivity in microglial cells within the arcuate nucleus has been associated with a reduction in TIDA activity, followed by an increase in serum prolactin levels (Gonzalez *et al.* 2004) indicating that microglial cells are the major cytokine contributing cells in this nucleus. Microglial cells can also produce nitric oxide besides cytokines (Chao *et al.* 1992, Nakamura *et al.* 1999). However, there are other studies to indicate that astrocytes are the main source of nitric oxide (Tolias *et al.* 1999). Nitric oxide reacts with superoxide to generate peroxynitrite, which is a powerful oxidant that induces nitration in tyrosine residues (Di Stasi *et al.* 1999, Balafanova *et al.* 2002, Galinanes & Matata 2002, Natal *et al.* 2008). Therefore, the nitric oxide produced in the arcuate nucleus could result in the formation of peroxynitrite, which specifically targets tyrosine residues within tyrosine hydroxylase and induces nitration of these residues. This causes steric hindrance and interferes with the catalytic activity of the enzyme causing impaired dopamine synthesis (Ara *et al.* 1998). Although chronic E2 exposure was able to increase nitration of this enzyme, aging by itself could promote this effect since MIS animals also had an increase in the ratio of nitrated TH/TH.



**Figure 11**  
Schematic representing changes in the arcuate nucleus and median eminence in response to E2 exposure and aging that culminates in hyperprolactinemia. Changes in the arcuate nucleus and median eminence are represented in separate boxes. Glia and TIDA neuronal cell bodies in the arcuate nucleus are represented by circles in which possible ongoing alterations are listed. Courtesy: Stephanie Pfeiffer, Educational Resource Center, UGA. A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0274>.

Confidence in the effect of E2 exposure is further increased by the fact that many of these changes are localized to the arcuate nucleus and not the substantia nigra. The main reason for this is that the substantia nigra has estrogen receptor-beta and E2 could act on this receptor or produce non-genomic effects to facilitate many of its beneficial effects in this brain region (Kuppers *et al.* 2000, Quesada *et al.* 2007).

In conclusion, chronic low-dose E2 exposure initiates an inflammatory cascade in the arcuate nucleus, which includes increases in cytokine followed by nitric oxide production that leads to formation of nitric oxide-related free radicals that nitrate tyrosine moieties on tyrosine hydroxylase. This leads to reduced functioning of this enzyme culminating in lower dopamine synthesis and increased secretion of prolactin that could place individuals at greater risk for developing mammary and pituitary tumors (Fig. 11). Although aging is associated with proinflammatory and oxidative stress mechanisms, our results suggest that there is no cumulative effect of aging and E2 exposure on TIDA neurons. Also, it appears that endogenous estrogens play an important role in augmenting the effect of exogenous E2 on TIDA neurons.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

#### Funding

This work was supported by funding from the NIH (National Institute of Aging) R01 AG 027697 to P S M. The authors thank Stephanie Pfeiffer, Educational Resource Center, University of Georgia, for the illustration (Fig. 11).

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Received in final form 26 October 2018

Accepted 31 October 2018

Accepted Preprint published online 1 November 2018