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Bisphenol S enhances gap junction intercellular communication in ovarian theca cells

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Abstract

Gap junction intercellular communication (GJIC) is necessary for ovarian function, and it is temporospatially regulated during follicular development and ovulation. At outermost layer of the antral follicle, theca cells provide structural, steroidogenic, and vascular support. Inter- and extra-thecal GJIC is required for intrafollicular trafficking of signaling molecules. Because GJIC can be altered by hormones and endocrine disrupting chemicals (EDCs), we tested if any of five common EDCs (bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), perfluorooctanesulfonic acid (PFOS), and triphenyltin chloride (TPT)) can interfere with theca cell GJIC. Since most chemicals are reported to repress GJIC, we hypothesized that all chemicals tested, within environmentally relevant human exposure concentrations, will inhibit theca cell GJICs. To evaluate this hypothesis, we used a scrape loading/dye transfer assay. BPS, but no other chemical tested, enhanced GJIC in

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

a dose- and time-dependent manner in ovine primary theca cells. A signal-protein inhibitor approach was used to explore the GJIC-modulatory pathways involved. Phospholipase C and mitogen-activated protein kinase (MAPK) inhibitors significantly attenuated BPS-induced enhanced GJIC. Human theca cells were used to evaluate translational relevance of these findings. Human primary theca cells had a ~40% increase in GJIC in response to BPS, which was attenuated with a MAPK inhibitor, suggestive of a conserved mechanism. Upregulation of GJIC could result in hyperplasia of the theca cell layer or prevent ovulation by holding the oocyte in meiotic arrest. Further studies are necessary to understand *in vitro* to *in vivo* translatability of these findings on follicle development and fertility outcomes.

Keywords

theca cells; bisphenol S; gap junction intercellular communication; connexin; ovary

1. Introduction

Nearly one out of every six couples suffer from infertility; a quarter of which remain etiologically unexplained, and a third are of female origin (Thoma et al., 2013; Practice Committee of the American Society for Reproductive, 2015). The basis for female infertility often relates to ovarian dysfunction and includes diseases that have steroid hormone imbalances such as polycystic ovarian syndrome, premature ovarian insufficiency, and luteal dysfunction (CDC, 2017). There is growing evidence that exposure to environmental contaminants may be a contributing factor to the rising infertility trend over the past four decades as they have been reported to affect hypothalamic-pituitary-gonadal axis regulation alter ovarian function including steroidogenic dysfunction, alteration in follicular development, oocyte maturation, and uterine dysfunction resulting in reduced implantation (Rattan et al., 2017) and, placental dysfunction (Strakovsky and Schantz, 2018; Gingrich et al., 2020).

The ovarian antral follicle is comprised of two distinct cell types that sustain oocyte development and maturation; granulosa and theca. Theca cells are steroidogenic cells at the outer layer of the follicle that provide structural and vascular support, and androgen and progesterone synthesis (Young and McNeilly, 2010). Prior to ovulation, theca cells synthesize testosterone that is transferred extracellularly into neighboring granulosa cells for estrogen synthesis. Intercellular communication within the follicle and transport of testosterone across follicular layers is thus essential for follicular growth and oocyte maturation (Orisaka et al., 2009). Regulated through gonadotropin-mediated events (Sommersberg et al., 2000; Norris et al., 2008; El-Hayek and Clarke, 2015), gap junction intercellular communication (GJIC) within the follicle is also critical for oocyte meiotic arrest and resumption. Granulosa cells allow intercellular transfer of cGMP to the oocyte which indirectly increases the local concentration of cAMP, holding the cell in a state of meiotic arrest via protein kinase A (PKA)-induced cell signaling (Shuhaibar et al., 2015). Reduction in cGMP, driven in part through a mitogen-activated protein kinase (MAPK)-dependent mechanism (Norris et al., 2008), allows phosphodiesterase 3A (PDE3a)-controlled cAMP hydrolysis resulting in meiotic resumption and ovulation (Mehlmann,

2005; Richards and Ascoli, 2018). Theca cells aid in control of the ovulatory process through a steroid feedback loop (Young and McNeilly, 2010) and by stimulation of proliferation and anti-apoptotic responses in granulosa cells (Orisaka et al., 2009). Upon ovulation, granulosa and theca cells undergo luteinization, a cellular differentiation process that includes an estrogen-to-progestogen shift in follicular steroidogenesis. This shift towards progesterone synthesis is essential for fertilization, embryo implantation, and pregnancy maintenance in mammalian species. To note, connexin 37 (Cx37) gap junctions have been implicated in the transfer of important, yet unidentified signals, from the oocyte to the granulosa cells to prevent luteinization prior to ovulation (Winterhager and Kidder, 2015). Theca cells functions are modulated by steroid hormones, growth factors, and bone morphogenetic proteins (BMP) (Young and McNeilly, 2010). Additionally, they are highly sensitive to a variety of stimuli, including nutrition (Williams et al., 2001), stress (Zhu et al., 2016), heat stress (Nteeba et al., 2015), and bacterial insult (Magata et al., 2014). Increasing evidence also suggests that endocrine disrupting chemicals (EDCs) can disrupt ovarian function (Patel et al., 2015; Craig and Ziv-Gal, 2018). However, studies specifically addressing the effects of EDCs on theca cells are scarce, but have identified that dioxins, genistein, tyrphostin and herbimycin can reduce progesterone synthesis (Gregoraszczuk et al., 1999; Grochowalski et al., 2001). In the rat, *in utero* exposure to di-2-ethylhexyl phthalate, a common plasticizer, reduced the size of the theca cell layer in offspring gestationally exposed (Meltzer et al., 2015). Although theca cells remain fairly understudied as a target of EDC toxicity, theca cells are critical for ovarian function whose alteration can lead to ovarian pathologies, such as polycystic ovarian syndrome, hyperthecosis, primary ovarian insufficiency, and premature ovarian failure, that can result in sub- or infertility (Richards et al., 2018).

Gap junctions are intercellular communication channels that are formed by apposition of connexin proteins organized into hexameric connexons. Once connexons from two adjacent cells dock end-to-end, intercellular channels are formed to allow diffusion of molecules <1 kDa in mass or <1.6 nm in diameter, a process known as gap junction intercellular communication. Assembly and activation of connexon channels can occur through multiple established pathways including protein kinase C (PKC) (Long et al., 2007), PKA (Pidoux et al., 2014), phosphatidyl choline-phospholipase C (PC-PLC) (Machala et al., 2003a; Upham et al., 2008) and MAPK (Warn-Cramer et al., 1996). Connexins are expressed in the ovary of mammalian species (Gershon et al., 2008), including humans (Furger et al., 1996; Wang et al., 2009). There are over twenty connexin genes in vertebrates, which are expressed in a tissue and a cell-specific manner (Sohl and Willecke, 2004b; Srinivas et al., 2018). Connexin 43 (Cx43) is the most highly expressed connexin in the ovary and it is essential for ovarian follicle formation in mice (Juneja et al., 1999; Ackert et al., 2001). However, Cx43 knockout mice are embryonic lethal, making reproduction in the absence of Cx43 difficult to study (Nishii et al., 2014) as no Cx43 ovarian conditional knockout mice have been developed to date. However, when compared to ovaries from $Cx43^{+/+}$ wildtype mice, cultured ovaries from $Cx43^{-/-}$ knockout mice resulted in folliculogenesis arrest at the primary (Juneja et al., 1999) or secondary follicle stage (Ackert et al., 2001). In the ovary, formation of gap junctions allows the development of a metabolic syncytium between the oocyte and its supporting cells (Kidder and Vanderhyden, 2010), but can also enable theca-to-granulosa

paracrine signaling via ATP release (Tong et al., 2007). Connexin expression in the antral follicle is a dynamic process. Specifically, Cx43 has reduced expression during the peri-ovulatory period (Okuma et al., 1996; Granot and Dekel, 2002; Sela-Abramovich et al., 2005; Borowczyk et al., 2006) enabling meiosis resumption (Norris et al., 2008) and the initiation of the ovulatory process (Borowczyk et al., 2006). In mice, theca cell gap junctions are primarily composed of Cx32 and Cx26 (Wright et al., 2001) and have significantly lower levels of GJIC than granulosa cells in the bovine ovary (Johnson et al., 2002). Theca-derived BMP4 and BMP7 contribute to the downregulation of Cx43 and gap junction intercellular communication in granulosa cells (Chang et al., 2013; Chang et al., 2014) through SMAD-dependent signaling (Chang et al., 2013; Rossi et al., 2016) in both mice and humans. Despite this, the role of gap junctions in theca cells is not yet well established.

GJIC plays a central role in coordinating intracellular signal transduction initiated by extracellular signals (endocrine, paracrine, and autocrine); in turn, controlling gene expression of neighboring cells via signaling molecules like cAMP, cGMP, and ATP (Mese et al., 2007; Trosko, 2011; Zong et al., 2016). This process can be modulated by EDCs with steroid hormone binding activity via non-genomic actions, such as cAMP and protein kinase pathway modulation (Vinken et al., 2009). Despite the significant role that intercellular communication plays in the ovarian follicle, to our knowledge the only EDC investigated for ovarian-specific GJIC outcomes is perfluorooctanesulfonic acid (PFOS) (Dominguez et al., 2016), which inhibited granulosa cell to oocyte GJIC (Dominguez et al., 2016). Additionally, bisphenol A (BPA) has been reported to reduce Cx43 and Cx26 mRNA expression in the mouse cumulus-oocyte cell complex (Acuna-Hernandez et al., 2018; Zhang et al., 2019a; Zhang et al., 2019b), and BPA analog bisphenol S (BPS) has been demonstrated to impair oocyte maturation and development (Zalmanova et al., 2017; Desmarchais et al., 2020) revealing the potential of other EDCs to influence GJIC. Taken together, these effects demonstrate the potential for EDCs to alter ovarian GJIC, potentially leading to detrimental reproductive outcomes. Therefore, the purpose of this study was to investigate the effects of known EDCs, specifically BPA, BPS, bisphenol F (BPF), triphenyltin chloride (TPT), and PFOS on GJIC in ovarian theca cells. BPA is a synthetic organic chemical, and the most widely used bisphenol in the manufacturing of epoxy resins and industrial and consumer plastics (Liao et al., 2012; Liao and Kannan, 2013). The recent ban of BPA from certain consumer products (Metz, 2016) has raised concerns about the safe use of other bisphenolic compounds like BPS and BPF. Additionally, we have included two non-bisphenolic EDCs of concern, TPT and PFOS in this study. We hypothesized that all five chemicals would reduce GJIC in theca cells. To test this hypothesis, we used primary theca cells from two monovulatory species: sheep and human. We further investigated the molecular pathways by which these chemicals alter GJIC in ovarian theca cells by using a signal-protein inhibitor approach.

2. Materials and Methods

2.1 Chemicals

All chemicals used in the study are listed in Table 1 and were dissolved in DMSO. All groups were administered the same solvent volume in media (0.1% DMSO). The doses of

chemicals used (BPA, BPS and BPF: 1 to 1,000 ng/ml, PFOS: 50 ng/ml, and TPT: 10 ng/ml) were chosen for their environmental exposure relevance as they fall within a magnitude of human exposures determined through biomonitoring studies (ranges of human exposure for BPA: 0.14 – 792 ng/ml in urine, BPS: 0.07 – 211.9 ng/ml in urine, BPF: 0.14 – 298.7 ng/ml in urine, PFOS: 4.6–168 ng/ml in plasma, and TPT: 0.06–204 ng tin/ml in urine) (Heitland and Koster, 2006; CDC, 2016; Fleisch et al., 2017; Worley et al., 2017; Pu et al., 2019).

2.2 Generation of primary sheep ovarian theca cells, purity, and luteinization

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Michigan State University and are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals and the current Animal Welfare Act. Ovine theca cells were used in this study as they represent an excellent *in vitro* model to study GJIC in the context of human chemical exposures and ovarian function as sheep and human are both monovulatory, and have similar ovarian sizes, steroid feedback regulatory mechanisms (Nippoldt et al., 1989; Kasa-Vubu et al., 1992), and connexin protein expression to humans (Borowczyk et al., 2006; Winterhager and Kidder, 2015). Isolation of theca cells was performed on eight multiparous Polypay × Dorsett breed sheep at gestational day 120 of pregnancy as previously described (Pu et al., 2019). High circulating progesterone during pregnancy allowed for collection of theca cells from a homogenous subset of follicles not subjected to follicular divergence or dominance. In brief, the theca interna cell layer of antral follicles was isolated by microdissection. Then, theca cells were dispersed using collagenase I (1 mg/ml) supplemented with 10 μg/ml deoxyribonuclease (DNAse I) in Ca^{2+}/Mg^{2+}-free buffer. The cell suspension was filtered, then fractioned on a discontinuous Percoll gradient (44% and 35% Percoll). Plated cells were maintained in basic medium consisting of DMEM/F12 media supplemented with 1% heat inactivated fetal bovine serum (FBS, Cat#: 35– 010-CV, Corning Inc., Corning, NY, USA), 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 39°C. Theca cell purity was determined using theca cell markers fibulin 5 (Hatzirodos et al., 2015) and vimentin (Qiu et al., 2018) by immunocytochemistry. Purity of all isolated primary cell cultures was > 95% (Pu et al., 2019). Theca cells were kept frozen at −80 °C until used for GJIC assays. Theca cells luteinization was performed as previously described (Pu et al., 2019). In brief, theca cells were grown in DMEM/F12 medium supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine, 1% heat inactivated FBS, 250 ng/ml ovine luteinizing hormone (LH; U.S. National Hormone and Peptide Program), and 50 ng/ml insulin-like growth factor-1 (IGF-1), and cultured for 72 h.

2.3 Ethics, exclusion criteria, and generation of human ovarian theca cells

Human ovarian tissue was obtained after written informed consent following Institutional Review Board (IRB: 17–1066 M) approval from Sparrow Health System and Michigan State University and is consistent with relevant guidelines and regulations. Ovaries were collected from healthy non-pregnant women (n = 3, aged 31 – 46 years) undergoing surgical uni- or bilateral oophorectomy. Patient parity varied between samples (0–5 births). No patients were taking antiandrogens, insulin sensitizers, or gonadotropins at the time of tissue collection. Exclusion criteria included polycystic ovarian syndrome, ovarian cancer, menopause, drug addiction, HIV, and/or hepatitis B or C diagnoses. Only follicles with a healthy appearance,

and ≥ 3 mm in size were chosen to pool for the generation of a theca cell line per individual. All ovaries were processed within 1 h of surgical removal and kept at 4°C. The theca interna cell layer of human antral follicles was isolated as previously described (Pu et al., 2019) and enzyme-digested as described for sheep. Changes to the enzyme digestion for human theca cells included more collagenase I (3 mg/ml) and DNase I (300 IU/ml). Theca cells were kept frozen at −80 °C until used for GJIC assays.

2.4 Cell viability assay

Cell viability was determined using an MTT assay as previously described (Palaniappan et al., 2013). In brief, theca cells (n = 8 primary cultures, with at least 3 replicates per experiment) were seeded into 96-well plates. Cells were treated with increasing concentrations of BPA, BPS, or BPF (0, 1, 10, 20, 50, 100, 200, 500, 1,000, and 10,000 ng/ml) for 72 h, or pathway inhibitors at concentrations and times (15 or 30 min) listed in Table 1. All chemicals tested were dissolved in DMSO to a final concentration of 0.1%. Vehicle group received 0.1% DMSO. Medium was then replaced with MTT working solution (50 μg/ml) and incubated for 4 h. Wells were rinsed, and DMSO added to each. Cell viability was determined by absorbance quantification at 570 nm using a microplate reader (SpectraMax M5e, Molecular Devices, LLC, Sunnyvale, CA, USA). Exposure groups were also visually assessed for cellular morphology differences.

2.5 Experimental design

To test if three bisphenols (BPA, BPS, and BPF), PFOS, and/or TPT alter GJIC, ovine pre-luteinized theca cells were subjected to a GJIC assay as outlined below. Once plated cells reached 90% confluency, media was replaced for exposure media consisting of basic medium supplemented with DMSO (0.1%, vehicle), BPA, BPS, BPF, TPT, or PFOS for 24 h, followed by a 2-h serum starving step, and subsequent gap junction communication assays using a scrape loading dye transfer assay (see below). Bisphenols (BPA, BPS, and BPF) exposure doses (1, 100, and 1,000 ng/ml) were chosen to cover a range of exposures from environmentally relevant to supraphysiological. Given that BPS altered GJIC, BPS was also tested at these additional doses: 10, 200, and 500 ng/ml. We then investigated if the observed GJIC BPS effect occurred in a theca cells independent of their stage (pre-luteinized, luteinizing, and post-luteinized). Luteinizing cells chemical exposure occurred concomitantly with LH during the last 24 h of the 72-h differentiation period, and luteinized cells were exposed after the 72-h luteinizing period in the absence of LH. The effect of BPS (at 200 ng/ml) exposure duration on GJIC was also assessed in pre-luteinized theca cells for 3, 6, 12, and 24 h.

To assess signal transduction mechanisms mediating changes in GJIC, specific gap junction pathway inhibitors (GF109203X, SB202190, D609, ET-18-OCH_3, H89, and U0126) and a PKA activator (CW008) were used. Pathways in which these inhibitors function and concentrations and time of exposure at which all test compounds were used as outlined in Table 1. Primary ovine theca cell cultures (n = 8) were used in the aforementioned experiments, with a minimum of 3 replicates per treatment group per experiment. Human theca cell GJIC experiments (n = 3 primary theca cell cultures per treatment group) followed

the same method to that described for ovine cells, at a fixed BPS exposure dose (200 ng/ml) and using a MAPK inhibitor (1 μM SB202190) for GJIC pathway analysis.

2.6 Gap junction intercellular communication assay

Theca cells were plated at 50 $\times 10^4$ cells/dish on a 60 mm-diameter tissue culture dishes (Corning Inc., Corning, NY, USA) and cultured in basic medium supplemented with 10% FBS. After 24 h, media was replaced with normal basic medium (containing only 1% FBS). Confluent cells were passed and seeded at 25 × 10^4 cells/dish in 35 mm-diameter tissue culture dishes and cultured in basic medium supplemented with 10% FBS. After 24 h, media was replaced with basic medium (1% FBS). Once cells reached 90% confluency, media was replaced for exposure media consisting of basic medium supplemented with DMSO (0.1%, vehicle), BPA, BPS, BPF, TPT, or PFOS for 24 h. To stimulate intercellular communication, cells were serum starved 2-h prior to the GJIC assay (Lin et al., 2003). For the gap junction pathway inhibitor experiments, inhibitors were added in the last 15 to 30 min (Table 1) of the serum starvation step. GJIC was assessed using the scrape loading/dye transfer technique as previously described (Upham, 2011). In brief, cells were washed with Ca^{2+}-Mg^{2+}-PBS and then lucifer yellow and rhodamine-dextran were added to each plate. With gentle pressure, a surgical scalpel blade was rolled through a cell monolayer. Passive diffusion of the lucifer yellow from the loaded cells into the adjacent cells was allowed for 5 min. Due to its high molecular weight and inability to pass through gap junction channels, the rhodamine-dextran was used to visualize the dye loaded cells (*not shown*). Cells were then washed with Ca^{2+}-Mg^{2+}-PBS, fixed with 4% neutral buffered formalin and stored at 4°C until imaging.

2.7 Image Analyses

To quantify theca cell GJIC, 6 to 10 images per primary cultured cell line per treatment group were captured using an Eclipse TE2000-U inverted microscope (Nikon, Toyko, Japan) with a CoolSNAP-ProCF camera (Media Cybernetics, Rockville, MD, USA). Images were quantified using Fiji image analysis software (Schindelin et al., 2012). The freehand selection tool was used to manually determine the area of co-joined lucifer yellow positive cells, and then averaged to determine the area of dye diffusion. A vehicle group was included in each run, and the average area of dye diffusion was normalized to the vehicle group.

2.8 Statistical analysis

All data are presented as a mean ± SEM. Appropriate transformations were applied, as needed, to account for normality of data. Comparisons among the treatment groups were analyzed by mixed model ANOVA with Tukey posthoc tests using experiment run as a covariate. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of bisphenols (BPA, BPS, and BPF) on cell viability

Cell viability was unaltered by any bisphenol (range: 1 to 10,000 ng/ml; Fig. 1). Additionally, TPT, PFOS, or inhibitors used at the concentration tested (Table 1) did not

affect cell viability (*data not shown*). Cell morphology was similar across treatment groups and was not affected by exposure to bisphenols, TPT, PFOS, or inhibitors at the concentrations tested.

3.2. BPS enhances gap junction communication in a cell stage- time- and dose-dependent manner

BPA and BPF, at all doses tested (ranging: 1 to 1,000 ng/ml), had no effect on GJIC in pre-luteinized theca cells (Fig. 2, *left and center panels*) following a 24 h exposure, and thus no further experiments were pursued with these two chemicals. The rhodamine-dextran dye control did not pass beyond the cells cut with the scalpel blade (not shown). PFOS and TPT, additionally, had no effect on GJIC (Fig. 2, *right panel*). However, pre-luteinized theca cells exposed for 24 h to BPS increased GJIC in a dose-dependent fashion (Fig. 3). GJIC increased starting at an exposure dose as low as 10 ng/ml, peaked at 200 ng/ml, and plateaued at higher doses.

To assess if exposure time affects the BPS-induced increase in GJIC in pre-luteinized theca cells, we used the lowest BPS dose that resulted in the strongest effect (200 ng/ml BPS resulted in ~2-fold increase in GJIC), for exposure times of 3, 6, 12, and 24 h. The increase in GJIC was time-dependent with 12 h and 24 h exposures resulting in the highest GJIC response (Fig. 3C).

To test whether BPS differentially modulates GJIC depending on the luteal stage of the theca cells, we used luteinizing and luteinized theca cells. In luteinizing theca cells, BPS also induced an increase in GJIC. Although less pronounced, BPS's effect was first observed at 200 ng/ml and displayed a dose-dependent increase up to the maximum dose tested (1,000 ng/ml) (Fig. 3E). In luteinized theca cells, the BPS effect followed a similar trend to the pre-luteinized theca cells but the effect was less robust (Fig. 3F).

3.3. Signal-protein inhibitor/activator evaluation of BPS-enhanced gap junction communication

A signal-protein inhibitor/activator approach was used to evaluate the molecular pathways potentially involved in BPS-induced upregulation of GJIC (Fig. 4A). First, PKA activator CW008 was used alone and in combination with BPS exposure as a positive control to enhance GJIC. CW008 exposure enhanced GJIC in pre-luteinized theca cells both single and in combination with BPS, although the combined effect was comparable to that of BPS alone (Fig. 4B). Additionally, TPA was used as a non-specific negative control due to its ability to inhibit GJIC by internalizing gap junction channels through a protein kinase C/extracellular receptor kinase 1,2 (PKC/ERK1/2)-dependent mechanism (Ruch et al., 2001; Leithe and Rivedal, 2004). The signal transduction pathway inhibitors (D609, ET-18-OCH_3, GF109203X, H89, SB202190, and U0126), were used to determine if phosphatidyl choline specific phospholipase C, phosphatidyl inositol specific phospholipase C, protein kinase C, protein kinase A, extracellular receptor kinase- mitogen activated protein kinase were involved in BPS-induced regulation of GJIC, and did not alter the effects of GJIC in the absence of BPS. Co-exposure of BPS and TPA resulted in GJIC like that observed in the vehicle group (Fig. 4C).

To evaluate the involvement of the PKA pathway, H89 was used as an inhibitor of PKA-induced connexin phosphorylation. H89 partially reduced (by ~23%) the BPS-induced enhanced GJIC (Fig. 4D). PKC pathway activation was tested using GF109203X as a panPKC inhibitor (Fig. 4E), ET-18-OCH_3 as a phosphatidylinositol-phospholipase C (PI-PLC)-dependent PKC inhibitor (Fig. 4F), and D609 as a phosphatidylcholine-phospholipase C (PC-PLC)-dependent PKC inhibitor (Fig. 4G). Both PLC-dependent PKC pathway inhibitors were able to significantly attenuate the BPS-induced enhanced GJIC by ~29 and ~31%, respectively. Finally, to understand the role of MAPK in BPS-induced upregulation of GJIC, a p38 MAPK inhibitor (SB202190), and a MEK1/2 inhibitor (U0126) were used. SB202190 was the only inhibitor used to result a full attenuation of the BPS effect (Fig. 4H), while U0126 was only able to partially prevent the BPS-induced enhanced GJIC to that of the vehicle control group by ~33% (Fig. 4I). No pathway inhibitors tested individually reduced GJIC below that of the vehicle control.

3.4. BPS enhances gap junction communication in human theca cells

GJIC in primary isolated human theca cells upon BPS exposure was also tested. At the time and dose optimized in ovine primary theca cell lines (24 h exposure to 200 ng/ml BPS), BPS was able to induce approximately a ~1.5-fold increase in GJIC, which was significantly attenuated with a MAPK inhibitor (Fig. 5).

4. Discussion

We demonstrated that BPS, but not four other common EDCs (BPA, BPF, TPT, and PFOS), enhanced GJIC in both a dose- and time-dependent manner in ovine primary ovarian theca cells. BPS-induced enhancement of GJIC was also evident in primary human theca cells. The lowest observed adverse effect level (LOAEL) for enhanced GJIC was 10 and 100 ng/ml BPS in pre-luteinized, and luteinizing and luteinized cells, respectively. Importantly, both LOAEL doses fall within environmentally relevant human exposures (Qiu et al., 2018; Zhao et al., 2018). Thus far, chemotherapeutic agents have been shown to enhance GJIC (Vinken et al., 2006; Liu et al., 2013; Xiao et al., 2013; Yang et al., 2014; Sovadinova et al., 2015; Babica et al., 2016a; Wu et al., 2016), while most EDCs (dichlorodiphenyltrichloroethane (Masten et al., 2001), polychlorinated biphenyls (Kang et al., 1996; Machala et al., 2003a), perfluorinated compounds (Upham et al., 1998; Hu et al., 2002; Upham et al., 2009), methoxychlor,vinclozolin (Babica et al., 2016b)), lipid by-products (octadecatetraenoate (Hasler et al., 1991)), cigarette smoke relevant polycyclic aromatic hydrocarbons (Tai et al., 2007; Upham et al., 2008; Osgood et al., 2014; Velmurugan et al., 2015; Osgood et al., 2017; Siegrist et al., 2019), cannabinoids (Δ(9)-tetrahydrocannabinol and cannabinol (Upham et al., 2003)), plant derivatives (licorice root; (Davidson and Baumgarten, 1988; Tanaka et al., 1999)), and pharmaceuticals (phenobarbital; (Klaunig et al., 1990; Ren and Ruch, 1996)) result in a dose- and time-dependent reduction of GJIC. For both PFOS and TPT exposures, only a single environmentally relevant dose was used. As a dose-dependent study was not performed for either of these chemicals, we cannot rule out that, similar to BPS, these chemicals may exert a dose-dependent effect on GJIC.

Epidemiological evidence linking infertility in humans and BPS exposure is currently only available in males (Ghayda et al., 2019), and not females. Other epidemiological associations from BPS exposure are restricted to metabolic outcomes like obesity (Liu et al., 2019) and type 2 diabetes (Ranciere et al., 2019). Our findings and those of others (Zalmanova et al., 2017; Nevoral et al., 2018; Desmarchais et al., 2020) highlights the need for future epidemiological studies to focus on evaluating if exposure to BPS can results in reduced fertility outcomes.

Reduced GJIC, *in vivo,* is recognized as a hallmark in the early stages of cancer, while late stage or metastatic cancers present with both down- and up-regulated GJIC, particularly during the vascularization of tumors (Aasen et al., 2017), including in the ovary (Hanna et al., 1999). Inhibition of GJIC has been associated with a chemicals' higher carcinogenic potential (Rosenkranz et al., 2000). On the contrary, substances that enhance GJIC, such as green tea micronutrients genistein and epicatechin (Sai et al., 2000; Ale-Agha et al., 2002) and other plant antioxidants (Nakamura et al., 2005a; Nakamura et al., 2005b; Upham et al., 2007; Sovadinova et al., 2015; Babica et al., 2016a), are hypothesized to have cancer preventive properties, and drugs enhancing GJIC have been viewed as novel chemotherapeutics (Yang et al., 2014; Wu et al., 2016). Despite this, in the context of the ovarian reproductive cycle where GJIC is temporospatially regulated (Kidder and Mhawi, 2002), enhanced GJIC could result in pathological outcomes like hyperplasia of the theca cell layer, a pathology seen in hyperandrogenism (Czyzyk et al., 2017). Importantly, a BPS-induced increase in GJIC, if also occurring intergranulosal or granulosa-to-oocyte, could impair follicle development and oocyte maturation. We are not aware of disease etiologies associated with altered GJIC in the ovary. However, cellular arrest before antral follicle development in Cx43-inhibited human granulosa cells (Winterhager and Kidder, 2015). Importantly, enhanced GJIC has been associated with the development of diabetic cardiomyopathy (Wang et al., 2017) and in the brain is associated with epileptic hypersynchronous neuronal activity (Xie et al., 2015). Upregulation of connexin mRNA expression and protein abundance is implicated in a model for Parkinson's disease (Xie et al., 2015) and also occurs following prion disease on-set (Lee et al., 2016) highlighting the role of GJIC imbalances during in disease states.

During ovulation, just prior to luteinization, there is a BMP4- and BMP7-mediated gradual loss in GJIC within the preovulatory antral follicle for oocyte maturation to occur (Okuma et al., 1996; Granot and Dekel, 2002; Sela-Abramovich et al., 2005; Borowczyk et al., 2006; Norris et al., 2008; Winterhager and Kidder, 2015). Therefore, BPS-induced enhanced GJIC occurring during the latest stages of preovulatory follicle growth, especially if GJIC is also altered in granulosa cells, could alter transfer of signaling molecules, like BMPs, preventing final oocyte maturation and progression into metaphase II. This may ultimately result in lower oocyte fertilization rates and reduced fertility. BPS also retained the ability to enhance GJIC during and after theca cell luteinization, a time when theca cells shift steroid synthesis from androgens to progesterone.

Estradiol and the xenoestrogen BPA have been previously reported to reduce GJIC via a down regulation in connexin mRNA expression in the ovarian cumulus-oocyte cell complex (Acuna-Hernandez et al., 2018; Zhang et al., 2019a; Zhang et al., 2019b). However, these

observations were made on the cumulus-oocyte cell complex in mice, an altricial litter-bearing small mammal with inherently different ovarian physiology than sheep, which is a precocial large mammal who typically bears one or two offspring. Additionally, these observations were made at doses exceeding 2 μM BPA, which exceeds not only BPA doses used in this study, but high-end human exposures (ranges for human exposure to BPA: 0.14 – 792 ng/ml in urine). BPS can also act as a xenoestrogen (Pelch et al., 2019), and the observed BPS-induced enhancement in GJIC could thus potentially be due to upregulation of connexin expression. However, connexin mRNA expression does not necessarily translate to a change in GJIC (Genetos et al., 2012), particularly in the context of EDC exposures (Zhang et al., 2019b), and therefore functional outcomes such as intercellular dye transfer should be assessed when studying changes in GJIC. Since enhanced GJIC occurs following exposure to BPS, the interplay between BPS, connexin expression, and the changing hormonal milieu throughout folliculogenesis and ovulation should be studied further.

To understand the mechanism by which BPS increases GJIC, we co-incubated theca cells with pharmacological inhibitors directed to specific signal-protein targets (Fig. 6). Our results demonstrate that BPS can act through multiple phosphorylation pathways. These pathways occur in parallel or upstream of those commonly implicated in GJIC regulation (Kurtenbach et al., 2014). PKA, PKC, and PLC pathways have redundant roles in the phosphorylation of connexins, or can indirectly regulate other pathways implicated in GJIC (Kanemitsu and Lau, 1993; Cesen-Cummings et al., 1998; Hossain et al., 1998; Ruch et al., 2001; Machala et al., 2003b; Upham et al., 2008). These redundant roles are likely why only a partial, albeit significant, attenuation of BPS-induced enhanced GJIC was achieved by any of the signal-protein inhibitors, both in sheep and human theca cells. To note, these phosphorylation checkpoints are implicated in other cellular processes such as invasion, differentiation, Ca^{2+} ion signaling and influx, and neuronal responsiveness, cancer metastasis, and the development of diabetes (Sotogaku et al., 2007; Morrison, 2012; Putney and Tomita, 2012; Sassone-Corsi, 2012; Tarafdar and Michie, 2014). Additionally, there are 21 known connexins expressed in humans (Sohl and Willecke, 2004a), at least 6 of which (Cx26, Cx32, Cx37, Cx40, Cx43, and Cx45) are present in the normal human ovary, and at least 11 (Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx40, Cx43, Cx46, and Cx50) have been reported in ovarian cancers (Gershon et al., 2008). Although the function of each connexin is not fully understood, expression of different connexins within the ovary can result in essential pathway redundancy needed to provide compensatory communication mechanisms upon specific pathway malfunction (Sinyuk et al., 2018). This makes the evaluation and identification of specific biochemical pathways altered in BPS-enhanced GJIC challenging.

All signal protein inhibitor experiments were conducted in pre-luteinized cells to avoid potential confounding factors that may be introduced during the luteinization protocol, such as active cellular differentiation and the presence of LH in the media. Since the BPS-induced enhanced GJIC was conserved across cell stages, we predict that this effect occurs through the same pathways regardless of the theca cells differentiation stage.

Bisphenols and perfluorinated chemicals, both common classes of EDCs, have recently been reported to reduce GJIC in the ovary both, functionally or through a reduction in connexin

expression (Acuna-Hernandez et al., 2018; Dominguez et al., 2019; Lopez-Arellano et al., 2019). To our knowledge, BPS is the first bisphenol congener and EDC reported to enhance GJIC. Importantly, gestational exposure to BPS in sheep reduces the population of progesterone-producing cells in the placenta (Gingrich et al., 2018). This was hypothesized to be due to an imbalance in cellular fusion at the commitment stage, which requires gap junction communication after cell apposition (Gingrich et al., 2018). Additional reproductive outcomes such as delayed onset of puberty (Shi et al., 2019), increased testosterone production (Shi et al., 2019), decreased ovary weight (Nevoral et al., 2018), and decreased number of antral and preantral follicles (Nevoral et al., 2018) are reported outcomes in female mice exposed to BPS *in vivo*. To our knowledge, neither GJIC or connexin expression was assessed or implicated in any of these BPS-related outcomes.

5. Concluding Remarks

This study provides a novel model to evaluate of the role of GJIC in theca cells. Using this model, we have demonstrated that BPS enhances GJIC in ovine ovarian theca cells. This effect is reproducible in primary human theca cells, highlighting the translational relevance of these findings. The inhibitor approach used demonstrates the promiscuity of BPS in altering multiple signaling pathways regulating GJIC in theca cells (Fig. 6). Importantly, since GJIC alterations can disrupt folliculogenesis, the role of BPS in mediating ovarian-mediated sub- or infertility should be evaluated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Bisphenol S, but not four other endocrine disrupting chemicals, alters GJIC in theca cells.
- Bisphenol S enhances GJIC in ovine and human theca cells.
- Bisphenol S-enhanced GJIC is partially mediated via the MAPK signaling pathway.

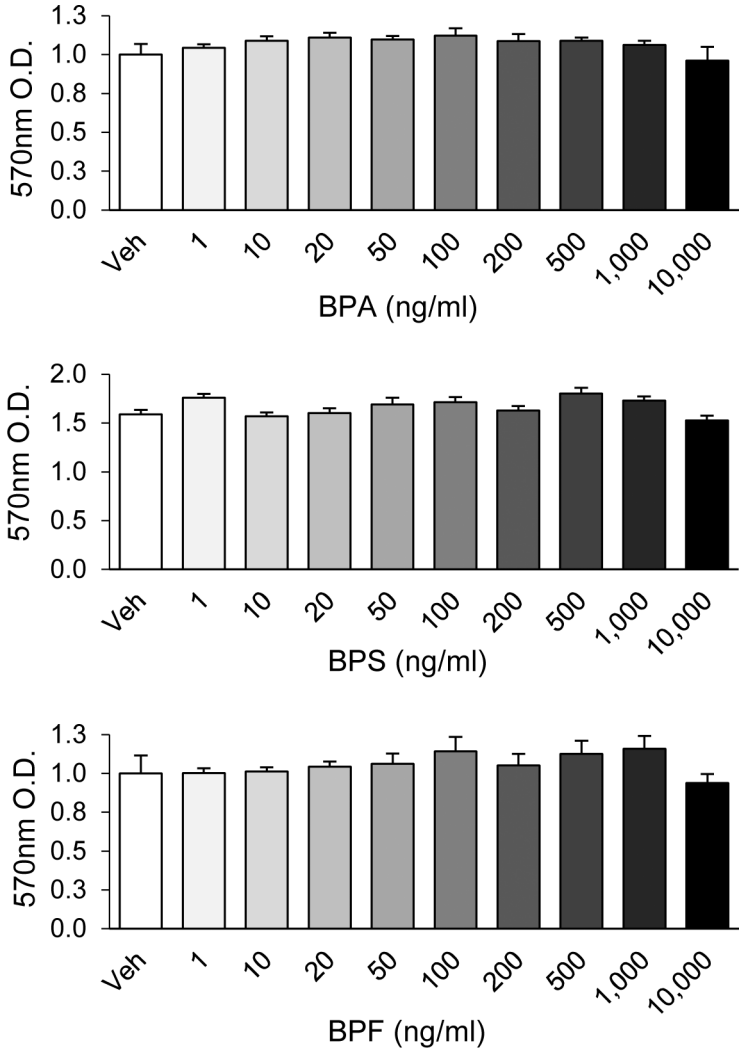

Figure 1.

Cytotoxicity assay for bisphenol A (BPA), bisphenol S (BPS), and bisphenol (BPF) in pre-luteinized ovine theca cells after 72 h of exposure (range: 1 to 10,000 ng/ml). Data are expressed as mean ± SEM. N = 3 primary theca cell cultures. No significant differences between exposure concentrations were detected. Veh: DMSO vehicle control. O.D.: optical density.

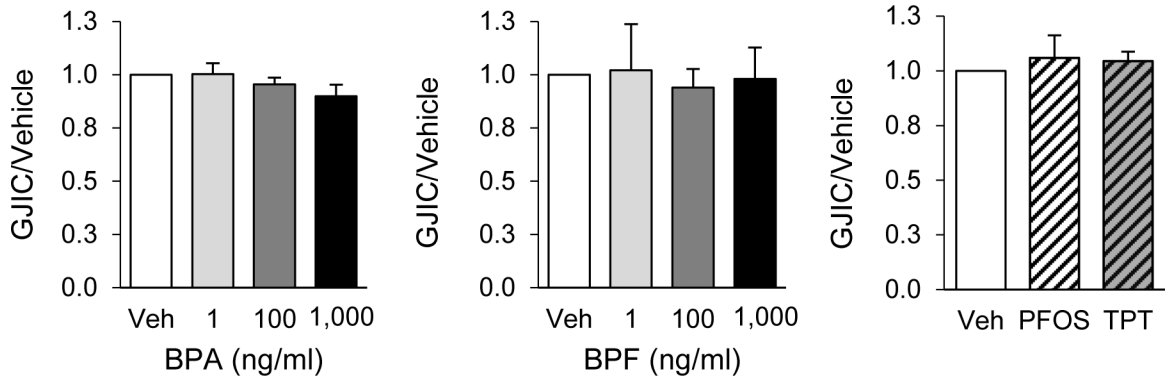

Figure 2.

Effects of exposure to common endocrine disrupting chemicals on gap junction intercellular communication (GJIC) in ovine primary theca cells. GJIC was assessed using the scrape loading dye transfer assay in pre-luteinized ovine theca cells exposed to bisphenol A (BPA), bisphenol F (BPF), perfluorooctanesulfonic acid (PFOS; 50 ng/ml), or triphenyltin chloride (TPT; 10 ng/ml). Data are represented as mean ± SEM. The effects were normalized to the vehicle group GJIC. At least 3 different theca cell cultures (each representing a biological replicate) were used for each chemical and dose. Veh: DMSO vehicle control.

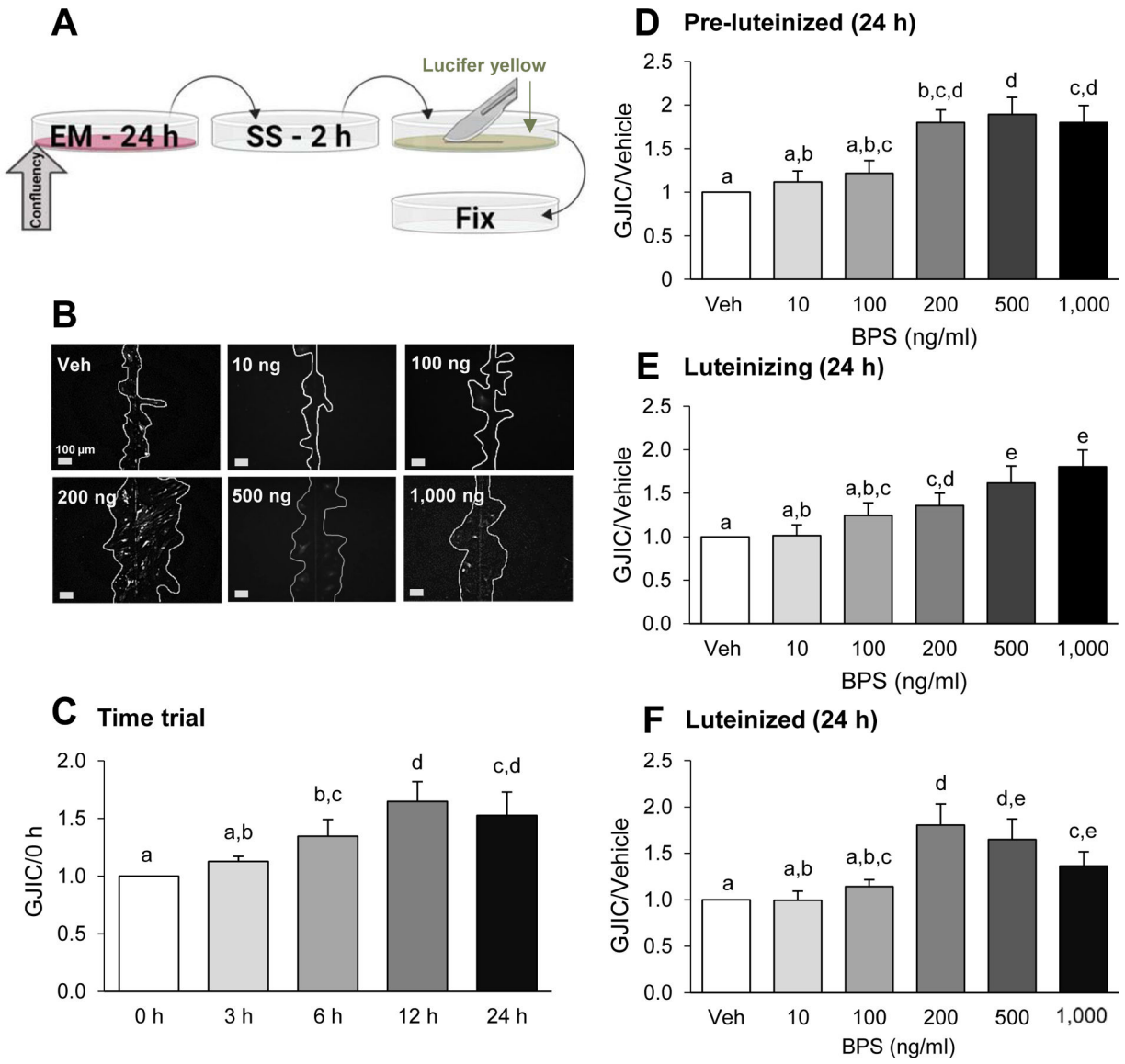

Figure 3.

Dose- and time-dependent effects of bisphenol S (BPS) exposure on gap junction intercellular communication (GJIC) in ovine primary theca cells. Scheme of exposure **(A)**: At ~80% confluency, cells are exposed to exposure medium (EM) containing BPS for 24 h, followed by a 2 h serum starvation (SS) period. Cells were then subjected to the scrape loading dye/transfer assay, using lucifer yellow dye (*arrow*) and a scalpel blade, before fixation (see text for additional details). Representative images from scrape loading dye transfer assay **(B)** used to assess GJIC in pre-luteinized ovine theca cells. White lines were drawn to facilitate visualization of the lucifer yellow intercellular transfer. BPS effect on GJIC (mean ± SEM) in a time-course in pre-luteinized theca cells **(C)** and in a dose-response in pre-luteinized **(D)**, luteinizing **(E)**, and luteinized **(F)** theca cells. The effect of BPS was normalized with the dye transfer in the vehicle control. At least 3 different theca cell cultures (each representing a biological replicate) were used for each time point, BPS

dose and cellular stage. Different letters denote statistical differences among treatment groups at $P < 0.05$. Veh: DMSO vehicle control.

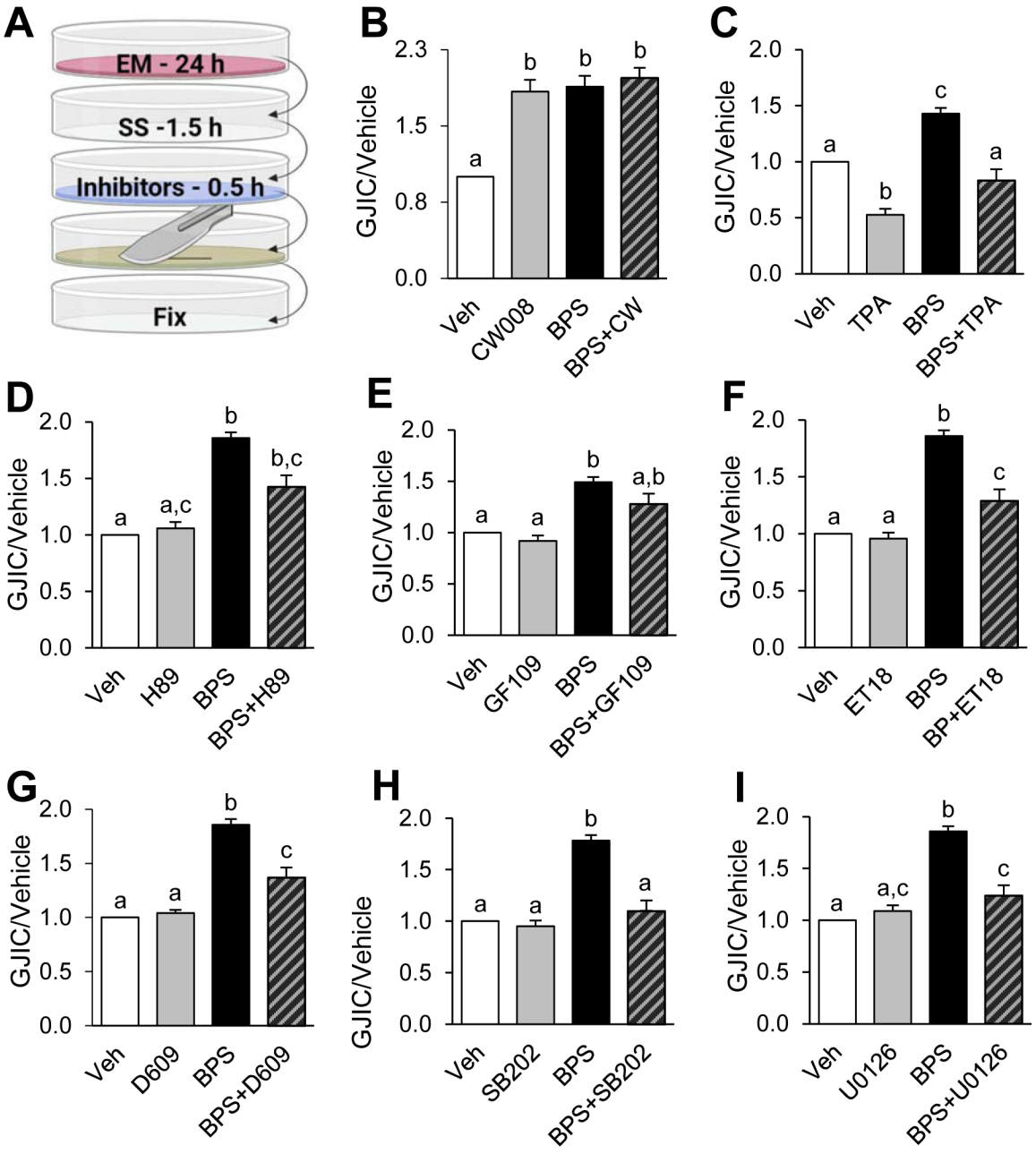

Figure 4.

Pathway identification of bisphenol S (BPS)-induced enhanced GJIC in pre-luteinized ovine primary theca cells using chemical modulators. Scheme of exposure to BPS and GJIC inhibitors **(A)**: At ~90% confluency, cells are exposed to exposure medium (EM) containing BPS followed by a 2 h serum starvation (SS) period, where inhibitors were added in the last 15 – 30 min. Cells were then subjected to the scrape loading dye transfer assay, which was run using lucifer yellow (*green plate*) and a scalpel blade, prior to fixation (see text for additional details). GJIC (mean ± SEM) in pre-luteinized ovine theca cells exposed to BPS with and without: positive control CW008 (PKA activator) **(B)**, negative control phorbol 12-myristate 13-acetate (TPA) **(C)**, or GJIC pathway inhibitors H89 (PKA inhibitor) **(D)**,

GF109203X (panPKC inhibitor) **(E)**, ET-18-OCH_3 (PI-PLC inhibitor) **(F)**, D609 (PC-PLC inhibitor) **(G)**, SB202190 (SB202, p38 MAPK inhibitor) **(H)**, or U0126 (MEK1/2 inhibitor) **(I)**. See Table 1 for inhibitor exposure times. GJIC is expressed relative to the vehicle group. At least 3 different theca cell cultures (each representing a biological replicate) were used for each inhibitor experiment. Different letters denote statistical differences among treatment groups at $P < 0.05$. Veh: DMSO vehicle control.

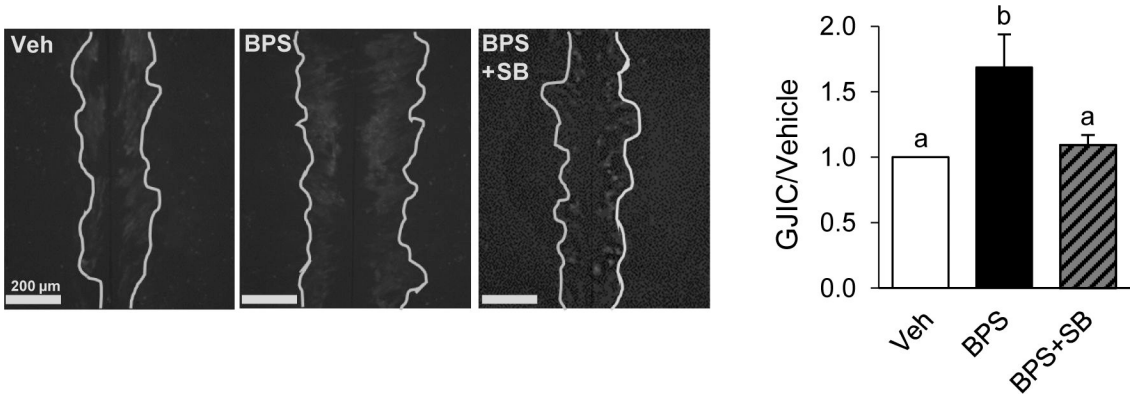

Figure 5.

Effect of bisphenol S (BPS) exposure on GJIC in human theca cells. Representative images from scrape loading dye transfer assay used to assess GJIC in pre-luteinized human theca cells. White lines were drawn to facilitate visualization of the lucifer yellow intercellular transfer. GJIC was assessed following exposure to vehicle control (Veh, 0.1% DMSO), BPS, or BPS in combination with MAPK inhibitor SB2020190 (SB, 1 μM). Data are represented as mean ± SEM and were normalized to dye transfer in the vehicle control. Different letters denote statistical differences among treatment groups at $P < 0.05$. Three different primary human theca cell cultures were used.

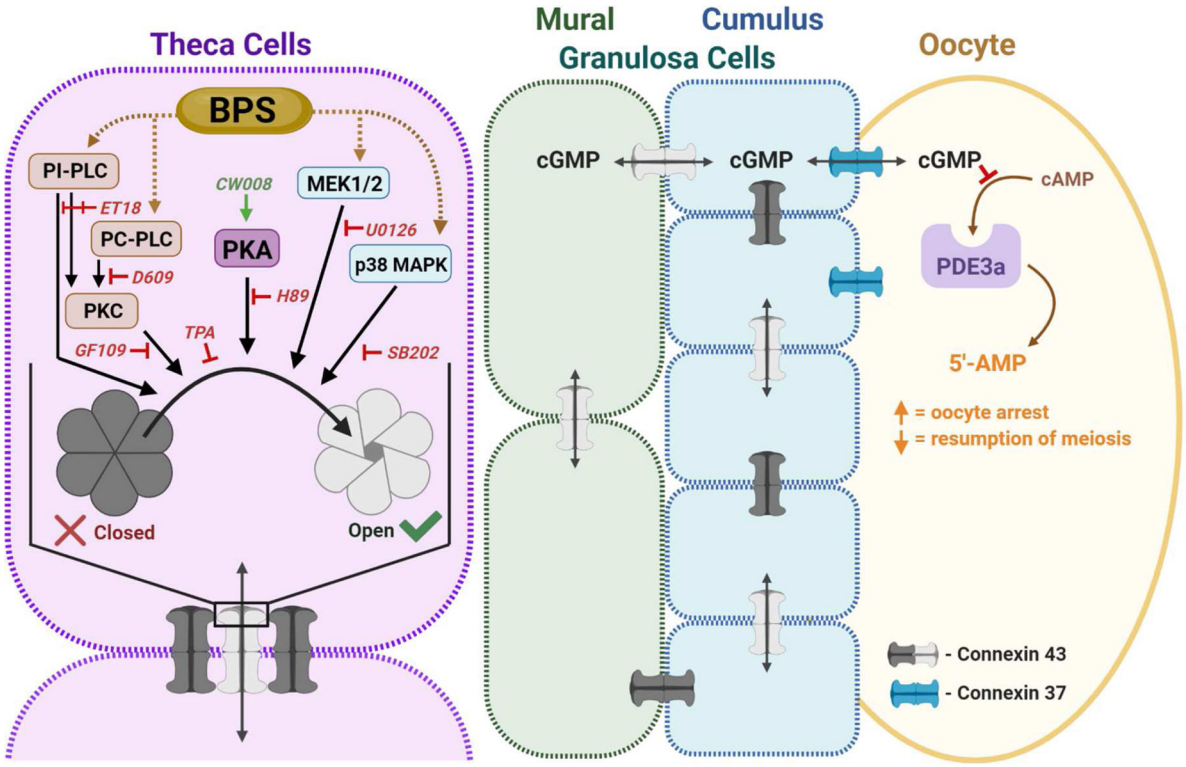

Figure 6.

Working model for BPS' modulation of theca cell GJIC. The antral follicle is formed by theca cells (*purple*), which provide physical and steroidogenic support to the underlying mural granulosa cells (*green*), cumulus granulosa cells (*blue*), and the oocyte (*yellow*). Connexin 37 and 43 form gap junction channels that connect inter-thecal, inter-granulosal and granulosa- to-oocyte cell communication, while theca-to-granulosa cell communication and hormone transport primarily occurs through paracrine signaling. Alterations in theca cell GJIC could affect the structural integrity and steroidogenic function of the follicle. GJIC in the ovary is temporospatially regulated, in part, by phosphodiesterase 3a (PDE3a), and needed for cellular arrest and meiosis resumption in the oocyte. Connexin phosphorylation signals opening, function, and recycling of gap junction channels. Pathways involved in GJIC regulation include protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) signaling. In theca cells, BPS can modulate GJIC through phosphatidylinositol-specific phospholipase C (PI-PLC) independent of PKC activation, as well as through MAPK/ERK kinase (MEK1/2). Like inter-thecal, inter-granulosal and granulosa-to-oocyte cell communication occurs through gap junctions, so we anticipate BPS could affect normal PDE3a-regulation of oocyte arrest and resumption of meiosis through dysregulated GJIC.ET18: ET-18-OCH_3, GF109: GF109203X, PC-PLC: phosphatidyl choline-specific phospholipase C, SB202: SB202190, and TPA: phorbol 12-myrisate 13-acetate.

Table 1.

Chemicals

Chemical *(target pathway)*	**Catalog #**	**Purity**	**Source**	**Concentration**	**Exposure**
Bisphenol A (BPA)	239658	99%	Sigma	1 – 10,000 ng/ml	24 h
Bisphenol S (BPS)	146915000	99.7%	Acros	1 – 10,000 ng/ml	24 h
Bisphenol F (BPF)	B47006	> 98%	Sigma	1 – 10,000 ng/ml	24 h
Perfluorooctanesulfonic acid (PFOS)	77282	98%	Sigma	50 ng/ml	24 h
Triphenyltin chloride (TPT)	45492	NR	Sigma	10 ng/ml	24 h
GF109203X (*panPKC inhibitor*)	NC9686383	98%	Enzo	1 μM	30 min
SB202190 (*p38 MAPK inhibitor*)	S7067	> 98%	Sigma	1 μM	30 min
D609 (*PC-PLC inhibitor*)	1437	> 98%	Tocris	50 μM	30 min
ET-18-OCH_3 (*PI-PLC inhibitor*)	3022	NR	Tocris	30 μM	15 min
H89 (*PKA inhibitor*)	B1427	> 98%	Sigma	40 μM	30 min
U0126 (*MEK1/2 inhibitor*)	1144	99%	Tocris	70 μM	30 min
CW008 (*PKA activator*)	5495	> 98%	Tocris	0.25 μM	24 h
Phorbol 12-myristate 13-acetate (TPA)	1201	> 99%	Tocris	5 nM	15 min
Lucifer yellow	PK-CA707–80015	> 99%	Promocell	0.5 mg/ml	5 min
Rhodamine-dextran	R8881	NR	Sigma	0.5 mg/ml	5 min

NR: Purity not reported.