Report by Dr. Marina O. Fernandez

COMMITTEE FOR AID AND EDUCATION IN NEUROCHEMISTRY (CAEN) CATEGORY 1B:

REPORT for Research supplies for use in the applicant's home laboratory –

International Society for Neurochemistry

ISN CAEN Grants-April 2018 round

Researcher: Marina Olga Fernandez, PhD

Institution: Fundación IByME

Period of award: July 2018-July 2019

Project title: **Exposure to Bisphenol A and Benzophenones: impact on brain inflammation and neuroendocrine circuits**

First of all, I would like to thank Dr. Alessandro Prinetti, the Committee for Aid and Education in Neurochemistry (CAEN) and the International Society for Neurochemistry for giving me the opportunity to continue my research in my home country. Doing research in Argentina is becoming increasingly challenging, as budget cuts are affecting not only the flow of grants for research, which tend to be scarce for new investigators like myself, but also the quality of life of researchers, due to the low salaries and increasing inflation rates. The development of this project would not have been possible without your financial support.

I would also like to point out that one MSc thesis was completed and another one is underway during the award period, thanks to your support:

2017-2018. Marigliano Camila. MSc Student. Major: Biotechnology, Universidad Argentina de la Empresa (UADE). Internship in the Laboratory of Neuroendocrinology, IByME-CONICET. MSc Research Project: Effects of Bisphenol A and Benzophenones 2 and 3 in GnRH neurons. Date of thesis defense: December 2018.

2018-2020. Riaño Gomez Juan Manuel. MSc Student. Major: Biological Sciences. School of Sciences, University of Buenos Aires (FCEN-UBA). Internship in the Laboratory of Neuroendocrinology, IByME-CONICET. MSc Research Project: Exposure to Bisphenol A and Benzophenones: impact on brain inflammation and neuroendocrine circuits. Thesis defense projected date: December 2020.

Background to project: Exposure to environmental toxicants during sensitive periods of development is associated with reproductive and metabolic alterations later in life. Bisphenol A (BPA), monomer of polycarbonate plastics, and Benzophenones (BPs), present in ultraviolet filters, are endocrine disrupting chemicals (EDC) that can be found in everyday products[1-4]. Babies and infants are exposed through bottles, sunscreens, breastmilk and formula, so understanding how exposure to these chemicals during early periods of life affects development of the nervous system is crucial. Perinatal exposure to BPA predisposes to obesity and metabolic syndrome in adulthood(*5, 6*) and it alters neuron and glia number in the prefrontal cortex [7]. It also alters specific neural populations involved in feeding. One study described that male mice exposed through pregnancy and lactation to BPA present increased expression in arcuate (ARC) agouti-related peptide (AgRP) and neuropeptide Y (NPY) after high-fat diet (HFD) feeding. Animals exposed perinatally to BPA exhibit decreased glucose tolerance when fed normal chow or HFD. Adult males also showed reductions in pro-opiomelanocortin (POMC)-immunopositive fiber density in the periventricular nucleus (PVN)[8]. Although classically it is considered that the developmental exposure is the most relevant for long lasting effects, many studies show that exposure during adulthood also have important effects on the central nervous system. Khadrawy and col.[9] demonstrated that adult rats exposed to BPA for 10 weeks showed a decrease in cortical neurotransmitters. Also, male adult exposure to BPs was associated with a decrease in fecundity[10] and semen quality[11].

Previous results from the lab demonstrated that neonatal exposure to BPA increases GnRH pulsatility from hypothalamic explants and induces ovarian and hormonal alterations that resemble what is observed in the polycystic ovarian syndrome[12, 13]. Also, more recent data shows that direct exposure to BPA inhibit Kisspeptin-induced GnRH expression in immature GnRH neurons, GN11 cells[14].

Our hypothesis was that exposure of mice to environmentally relevant doses of BPA and two BPs (BP2 and BP3) altered hypothalamic inflammation in cellular networks involved in the control of energy homeostasis and reproduction. We initially tested direct effects of these compounds on hypothalamic explants and neuronal cultures, to better understand the mechanisms of action.

A) We firstly evaluated direct effects of the Endocrine Disruptors (EDC) on GnRH expression and secretion in cultures of hypothalamic explants and GnRH neurons (GN11 and GT1-7 cells), in order to analyze effects on cell physiology. We will briefly describe the methods used for this part:

Animals: Adult Balb/c mice (8-10 weeks old from the La Plata vivarium) were used for this study. Animals were rapidly sacrificed and whole hypothalami were dissected. Individual hypothalamic were places in 96 well plates (one hypothalamus per well) and cultured in Krebs-Ringer-Bicarbonate buffer. Hypothalami were stimulated for 6 h with BPA, BP2, BP3 (EDC, $1x10^{-9}$ M, Sigma) or medium. Media were collected for GnRH measurement (RIA) and hypothalamic processed for RNA preparation.

Cells: GN11 and GT1-7 cells were cultured in DMEM with 4.5g/L glucose (high glucose) and 548 mg/L L-glutamine, with 10% FBS, 1% penicillin/streptomycin and 110 mg/L sodium pyruvate (complete medium). Cells were plated in 6 or 12-well plates in complete medium for gene expression analysis or in 24-well plates for GnRH secretion. After 24 hours, medium was replaced by DMEM high glucose, with glutamine, penicillin/streptomycin, sodium pyruvate, and 10% charcolyzed FBS (stimulaton media), and the appropriate EDC were added ($1x10^{-9}$ M, Sigma). After 24 hours, media was changed, stimuli renewed and Kiss (1x10-9M) or vehicle added. Four hours later, media were collected and RNA extracted using TriReagent (Molecular Research Center, OH, USA). For GPR54 gene expression in GT1-7, cells were plated (12-well plates) in complete medium; 24 hours later, medium was replaced by stimulation media and cell stimulated with the EDC for 12 or 24 hours. After this period, media were removed and RNA extracted using TriReagent. For GnRH secretion, after 24 h incubation with the EDC, media was changed to Krebs-Ringer-Bicarbonate buffer, stimuli renewed and cells further stimulated with Kiss (1x10-9M) or vehicle for 1 h. Media were collected and GnRH analyzed by RIA.

Proliferation assay: Cells were plated in 96-well plates (20000 cells/well) in complete medium. After one day, medium was replaced with DMEM high glucose, with glutamine, penicillin/streptomycin, sodium pyruvate, and 0.1% BSA. Cells were stimulated with BPA, E2, BP2 or BP3 ($1x10^{-9}$ or $1x10^{-7}$ M), ICI 182780 (ICI, an ERα-β antagonist, $1x10^{-6}$ M, Sigma) or combinations of the stimuli. After 24 hours, MTS (Promega, WI, USA)-PMS (Sigma) were added to the wells according to manufacturer's instructions. Absorbances at 490 nm were measured and results registered as Abs490(sample)/Abs490(Control).

Gene expression: RNA (2ug) was reverse transcribed and Real-Time PCR performed using HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne) in a Bio Rad CFX96 Real-Time System. Cyclophilin B (*Ppib*) as housekeeping gene and results were analyzed using the mathematical model of Pfaffl et al (2). Real-Time primers are included in the table:

Gene	Fwd 5'->3'	Rev 5'->3'
Gfap	GCGAAGAAAACCGCATCACC	GTGACTTTTTGGCCTTCCCC
Gpr54	TGCAA ATTCGTCAAC TACATC	GGAACACAGTCACATACCAG
Gnrh	GAACCCCAGCACTTCGAATGT	TGGCTTCCTCTTCAATCAGACTTT
IL-1β	AACCTGCTGGTGTGTGACGTTC	CAGCACGAGGCTTTTTTGTTGT
IL6	CCTCTCTGCAAGAGACTTCCAT	ACAGGTCTGTTGGGAGTGGT
IL18	TCTTGGCCCAGGAACAATGG	ACAGTGAAGTCGGCCAAAGT
Ppib	GACCCTCCGTGGCCAACGAT	ACGACTCGTCCTACAGATTCATCTC

Results 1: Effects of EDC on mature GnRH neurons

1a. GT1-7 cells:

When GT1-7 cells were exposed to all EDC for 24 h, the increase in GnRH gene expression in response to Kiss was inhibited (Figure 1A). Secretion in response to Kiss, on the other hand, was similar in control and EDC-exposed cultures, in all cases there was a small increase in secretion in response to Kiss (Figure 1B).

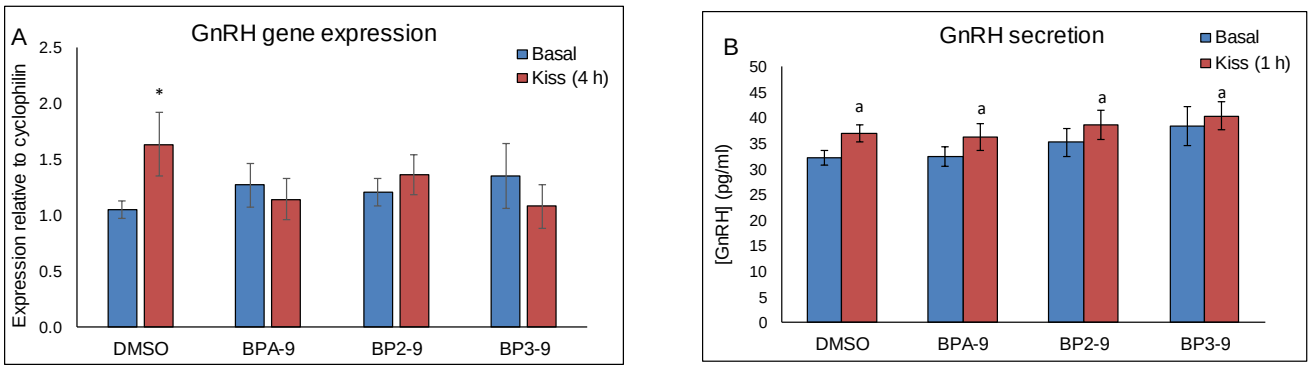

Figure 1: GnRH gene expression and secretion in GT1-7 cells. Cells were exposed to the EDC ($1x10^{-9}$ M) for 24 h, and then to Kiss $1x10^{-9}$ M for the indicated time. A) GnRH gene expression. Repeated measures two-way ANOVA: Interaction $p<0.05$, *=different from basal $p<0.01$, n=6. B) GnRH secretion. Repeated measures two-way ANOVA: Interaction ns. Main Effect Time $p<0.005$. a=different from basal, n=7.

Later, GPR54 (Kiss receptor) expression levels were evaluated in cultures exposed for 12 and 24 h to the EDC, to better understand the mechanisms by which exposure impacted GnRH expression. Twelve-hour exposure to BPA decreased GPR54 expression, while there was no change in cultures exposed to BP2 or BP3.

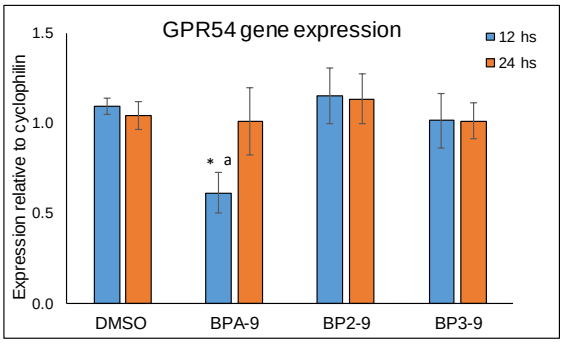

Figure 2: GPR54 gene expression in GT1-7 cells. Cells were exposed to the EDC ($1x10^{-9}$ M) for the indicated time. Repeated measures two-way ANOVA: Interaction $p<0.01$. *=different from DMSO-12 h $p<0.05$, a=different from BPA-24h, $p<0.001$, n=4

These results show a possible mechanism for BPA action on Kiss-induced GnRH expression; however, BP2 and BP3 mechanisms of action for the reduction in Kiss-induced GnRH expression remains to be resolved.

Gene expression of IL18 (a pro-inflammatory cytokine expressed in GnRH neurons) was analyzed in GT1-7 cells after exposure to the EDC. Twenty-four-hour exposure to BPA induced a significant increase in IL-18 (Figure 3A), whereas exposure to neither BP2 nor BP3 had a significant effect, although there was a trend towards an increase (Figure 3B). We are currently adding more cases and analyzing more time points to further evaluate the effects.

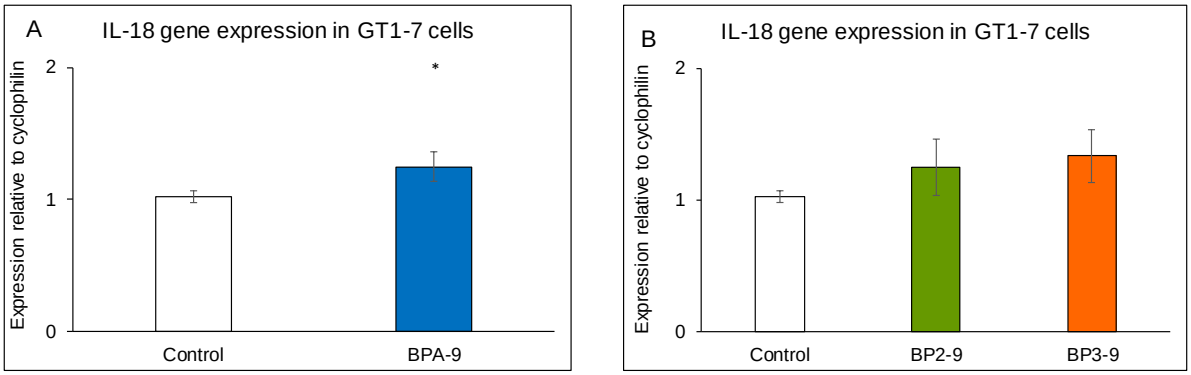

Figure 3: IL-18 gene expression in GT1-7 cells. Cells were exposed to the EDC ($1x10^{-9}$ M) for twenty-four hours, n=7. A) Effects of BPA vs Control. Student's t-test: * $p<0.05$. B) Effects of BP2 or BP3 vs Control: Anova ns.

When IL-6 was analyzed in this cell line, we observed that 24-hour BPA exposure reduced its expression (Figure 4). BP3, on the other hand, increased its expression after 12 hours of exposure, while BP2 had no effect. More cases and times of exposure are under evaluation. IL1β was not detectable in this cell line.

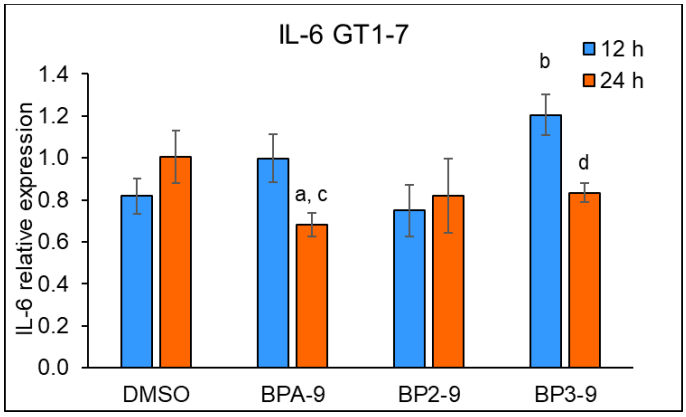

Figure 4: IL-6 gene expression in GT1-7 cells. Cells were exposed to the EDC ($1x10^{-9}$ M) for the indicated time. Repeated measures two-way ANOVA: $p<0.05$, a: different from DMSO-24 h; b: different from DMSO-12 h; c: different from BPA^{-9}-12 h; d: different from $BP3^{-9}$-12 h; n=4

1b. Hypothalamic explants from adult Balb/c mice

Whole hypothalami from adult Balb/c mice were incubated with the EDC ($1x10^{-9}$M) for 9 hours. BPA-exposed hypothalami expressed significantly higher GnRH mRNA levels than control-exposed ones (Figure 5A). In BP2 or BP3-exposed hypothalami GnRH gene expression was also higher compared to controls, although this difference was not significant (Figure 5B). On the other hand, none of the EDC had any effect on GnRH secretion (Figure 5C).

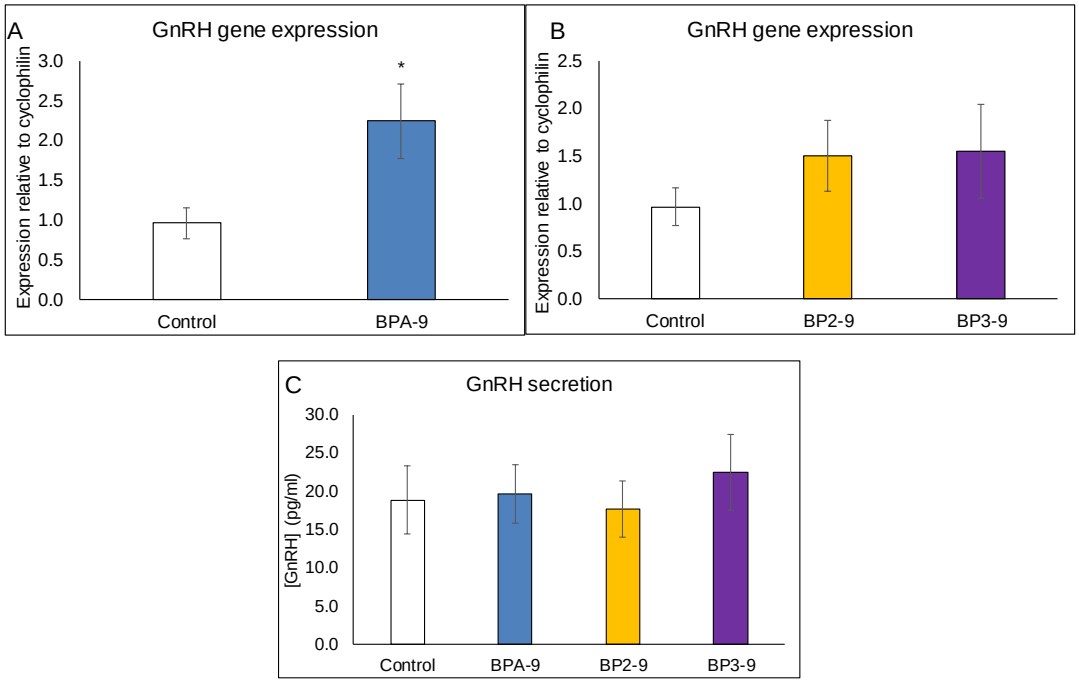

Figure 5: GnRH gene expression and secretion in whole hypothalami from adult mice. Hypothalami were exposed to the EDC ($1x10^{-9}$ M) for 6 h. A) GnRH gene expression after 6 h BPA exposure. T-test, $p<0.05$. *=different from control, n=9. B) GnRH expression after 6 h BP2 or BP3 exposure. ANOVA ns, n=9. C) GnRH secretion after 6 h exposure to the indicated EDC. ANOVA ns, n=8

As GnRH gene expression was increased in the hypothalami exposed to BPA, GPR54 was measured in Control and BPA-exposed hypothalamic. There was no significant difference between these two groups (Figure 6).

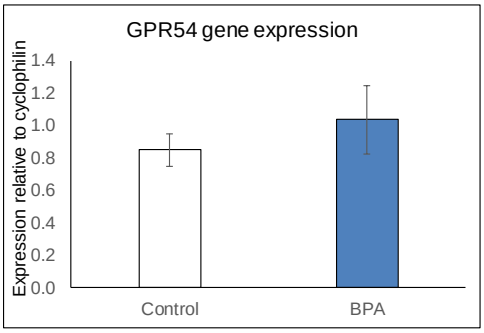

Figure 6: GPR54 gene expression in whole hypothalami from adult mice. Hypothalami were exposed to the BPA ($1x10^{-9}$ M) for 6 h. ANOVA: ns, n=9

Inflammatory and astrocyte-specific markers were later analyzed in the hypothalami exposed to the EDC. GFAP was increased in hypothalamic explants exposed to BPA (Figure 7A), while exposure to BP2 and BP3 exposure did not change its expression (Figure 7B).

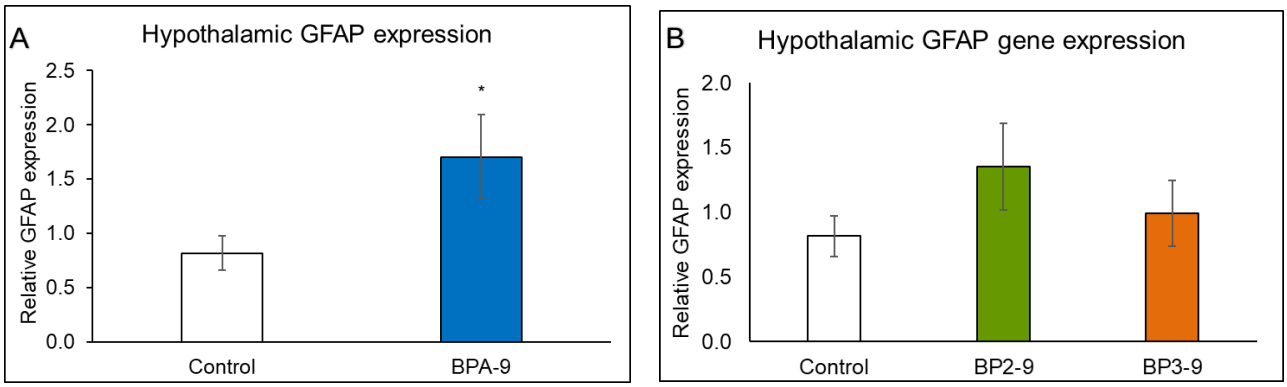

Figure 7: GFAP gene expression in whole hypothalami from adult mice. Hypothalami were exposed to the EDC ($1x10^{-9}$ M) for 6 h. A) GFAP gene expression after 6 h BPA exposure. T-test, $p<0.05$. *=different from control, n=9. B) GFAP expression after 6 h BP2 or BP3 exposure. ANOVA ns, n=9.

When IL-18 was analyzed, no differences were observed in neither BPA-exposed nor BPs-Exposed hypothalamic (Figure 8A and B).

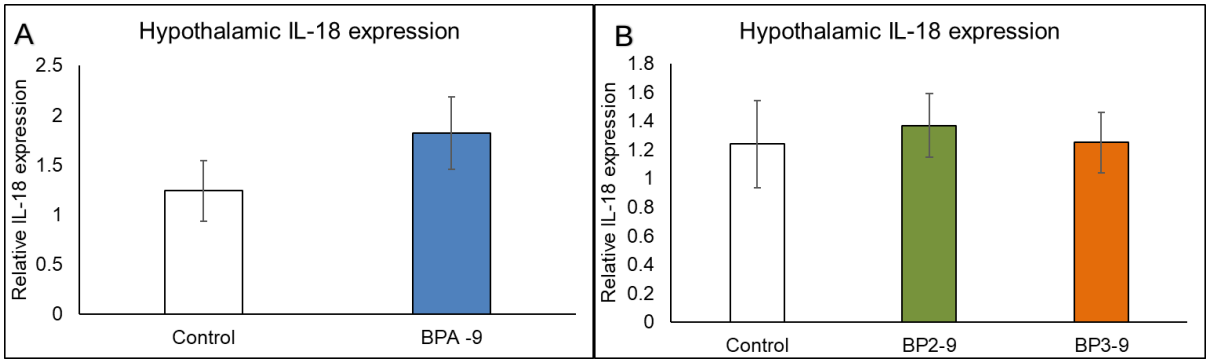

Figure 8: IL-18 gene expression in whole hypothalami from adult mice. Hypothalami were exposed to the EDC ($1x10^{-9}$ M) for 6 h. A) IL-18 gene expression after 6 h BPA exposure. T-test, ns, n=9. B) IL-18 expression after 6 h BP2 or BP3 exposure. ANOVA ns, n=9.

Regarding IL-1β expression, there was no statistical difference between EDC-exposed hypothalami and control-exposed hypothalami (Figure 9A and B)

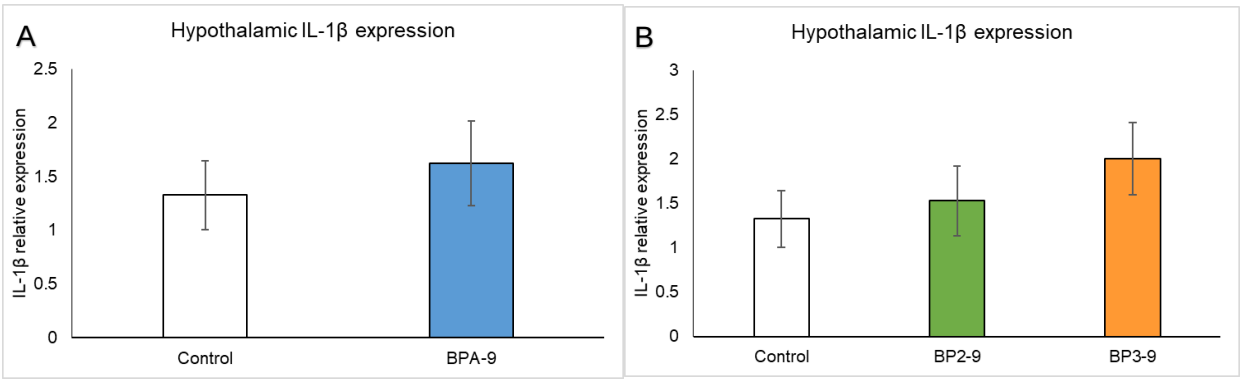

Figure 9: IL-1β gene expression in whole hypothalami from adult mice. Hypothalami were exposed to the EDC ($1x10^{-9}$ M) for 6 h. A) IL-1β gene expression after 6 h BPA exposure. T-test, ns, n=8. B) IL-1β expression after 6 h BP2 or BP3 exposure. ANOVA ns, n=8.

Regarding IL-6 expression, there was no difference in BPA- or BP3-exposed hypothalami and control-exposed hypothalami (Figure 10A and C). On the other hand, BP2-exposed hypothalami showed reduced IL-6 expression that was not statistically significant (Figure 10C).

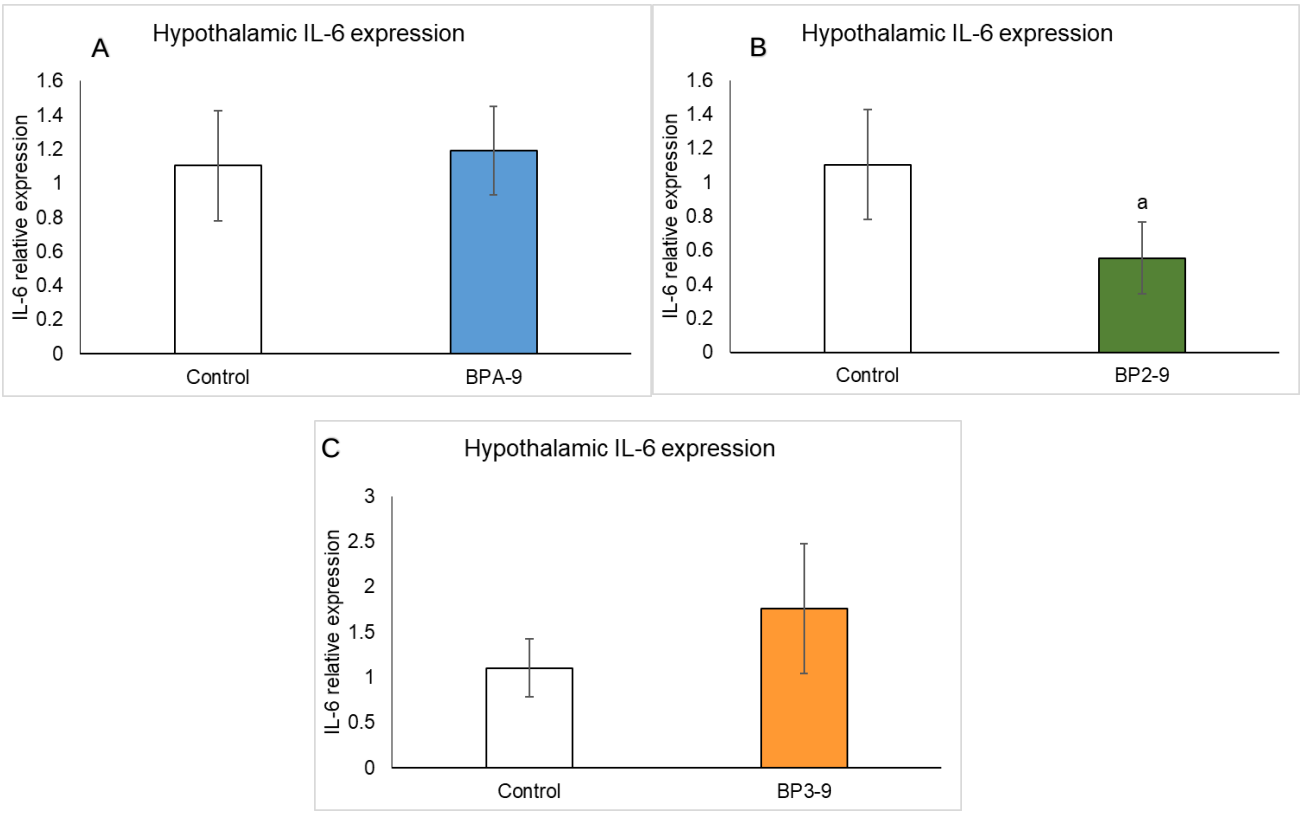

Figure 10: IL-6 gene expression in whole hypothalami from adult mice. Hypothalami were exposed to the EDC ($1x10^{-9}$ M) for 6 h. A) IL-6 gene expression after 6 h BPA exposure. T-test, ns, n=8. B) IL-6 expression after 6 h BP2 a: T-test p=0.08, n=8.C) IL-6 gene expression after BP3 exposure. T-test ns, n=8.

Conclusions1: Our results show that exposure to endocrine disrupting chemicals (EDC) at low concentrations inhibit kisspeptin-induced GnRH gene expression in GT1-7 cells, without altering GnRH secretion. In the case of BPA-exposed cells, this could be in part due to a reduction in GPR54 gene expression, whereas the mechanism of action remains to be found in cells exposed to BP2 and BP3. IL-18, a pleiotropic cytokine that is expressed in GnRH neurons, is increased in BPA-exposed cells, whereas IL-6 was decreased by BPA as well as BP3 after 24-hour exposure. In whole hypothalamic exposed to BPA for 6 hours, there was an increase in GnRH as well as GFAP gene expression, whereas there was no change in IL-1β, IL-18 or IL-6. Neither BP2 nor BP3 changed GnRH, GFAP, IL-1β or IL-18 expression; however, hypothalami exposed to BP2 showed a reduction in IL-6 that was not statistically significant. These EDC can bind to steroid hormone receptors, so one mechanism for the modulation of cytokines could be binding to estrogen receptor. To verify this hypothesis, experiments using the estrogen receptor antagonist ICI 182780 are underway using GT1-7 cells as well as whole hypothalami.

Results 2: Effects of EDC on immature GnRH neurons (GN11 cells)

Effects of EDC were also evaluated in immature GnRH neurons, GN11 cells, kindly donated by Dr Pamela Mellon, UCSD, USA. Previous results showed that twenty-four-hour exposure to BPA, BP2 and E_2 inhibited kisspeptin-induced GnRH gene expression (Figure 11A and B), while BP3 did not have such effect.

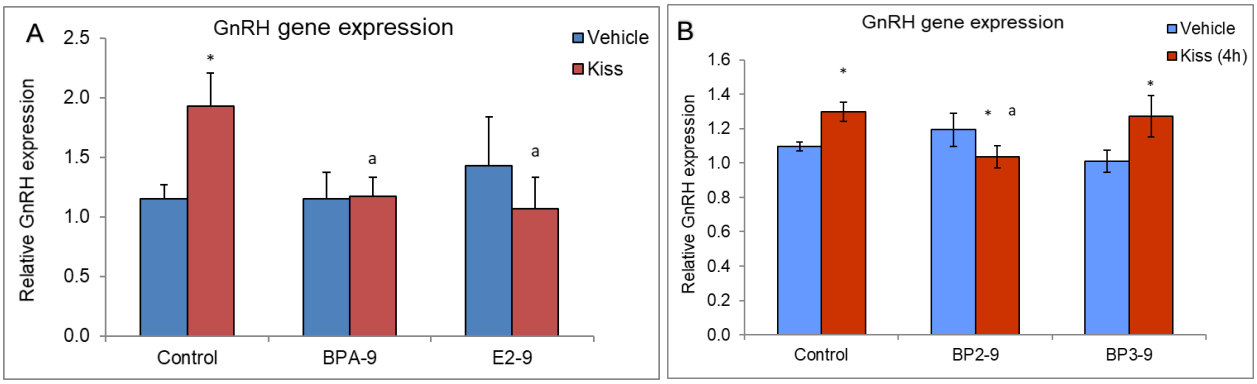

Figure 11: GnRH gene expression in GN11 cells. Cells were exposed to the EDC ($1x10^{-9}$ M) for 24 h, and then to Kiss $1x10^{-9}$ M for 4 h. A) Effects of BPA and E_2 on GnRH gene expression. Repeated measures two-way ANOVA: Interaction $p<0.05$, *=different from Vehicle $p<0.02$, a= different from Control-Kiss $p<0.05$, n=6. B) Effects of BP2 and BP3 on GnRH gene expression. Repeated measures two-way ANOVA: Interaction $p<0.05$, *=different from Vehicle $p<0.02$, a= different from Control-Kiss $p<0.05$, n=8.

We are currently evaluating GPR54 and cytokine expression after 12 and 24 hours of EDC exposure. We observed that 24-hour exposure to BP2 or BP3 did not change GPR54, similar to what we observed in GT1-7 cells.

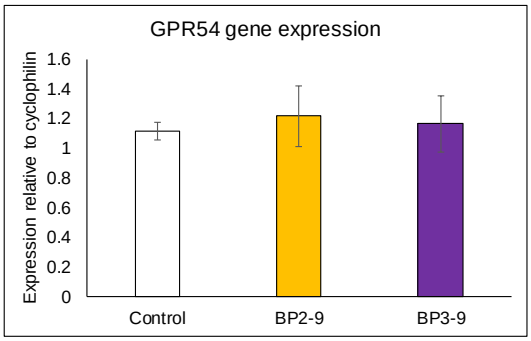

Figure 12: GPR54 gene expression in GN11 cells. Cells were exposed to the BP2 or BP3 ($1x10^{-9}$ M) for 24 h. ANOVA: ns, n=10

We later analyzed proliferation after 24-hour exposure to the different EDC. All compounds increased GN11 cell proliferation both at $1x10^{-9}$ and $1x10^{-7}$ M, and this was only reversed by the estrogen receptor antagonist ICI 182780 in the case of BPA $1x10^{-9}$ (Figure 13).

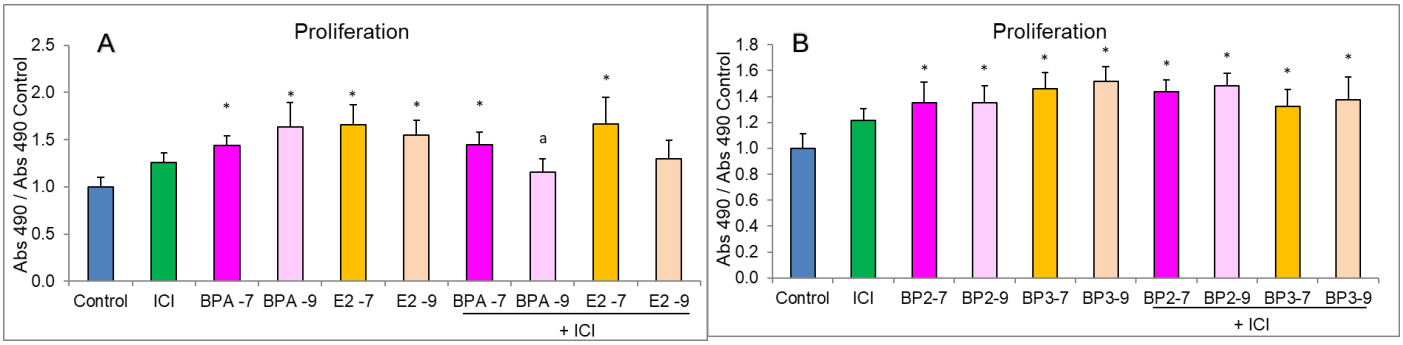

Figure 13: Cell proliferation in GN11 cells. Cells were exposed to the EDC ($1x10^{-9}$ M, $1x10^{-7}$) for 24 h, alone or in combination with ICI 182780 A) Effects of BPA and E_2 on GN11 cell proliferation. Repeated Measures ANOVA * = different from control, $p<0.05$, a=different from BPA-9, $p<0.05$, n=9. B) Effects of BP2 and BP3 on GN11 cell proliferation. Repeated measures ANOVA: *=different from Control $p<0.05$, n=8.

Conclusions2: EDC increased GN11 cell proliferation and this is only reversed by the ER antagonist ICI 182780 in the case of BPA $1x10^{-9}$ M. BPA, BP2 and E_2 also decreased Kisspeptin-induced GnRH expression. These results show that, at least for these effects, BPA and BP2 behave as E_2. Experiments to evaluate effects of the EDC on cytokine expression in this cell line are underway and will be finished soon.

B) After evaluating if the EDC had effects on GnRH expressing cell lines, we started to evaluate if the in-vivo exposure to the EDC induced brain inflammation and alterations in glucose metabolism. This part is underway, so we will present an overview of the methods and results obtained so far.

Methods: Adult (60-90 days) C57BL/6 male mice were used for this part. Animals were exposed orally for 5 days to 250 µg/kg BP2 or BP3 in vehicle or vehicle alone (Vehicle: 10%ETOH/90%PBS) using a pipette tip. After the last exposure, animals were fasted for 2 hours and glucose was measured with a glucose meter. Mice were rapidly euthanized by decapitation, and serum, brain and pituitary were obtained for analysis. Pituitaries were fixed in formalin and brains rapidly frozen in dry ice. Serum insulin was determined by radioimmunoanalysis. For gene expression analysis, 500 µm-thick frozen micropunches of select brain regions (AVPV/PeN [Plates 28 –32 of Paxinos and Franklin Mouse Atlas], ARC [Plates 44 – 48]) were obtained as described by N. P. Di Giorgio *et al.*, *Endocrinology* **155**, 1033 (2014). We are currently in the process of extracting the mRNA to analyze gene expression in this areas.

Results:

We did not find any significant difference in glucose or insulin serum levels after two-hour fasting (Figure 14). Messenger RNA samples from brain micropunches are being extracted and will be retrotranscribed for qPCR soon. A separate set of animals will be exposed to the same EDC for immunohistochemistry.

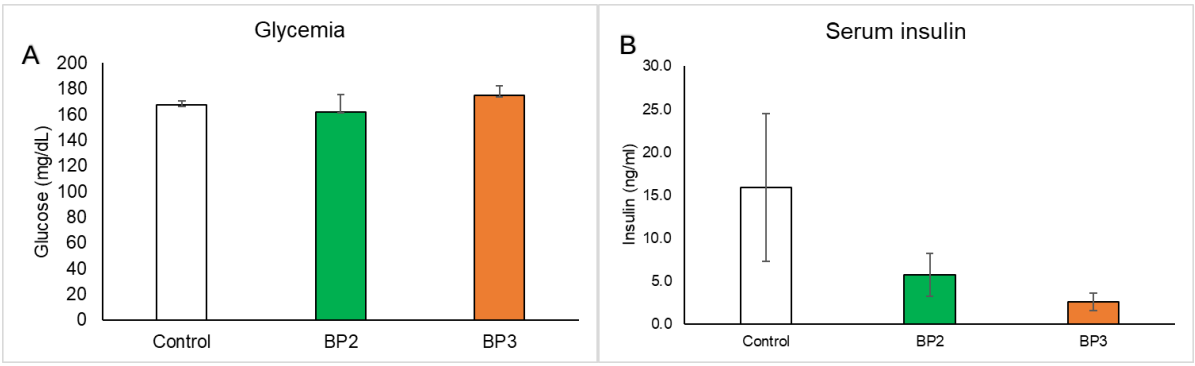

Figure 14: Serum glucose and insulin after 2-hour fasting. ANOVA: ns, n=7-8 (glycemia), n=6 (insulinemia).

The results showed in this report were presented in national and international conferences, and they are part of a manuscript under preparation.

Abstracts:

2019. **In Vitro Exposure to Bisphenol A, Benzophenones 2 or 3, Have Different Effects on GnRH Gene Expression and Secretion in Mature and Immature GnRH Neurons**
Camila Marigliano Paula Arocena, Juan Riaño Gómez, Carlos Libertun, Victoria Lux-Lantos, and Marina Fernandez,
ENDO2019, 101st Annual Meeting of the Endocrine Society
New Orleans, USA, March 23 – 26th, 2019
Journal of the Endocrine Society, Volume 3, Issue Supplement_1, April-May 2019, MON-031, https://doi.org/10.1210/js.2019-MON-031

2018. **In-vitro exposure to Bisphenol A and Benzophenones 2 and 3 alter GnRH gene expression in mature GnRH neurons.**
Marigliano C, Libertun C, Lux-Lantos V, Fernandez M
XX Annual Meeting of the Sociedad Argentina de Biología (SAB)
Buenos Aires, Argentina. December 5-7 2018
Oral presentation, Conference Book.

2018. **In-vitro exposure to Bisphenol A increases GFAP Gene Expression in Whole Hypothalami and IL-18 Gene expression in GT1-7 Cells**
Fernandez M, Marigliano C, Riaño-Gomez J, Libertun C, and Lux-Lantos V
XX Annual Meeting of the Sociedad Argentina de Biología (SAB)
Buenos Aires, Argentina. December 5-7 2018
Oral presentation, Conference Book.

Manuscript in preparation:

In Vitro Exposure to the endocrine disruptors Bisphenol A, Benzophenone 2 or 3, Have Different Effects on GnRH Gene Expression and Secretion in Mature and Immature GnRH Neurons. Paula Arocena*, Camila Marigliano*, Juan Manuel Riaño Gomez*, Eleonora Sorianello, Carlos Libertun, Victoria Lux-Lantos and Marina Fernandez. *equal contributions.

I would like to sincerely thank Dr. Alessandro Prinetti and the Committee for Aid and Education in Neurochemistry (CAEN) for supporting this research project. The ISN-CAEN has been duly acknowledged in congress presentations, seminars and meetings and will be properly acknowledged in our publications related to this project.

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14. Fernandez Marina O, Arocena Paula, Lux-Lantos Victoria, Libertun Carlos. In-Vitro Effects of Bisphenol A in Immature GnRH Neurons. Endocrine Review 38(3 Supplement). 2017.

Financial report:

I would like to thank the International Society for Neurochemistry for giving me the opportunity to continue my research in my home country. Funds were mainly used for reagents for molecular biology, plastics for in-vitro culture and general lab supplies. We also bought antibodies for immunohistochemistry and western blot. Total awarded: U$D 4800.

Item	U$D spent
Molecular Biology Reagents (qPCR mixes and cDNA synthesis kits)	1800
Antibodies	2000
General lab supplies	600
Cell culture supplies	400

Picture in the lab: