GPER CONTROL AND FUNCTIONS

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GPER CONTROL AND FUNCTIONS

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EDITORIAL
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Editorial: GPER: Control and Functions

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Editorial on the Research Topic

GPER: Control and Functions

Since the pioneering work of Elwood V. Jensen (1920–2012), which led to the discovery of estrogen-binding "substances" shortly afterwards called estrophilin, the concept of estrogen receptor (ER) has evolved considerably (1–3). Initial reports localized ERs in the nuclear compartment of cells of reproductive tissues after a translocation process from the cytoplasmic membrane to promote transcription (4, 5). Until the cloning of ERβ in 1996 in rat prostate and ovary (6), only one receptor, named ERα, was known to bind the endogenous female hormone estradiol. In the following decades, at least three additional estrogen receptors were identified and cloned, i.e., GPER (7, 8), ERα46 (9), and ERα36 (10). ERα46 and 36 result from an alternative RNA splicing process of the gene *ESR1* encoding ERα (66 kDa), whereas GPER has its own transcript. The fact that estrogen receptors were discovered in the cytosol and cytoplasmic membrane of many different cell types, confirmed not only their ubiquitous character but also trafficking mechanisms in charge of the control of transcription. In the light of these observations, estrogen-mediated cellular signaling quickly became much more complex than initially claimed. In connection with these findings, two principal signaling processes were established, one initiated in the nucleus and the other at the cytoplasmic membrane.

Among estrogen receptors, GPER appears as the most atypical as it belongs to the family of class A (rhodopsin) G protein-coupled receptors (GPCRs) (11). Found in the cytoplasmic membrane, it can translocate to the membrane of the endoplasmic reticulum to exert specific functions (12) or to the trans-Golgi network for down-regulation (13). Based on what we know about the structure and functions of the classical estrogen receptor ERα, this discovery was extremely surprising and stimulated conflicting debates about the role of GPER, i.e., whether it directly binds estradiol or functions as a protein partner of ERα, similar to coactivators. While the latter scenario is not definitively excluded, depending on the context, a network of observations supporting the direct interaction of estradiol with GPER prompted its renaming from GPR30 (Luo and Liu). Since GPER binds the female hormone estradiol, one "basic" question is: does GPER play a role in sexual dimorphism? The answer is far from definitive, with sex differences in GPER distribution between males and females observed in some studies but not others (14). GPER-mediated sexual dimorphism may lie in providing differences between males and females in the social and behavioral network, as explained by Dovey and Vasudevan. In specific regions of the central nervous system (hypothalamus and amygdala), sex differences in the distribution of GPER impact synaptic plasticity and as such, the perception of anxiety, social and object recognition, and spatial memory (Kumar and Foster). In this

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regard, changes in the interaction of females with their environment during the estrous cycle could be explained, at least in part, by GPER expression fluctuations in the central nervous system during this same period, as explained by Llorente et al. Functional crosstalk with classical estrogen receptors (principally ERα and ER36) and tyrosine kinase receptors (principally EGFR) has also been established (15). As such, it is not surprising that GPER interferes with kinase cascades and calcium flux, with consequences in the cardiovascular system, as explained by Tran, as well as on cell growth and neuronal transmission (Kumar and Foster). In this regard, it should be stressed that the submembrane part of GPER encompasses four Ca^{2+}-calmodulin-binding sites, an observation that contributes to making this protein atypical (16). Such mechanisms could also play a role in glucose metabolism and obesity, opening new and exciting clinical opportunities.

As observed with the classical estrogen receptor ERα, endocrine-disrupting chemicals such as bisphenols, dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and phytoestrogens (e.g., genistein) promote cell proliferation and migration through GPER, as reviewed by Périan and Vanacker. Such observations impose the development of a low-to-middle throughput method to detect endocrine disrupting agents acting through GPER. Such method is now available (Périan et al.). In this context, an impact of soy isoflavones on promoting glial cell migration through GPER has been evidenced (Ariyani et al.). Strikingly, tamoxifen, which is widely used to fight estrogen-dependent breast cancer by directly interfering with the estradiol-binding site of ERα, up-regulates GPER and enhances cell proliferation, an observation that could explain, at least in part, tamoxifen resistance, as highlighted by Molina et al.

Hence, GPER appears not only as a key pleiotropic actor of mammalian hormone homeostasis but also as a promising target for the modulation of related physiological and pathological actions. However, the lack of crystal structure for GPER remains an obstacle to the development of modulators. Computational (virtual) approaches consisting of multiple protein sequence alignment combined with molecular docking of compound libraries have been proposed to identify new potential modulators or model explaining the mode of binding of active molecules (Grande et al.).

In this Research Topic celebrating 25 years since the discovery of GPER, many aspects of the functional role of GPER will be discussed.

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All authors have contributed to the article and have approved the submitted version.

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MINI REVIEW
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Does GPER Really Function as a G Protein-Coupled Estrogen Receptor *in vivo*?

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Estrogen can elicit pleiotropic cellular responses via a diversity of estrogen receptors (ERs)—mediated genomic and rapid non-genomic mechanisms. Unlike the genomic responses, where the classical nuclear ERα and ERβ act as transcriptional factors following estrogen binding to regulate gene transcription in estrogen target tissues, the non-genomic cellular responses to estrogen are believed to start at the plasma membrane, leading to rapid activation of second messengers-triggered cytoplasmic signal transduction cascades. The recently acknowledged ER, GPR30 or GPER, was discovered in human breast cancer cells two decades ago and subsequently in many other cells. Since its discovery, it has been claimed that estrogen, ER antagonist fulvestrant, as well as some estrogenic compounds can directly bind to GPER, and therefore initiate the non-genomic cellular responses. Various recently developed genetic tools as well as chemical ligands greatly facilitated research aimed at determining the physiological roles of GPER in different tissues. However, there is still lack of evidence that GPER plays a significant role in mediating endogenous estrogen action *in vivo*. This review summarizes current knowledge about GPER, including its tissue expression and cellular localization, with emphasis on the research findings elucidating its role in health and disease. Understanding the role of GPER in estrogen signaling will provide opportunities for the development of new therapeutic strategies to strengthen the benefits of estrogen while limiting the potential side effects.

Keywords: GPR30, GPER, estrogen receptor, estrogen, physiological role

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INTRODUCTION

Estrogen, more specifically, 17β-estradiol (E2), is a female sex hormone, which is essential for not only the development of the female reproductive organs but also the secondary sex characteristics (1). In addition, this hormone plays a critical role in the development and function of the male reproductive tract (2). Moreover, E2 plays important physiological roles in almost every part of the body, including the nervous system (3), immune system (4, 5), skeletal tissue (6, 7), musculature (8–11), as well as the endocrine system (12, 13). E2 exerts the comprehensive physiological effects by interacting with estrogen receptors (ERs) and subsequently, activating various signaling cascades that extend from seconds to hours (14, 15). In this review, we provide a brief overview of estrogen signaling and describe the characteristics of its receptors, emphasizing on GPR30, presumably a G protein-coupled ER (GPER). We focus on discussing studies aimed at elucidating the potential

physiological and pathological roles of GPER in regards to its estrogen binding properties and in mediating the actions of E2 *in vivo*. In addition, this review also summarizes recent research that supports E2-independent effects of GPER in various tissues.

ESTROGEN RECEPTORS

Steroid hormones are synthesized in the ovaries (E2, progesterone), testes (androgens, testosterone), and adrenal glands (cortisol, androgens). E2 is a critical steroid hormone that was originally believed (in the 1960's) to exert its physiological effects through a nuclear ER, later termed as ERα, which was identified in the rat uterus (16–18). About three decades later, the first ERα knockout mouse model was generated (19). The second ER, ERβ, was identified in the rat prostate in 1996 (20). ERs are ligand-regulated nuclear transcriptional factors that are believed to mediate a wide array of biological actions of E2.

Besides these classical nuclear ERs, which can initiate transcriptional events in the promoter regions of target genes, E2 is also reported to engage in rapid non-genomic signaling events (21, 22). Several studies have shown that E2 triggers a variety of intracellular signaling events, including mobilization of intracellular calcium in MCF-7 breast cancer cells (23), production of cyclic adenosine monophosphate (cAMP) in primary rat uterine cells (24), activation of mitogen-activated protein kinases p38 in MCF-7 cells and ROS17/2.8 rat bone cell line (25, 26), and activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) in human neuroblastoma cells (27). The underlying mechanisms for E2 exerting these rapid cellular actions appear to be complex that may involve ERs, the variants of ERα, and unknown E2 receptors (22, 28). Cellular signal transduction can occur as a result of E2 activating G proteins, which then lead to the modulation of downstream cellular pathways (29–31). Thus, a potential role for G protein-coupled receptors (GPCRs), which utilize E2 as ligand, has been proposed as an important route through which E2 exerts cellular functions.

GPER, AN ATYPICAL G PROTEIN-COUPLED RECEPTOR

Discovery of GPER

As early as the 1960–1970s, two independent studies reported the rapid cellular effects of E2 on cAMP synthesis (32) and calcium mobilization (33). These acute effects evoked by E2 are transmitted through enzymes and ion channels via the activation of membrane-associated ERs that may not involve transcription, which are thereby referred as non-genomic or extra-nuclear signaling pathways (34, 35). In 1997, a novel seven transmembrane-domain GPCR, named GPR30, was first identified and cloned (36), which showed high sequence homology to the interleukin 8 receptor and the angiotensin II receptor type 1 (37, 38). Therefore, it was initially speculated that the endogenous ligand activating GPR30 is a chemokine or peptide (37, 39). However, chemokines and/or peptides failed to evoke responses in GPR30 transfected cells (37, 39), suggesting that GPR30 might be an orphan GPCR without cognate endogenous ligands. In 2004, Maggiolini et al. performed gene expression analysis of SKBr3 cells lacking ERs. The results indicated that the proto-oncogene c-fos was upregulated in response to E2. Interestingly, the upregulation of c-fos by E2 was blocked when the endogenous GPR30 expression was silenced (40). In another study that used breast cancer cell lines, GPR30 expression was positively correlated with ERα expression, suggesting these two receptors might be regulated by the same regulatory mechanism or transcription factors (36). The orphan fate of GPR30 reached a turning point in 2005 (41). Two independent research groups provided data demonstrating that E2 directly binds to GPR30, which thus acts as a membrane-bound ER (30, 31). In 2007, the physiological role of GPR30 *in vivo* was first examined in rats (42). The results showed that administration of E2 induced GPR30 expression and attenuated hepatic injury via protein kinase A (PKA)-mediated mechanism in rats. Consistently, knockdown of GPR30 but not ERα attenuated the E2-dependent activation of PKA in hepatocytes isolated from rats. Therefore, GPR30 was officially named as GPER by the International Union of Basic and Clinical Pharmacology in 2007 (43). The characteristics of all three known ERs are summarized in **Table 1**.

With the discovery of GPR30 as a novel ER (GPER), growing evidence has emerged to describe the rapid action of E2 via GPER (15, 30, 36, 53). A search in PubMed in January 2020 with the keywords "GPR30 or GPER and estrogen" yielded 1,280 publications since 1997, with 88.6% (1, 54) published during the past decade. This area has attracted a surge of interest recently and represents one of the most active area in the field of E2 research.

GPER Expression in Tissue

The expression of GPER protein is not only restricted to E2-responsive tissues, as originally speculated. It is also present in many other tissues in humans (36–39, 55, 56) and rodents (57–62), such as brain, placenta, lung, liver, prostate, ovary, pancreatic islets, adipose tissue, vasculature, muscle, skeleton, as well as immune cells (63, 64). Interestingly, it appears that the expression pattern of GPER is age-, species-, gender-, or tissue-dependent. For example, the mRNA expression of GPER in skeletal muscle tends to be higher in premenopausal women compared to post-menopausal women (65). In mouse skeletal muscle, GPER mRNA abundance is almost 4-fold greater in females than that in males (57), with greater expression of GPER mRNA in female soleus than in extensor digitorum longus muscle (EDL) (66). GPER is also highly expressed in human bone tissues, and thus it may mediate the action of E2 on preserving bone density (67), suggesting a potential therapeutic strategy to prevent or alleviate menopausal osteoporosis by targeting GPER. Moreover, a high density of GPER was detected in the brain of hamster, including hypothalamus, thalamus, cerebellum, and amygdala, and the expression pattern of GPER behaved in a sexually dimorphic fashion in both young (post-natal 7 days) and adult (post-natal 60 days) animals (68). The gene expression of GPER was significantly higher in adult female hypothalamus than that of adult male, whereas the opposite expression pattern was observed in thalamus in young hamster. Similarly, the expression

TABLE 1 | Characteristics of ERs (44–52).

ER characteristics	ERα	ERβ	GPER
Category	Nuclear steroid hormone receptor superfamily		G protein-coupled receptor superfamily
Location	Nucleus	Nucleus	Membrane-associated
Size	595 aa	530 aa	375 aa
Numbers of isoforms	3	5	1
Chromosome region	6q25.1	14q23.2	7p22.3
Structure	DNA-binding domain, ligand-binding domain, N-terminal domain		7 transmembrane α-helical regions, 4 extracellular and 4 cytosolic segments
Distribution in tissues	Hypothalamus, hippocampus, testes, ovary, endometrium, uterus, prostate, kidney, liver, breast, epididymis, muscle, adipose tissue	Testes, ovary, prostate, vascular endothelium, bladder, colon, adrenal gland, pancreas, muscle, adipose tissue	Central and peripheral nervous system, uterus, ovary, mammary glands, testes, pancreas, kidney, liver, adrenal and pituitary glands, cardiovascular system, adipose tissue

pattern of GPER mRNA displayed contrary trend in cerebellum and amygdala areas in young hamster between male and female (68). However, it is presently unclear whether GPER shows a similar expression pattern in humans. Interestingly, GPER expression is developmentally regulated. In the mammary gland, GPER abundance is lower in the elongating ducts during puberty and then increases through periods of sexual maturity (15). In the cartilage of the human growth plate, GPER expression decreases as puberty progresses in both genders (69). Studies have shown that GPER expression level in mammary ductal epithelia is dependent on estrous cycle (15), and consistently, the highest GPER mRNA expression level was found on day 3 of estrous cycle and then declined to the lowest level on day 12 in equine endometrium (70). Results from another study examining GPER expression in hamster ovarian cells during estrous cycle exhibited similar pattern that GPER mRNA and protein abundance reached the peak levels on day 3 of estrous cycle and decreased on day 4. These findings are very important, as they provide a basis for investigating the physiological or pathological roles of GPER including cancer development, immune regulation, and reproductive, cardiovascular, as well as metabolic functions (64, 71).

GPER Localization in Cells

GPER is a seven transmembrane GPCR and therefore it is presumed to be located on the plasma membrane (72) as are most GPCRs (30, 73). Indeed, it has been shown that GPER induces signaling via activation of Gαs or Gαi (15, 30), strongly suggesting that this receptor is associated with the plasma membrane. Interestingly however, several studies provide evidence showing that a larger fraction of total cellular GPER is localized in intracellular compartments. Revankar et al. used fluorescent E2 derivatives (E2-Alexas) to visualize the extra- and intracellular binding properties of GPER in COS-7 (monkey kidney fibroblast) cells. Surprisingly, the confocal images revealed that E2-Alexas failed to label the plasma membrane but predominantly bound to endoplasmic reticulum (31). In addition, E2-Alexas- or antibody-stained GPER is also co-localized in the Golgi apparatus and nuclear membrane in GPER expressing cancer cell lines (31). Similarly, the predominant intracellular staining pattern of GPER was also observed in human umbilical vein endothelial cells (74), vascular smooth muscle cells (74), and pancreatic islet cells (75, 76). Intriguingly, fluorescent microscopy and western blotting evidenced that GPER was present in mitochondria in undifferentiated C2C12 myoblasts, but was found in cytoplasm in differentiated C2C12 myotubes that modulates E2 actions (77). However, some other studies reported that GPER is mainly localized to the plasma membrane of uterine epithelia (78), myometrium (79), renal epithelia (80–82), and hippocampal neurons (73, 83), though an intracellular expression of GPER has also been reported in neurons (60). Therefore, the cellular distribution of GPER apparently varies depending on species, tissue, and cell types. Interestingly, several studies indicated that GPER is activated intracellularly, which then diffuses across cell membranes and initiates cellular signaling (31, 84, 85). These results indicate that GPER is an atypical GPCR, and its intracellular location may dynamically change in response to specific environmental cues and also could be tissue-dependent. Thus, a role for GPER as a plasma membrane-based ER is still controversial, and the exact mechanism by which GPER acts in response to E2 remains elusive.

GPER Ligands

As discussed above, studies utilizing E2-Alexa or a fluorescent derivative of E2 demonstrated intracellular localizations of GPER (31, 86). Measurement of steroid binding to membrane-associated receptors is challenging because of the lipophilic nature of steroids and relatively low levels of membrane proteins that cause high background binding. Nevertheless, results from ligand binding assays demonstrated that GPER is a specific receptor for E2 with estimated binding affinities of 3–6 nM (30, 31), which is however much lower as compared with its binding affinities for classical ERs that are in the range of 0.1–1.0 nM (87).

In addition to E2, compounds with estrogenic activity can be found in a large variety of natural sources such as plants (e.g., soy) and fungi (88). With the rapid development of synthetic estrogenic substances, it is not surprising that a large number of estrogenic compounds have been shown to interact with GPER. Tamoxifen, for instance, is a well-known selective ER modulator and found to act as a GPER agonist (31, 89). Interestingly,

stimulation with 4-hydroxytamoxifen, the active metabolite of tamoxifen, failed to activate PI3K in ERα positive cells but did activate PI3K in GPER expressing cells (86). Another widely used selective ERα/β antagonist, ICI182,780 (ICI), was also shown to bind to GPER (30) and activate this receptor (90). In GPER-transfected MDA-MB-231 breast cancer cells (ERα-deficient), ICI can activate ERK1/2 (90), confirming its effect as a GPER agonist. Consistently, another recent study demonstrated that raloxifene, a selective ER modulator, also elicited cellular response via GPER in ERα-deficient endometrial carcinoma Hec50 cells (91). In addition, numerous synthetic estrogenic compounds have been shown to bind and/or activate GPER, including zearalonone, non-phenol, kepone, p, p′-DDT, o, p′-DDE, 2, 2′, 5′, -PCB-4-OH (92), and bisphenol A (93, 94). Finally, several lines of research have demonstrated the agonistic actions of some plant-derived polyphenolic compounds toward GPER, including genistein (40, 92, 95, 96), quercetin (40), equol (97), resveratrol (98), oleuropein, hydroxytyrosol (99), and daidzein (100). However, it should be noted that the results from these studies were obtained exclusively from *in-vitro*-based assays using cancer cells or clonal cells with artificially over-expressed GPER, and whether and how they exert estrogenic effects as well as the target tissue *in vivo* are still unknown. Hence advancing the field of GPER research using these estrogenic compounds is fraught with complications. Fortunately, a highly selective GPER agonist, G-1, was synthesized in 2006 (101) and further studies of GPER action are greatly facilitated by this compound.

G-1 showed high binding affinity for GPER (Kd = 10 nM) without binding to ERα/β at concentrations as high as 10 μM (102). Three years later, a subsequent study identified a highly selective GPER antagonist, G15, with a similar structure as G-1 but lacking the ethanone moiety (103), which displayed a minimal binding to ERα/β (Kd >10 μM) (104). Another GPER specific antagonist G36 was generated to restore the steric bulk of G-1 and the ER counter selectivity (102). These selective modulators of GPER have been used in over 200 studies to evaluate GPER actions in a variety of cellular and animal models. More recently, a first peptide GPER ligand corresponding to part of the hinge region/AF2 domain of the human ERα was identified, which acts as an inverse agonist of GPER to suppress mitogenic signaling and inhibit breast cancer cell growth (105, 106). In addition, two novel GPER specific agonists, GPER-L1 and GPER-L2, were synthesized in 2012 with binding affinities of ∼100 nM (107). The same year, a synthetic molecule, named as MIBE, was reported to bind and block both ERα and GPER activity in breast cancer cells (108). Recently, a small molecule with high binding selectivity to ERα/β over GPER, termed AB-1, was generated, which may further aid distinguishing the roles of ERα/β and GPER in E2 signaling (109). Intriguingly, the widely used ERα specific agonist, propyl pyrazole triol (PPT), has been reported to act as GPER agonist at concentrations as low as 10–100 nM. On the contrary, the ERβ specific agonist, diarylpropionitrile (DPN), had no effect on GPER at concentrations up to 10 μM (91). Therefore, the results from studies regarding the use of these compounds aimed at modulating ER actions should be interpreted carefully with respect to the concentrations of these compounds.

GPER IN HEALTH AND DISEASE

With the increasing spectrum of research on GPER *in vitro*, many critical questions remain: what is the physiological role of GPER? Does GPER really serve as a GPER and act independently or collaborate with the classical ERs? Will drugs targeting GPER be more effective than those targeting ERα/β for treatment of disease? Although GPER was officially named by the International Union of Basic and Clinical Pharmacology in 2007 (43), deciphering the physiology role(s) of GPER as a novel ER in health and disease remains challenging, which is due to the complex nature of E2-initiated cellular events that involve multiple receptors, various cellular signaling cascades, direct or indirect binding of the E2-ER complex to DNA, and regulation of gene expression. While these aspects are beyond the scope of this review, various mechanisms of E2 signaling are summarized in **Figure 1**. The readers can refer to other review papers on this topic [see (90, 110, 111) for more detailed information]. In the following section, recent studies regarding the physiological roles of GPER in different tissues and disease are discussed.

GPER in Reproductive System

Since GPER is believed to be an ER, its action in the reproductive system attracted considerable attention. Early studies investigating the action of GPER were performed in various cancer cells derived primarily from reproductive tissues, including breast (30, 36, 36), ovary (112–114), endometrium (89, 115, 116), testis (117, 118), prostate (119), as well as thyroid tissues (95, 120). Since GPER was first identified and cloned in breast cancer cells, much early research has focused on exploring the role of GPER in various types of cancer, which has been reviewed thoroughly elsewhere (121–123) and will be briefly discussed in this space. For instance, Upon stimulation with E2, estrogenic compounds (e.g., genistein, hydroxytamoxifen) or selective GPER agonist G-1, GPER enhanced cancer cell proliferation in the classical-ER-negative breast cancer cells (30) and in the thyroid (95), endometrial (89), and ovarian cancer cells (113), suggesting that GPER may contribute to E2-induced cancer growth. Research by De Francesco., el al. provides novel insight into the mechanism by which activation of GPER triggers cancer cell proliferation (124). Specifically, their research demonstrated that E2 and GPER specific agonist G-1 upregulated HIF1α-dependent vascular endothelial growth factor expression in ER-negative breast cancer cells and cancer-associated fibroblasts, which led to angiogenesis and breast cancer progression as shown in a mouse xenograft model of breast cancer. Interestingly, It was shown that GPER specific agonist G-1 suppressed SKOV-3 and OVCAR-3 cell proliferation and activated caspase-dependent cell apoptosis, indicating that GPER may act as a tumor suppresser for ovarian cancer (125). In line with this finding, several reports also discovered such contrary effects of GPER in reproductive cell lines. Activation of GPER by E2 or G-1 suppressed human bladder urothelial cell

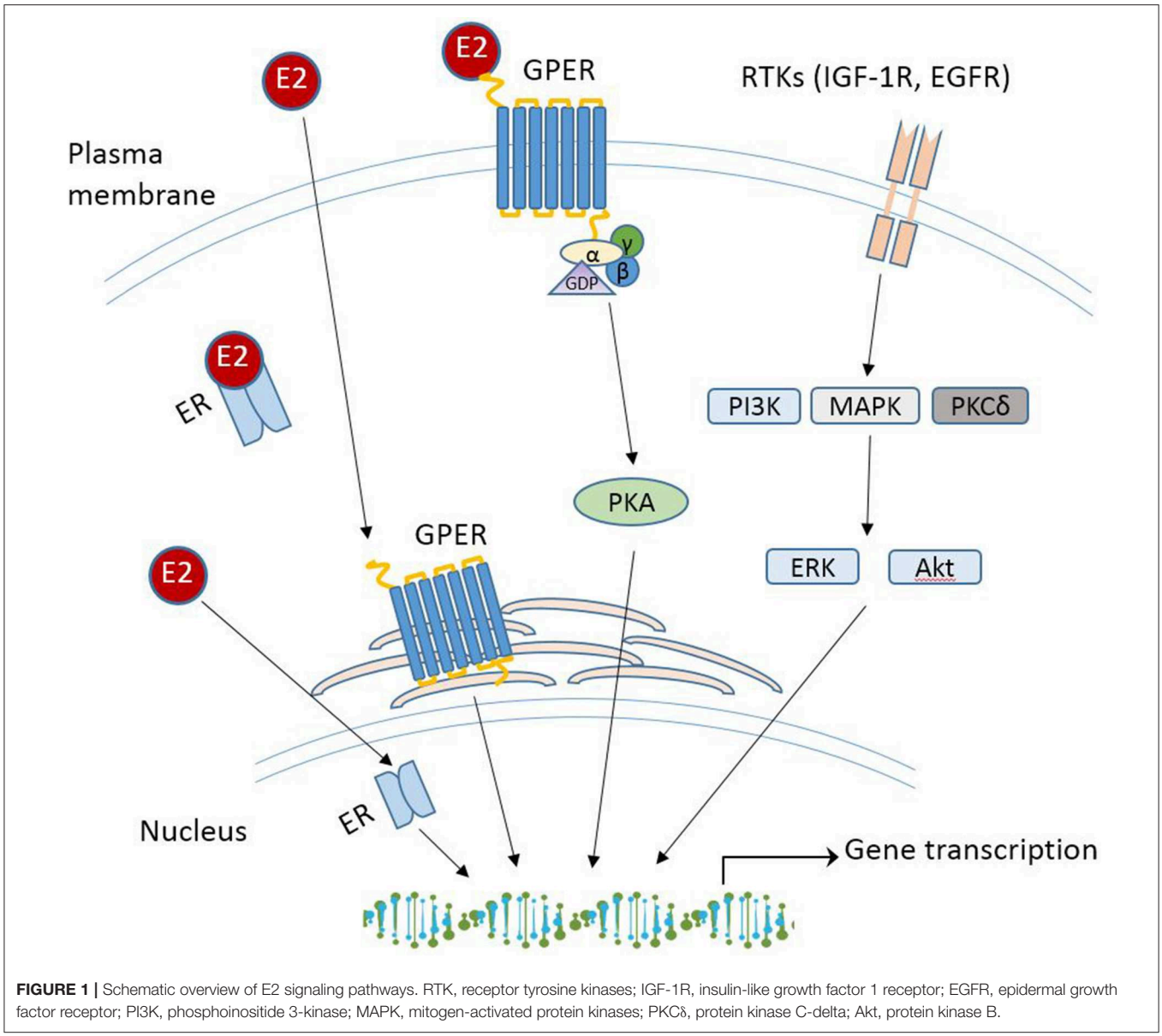

FIGURE 1 | Schematic overview of E2 signaling pathways. RTK, receptor tyrosine kinases; IGF-1R, insulin-like growth factor 1 receptor; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinases; PKCδ, protein kinase C-delta; Akt, protein kinase B.

proliferation via down-regulating the activation of protein-1 (AP-1) (126), which is one of the major regulators of cell proliferation (127). Similarly, in the classical ER negative human breast cancer cell lines SkBr3 and MDA-MB-231, activation of GPER by G-1 inhibited cell proliferation and induced G2 cell-cycle arrest *in vitro* and suppressed ER negative breast cancer growth *in vivo* (128). In ovarian-like granulosa tumor cells, E2 activates GPER-protein kinase C signaling, which then phosphorylates forkhead transcription factor 2 to promote cell apoptosis (129). In human, activation of GPER by G-1 enhanced contractile responses to oxytocin in the myometrium during labor (79). In addition, GPER together with ERα regulates the proliferative and/or apoptotic pathways involved in spermatogenesis via the EGFR/ERK/c-jun pathway in male rodent reproductive development (130, 131). However, the physiological relevance of these *in vitro* findings are unknown. Actually, in contrast to these *in vitro* findings, activation (101, 132) or deletion of GPER (57, 58) displayed no effect on the development of reproductive organs in mice, which is in clear contrast to the established phenotype of these animals lacking ERα or E2. This result suggests that GPER may be either not endogenously activated by E2 or not involved in mediating estrogenic actions of E2 in reproductive organs. Interestingly, studies from ovariectomized mice indicated that activation of GPER inhibited E2-induced uterine epithelial cell proliferation, which was associated with the reduced E2-stimulated ERα phosphorylation (78), Therefore, GPER inhibition of E2-stimulated cell proliferation may be mediated via suppressing the phosphorylation of ERα, which is important for various E2-stimulated transcriptional events (54), suggesting a possible "yin-yang" relationship between

these two receptor. These findings demonstrate the complex roles of GPER in reproductive system and further investigation is needed.

GPER in Cardiovascular System

Increasing evidence shows that GPER exerts cardioprotective effects. In mouse models, It was shown that global deletion of GPER increased blood pressures (57), atherosclerosis progression and systemic inflammation (74). GPER also plays a direct cardioprotective role, as mice with a cardiomyocyte-specific deletion of GPER displayed abnormal cardiac structure and impaired systolic and diastolic function (133). GPER may mediate a direct vasodilatory effect of E2 in vasculature. As a selective antagonist of the classic ERα/β, ICI exhibited agonistic action on GPER and promoted the dilation of coronary artery in porcine (134). Interestingly, acute infusion of GPER selective agonist G-1 decreased blood pressure in male rats, while long-term injection of G-1 decreased mean arterial pressure in the hypertensive ovariectomized female rats, suggesting that activation of GPER potentially protects E2-insufficient females from hypertension (135). In line with the results from using animal models, stimulation by G-1 dilated human internal mammary arteries and notably, the relaxant effects of G-1 were more potent than that of E2 in precontracted human aorta and carotid artery (136). The exact mechanism for blood pressure-lowering action of GPER is not clear. Studies from GPER knockout mice suggest that GPER can directly stimulate nitric oxide (NO) production from endothelial cells (ECs) and subsequent vessel dilatation (137, 138). Indeed, treatment with GPER selective antagonist G36 suppressed E2-induecd NO release in human ECs, whereas activation of GPER with G-1 promoted endothelial NO synthase phosphorylation (138), suggesting that GPER is at least partially responsible for NO-mediated vasodilatory action of E2. Another study demonstrated that activation of GPER inhibited endothelin-triggered vasoconstriction via reducing vascular smooth muscle cell Ca (2+) sensitivity (139). Further, GPER was shown to protect against angiotensin (Ang) II-induced hypertension through suppressing NADPH oxidase 4-dependent oxidative stress via activation of cAMP signaling pathway (140), suggesting that this receptor also exerts an antioxidant role. Therefore, GPER could potentially be a target for developing strategy to promote cardiovascular health.

GPER in Nervous System

Estrogen has many beneficial effects in the brain, which include improving cognitive performance (141), opposing the early occurring hippocampal damage (142), increasing neuronal connectivity (143), and preventing or slowing age-related cognitive decline (144). Although these protective effects of E2 are largely attributed to the classical ERα/β, increasing evidence demonstrates that GPER also plays potential role(s) in E2-mediated neurological functions. As stated before, GPER is expressed throughout the central and peripheral nervous system of male and female rodents and humans (49). Acute administration of E2 or GPER selective agonists STX or G-1, improved neuron survival rate by 40–45% compared to control in ovariectomized female rats (145). In contrast, G-1 promoted apoptosis of rat embryo cortical astrocytes exposed to oxygen and glucose deprivation, whereas the addition of the GPER antagonist G-15 suppressed this effect, suggesting a direct impact of GPER on the viability of cortical astrocytes (146). Interestingly, administration of G-1 counteracted iron- and ovariectomy-induced memory impairments in female rats (147). Therefore, GPER could be a novel target in treatment of neurodegenerative diseases, such as memory disorders, Alzheimer's disease and ischemic stroke. Although the results from limited studies using selective chemicals of GPER consistently demonstrate a neuronal effect of GPER, it remains to be determined whether GPER truly acts as an E2 receptor. As reported, infusion of G-1 or E2 promoted memory function in ovariectomized female mice, but G-1 activated the c-Jun N-terminal kinase while E2 stimulated ERK1/2. In addition, G15 failed to block the activation of ERK1/2 induced by E2, but infusion of G15 to the dorsal hippocampus impaired memory formation and object recognition (148). These data suggest that the benefits of hippocampal GPER on memory function is not mediated by E2. Thus, the role and precise sites in neurons responsible for GPER action need to be elucidated, which may be achieved by using tissue-specific knockout animal models.

GPER and Glucose Metabolism

While the classic ERs have been known to play a role in mediating E2 effects on glucose metabolism and metabolic diseases, the metabolic action of GPER remains to be determined. The generation of GPER knockout (GPRKO) mice facilitates our understanding of the physiology role of GPER. Martensson et al. showed for the first time that GPRKO female mice displayed hyperglycemia, impaired glucose tolerance, and reduced body weight and bone growth, whereas GPRKO male mice were metabolically normal (57), thus demonstrating a gender-dependent effects of GPER on glucose homeostasis and animal growth. The potential anti-diabetic effect of GPER was revealed from studying the ERα/β double knockout (DKO) mice treated with streptozotocin (STZ) (149), in which E2/ERα/β signaling was removed, thereby allowing to determine only GPER-mediated action of E2. The results indicated that ovariectomized ERα/β DKO mice were more susceptible to STZ-induced islet apoptosis and diabetes as compared with sham-operated ERα/β DKO mice, but the STZ-induced islet apoptosis and diabetes in ovariectomized ERα/β DKO mice were attenuated by E2 replacement therapy (149), suggesting that E2/GPER signaling is protective against STZ-induced insulin deficient diabetes. Indeed, the authors further demonstrated that female GPRKO mice were predisposed to insulin-deficient diabetes due to increased β-cell apoptosis. In accordance with this *in vivo* result, GPER agonist G-1 directly protected mouse and human islets against oxidative stress-induced apoptosis, and E2 still promoted pancreatic β-cell survival in ERα/β DKO mice exposed to STZ (150). Taken together, these results showed that in the absence of the classical ERα/β, E2 may signal through GPER to protect against STZ-induced islet apoptosis. Consistently, data from several other studies showed that deletion of GPER resulted in a reduced insulin secretion from pancreas, suggesting that GPER indeed

plays a role in maintaining metabolic functions via regulating insulin secretion in mice (149, 151, 152). Furthermore, the protective effect of E2 on pancreatic β-cells can be mimicked by GPER agonist, genistein (153). Interestingly, we recently found that deletion of GPER protected female mice from high-fat diet (HFD)-induced obesity and hyperglycemia (154). After 15 weeks of HFD feeding, their blood glucose levels gradually diverged with GPRKO displaying significantly lower fasting and non-fasting blood glucose levels as compared with those in WT while their insulin sensitivity was not different. The reason for these discrepancies are not clear. It should be noted that our study used GPER mice in 129 background in contrast to C57BL/6 GPER mice as used in other studies. Other factors, such as the genetic knockdown or knockout strategy, the breeding strategy, and the environment can have unexpected influence on the phenotypes as well. Of the note, certain maternal and/or experimental diets contain significant amount of phytoestrogens (i.e., soy protein or alfalfa meal) (155), which could modulate the estrogenic activity and therefore could profoundly alter the related outcome of a study given the well-documented various effects of dietary phytoestrogens in rodent models (156, 157).

GPER and Obesity

While the classical ERs have been well-investigated regarding their roles in mediating E2 effects on fat metabolism and metabolic diseases, little is known about metabolic action of GPER as well as the possible complex interactions among the three ERs in different cell types. E2 and STX, a synthesized non-steroidal compound acting as a GPER selective agonist (158), rapidly attenuated the baclofen response in hypothalamic arcuate POMC neurons in WT, ERαKO, ERβKO, and ERα/β DKO mice, and prevented excessive body weight gain in ovariectomized guinea pigs, suggesting a potential role of GPER in energy metabolism in females (159). Multiple studies have investigated the role of GPER in regulating body weight and fat deposits. The first such study reported an increase in body weight and visceral adiposity in both male and female GPRKO mice as compared with those in WT mice (151). In addition, Davis et al. reported similar observations that KO mice were heavier than the WT littermates fed a standard chow diet (STD), although this difference between female mice occurred 5 weeks later as compared to male mice (51). However, others found no significant effect of GPER on body weight of both female and male mice (52). Data from a recent study showed an increased body weights in both male and female GPRKO mice caused by increased fat mass with enlarged adipocytes when fed a phytoestrogen free low fat diet (51). However, Martensson et al. reported contrary results that female GPRKO mice exhibited slightly lower body weights as compared with WT, whereas no such differences were observed in male GPRKO mice (57). The reasons for these disparate results are not clear. However, these studies were not designed for investigating the roles of GPER in obesity development. As female mice in these studies were used at their young ages and fed a STD, they remain lean without apparent metabolic abnormalities, which therefore are not sufficient to reveal the role of GPER in obesity development that is typically caused by high calorie intake.

We recently performed relatively long-term study with detailed analyses of body weight and body composition of female GPRKO mice either maintained on a STD or exposed to a phytoestrogen-free HFD (154). There were no differences in their body weight, fat mass, and all other measured metabolic phenotypes between WT and GPRKO either male or female mice on a STD. However, after 23 weeks of HFD feeding, female GPRKO mice gained 61% of their starting body weight while WT female mice increased by 85% with no difference in energy intake between two groups. At 20 weeks, the fat mass of WT was 1.8-fold of that in GPRKO mice with only slightly higher lean body mass in GPRKO animals, suggesting that the difference in body weight between GPRKO and WT female mice was primarily due to their fat mass difference. Interestingly, no such differences in metabolic phenotypes were observed between WT and GPRKO male mice fed a HFD. In addition, the inguinal, gonadal, and perirenal fat pads from GPRKO mice weighted less than those in WT female mice, while the pancreas from GPRKO female mice was slightly heavier than WT mice. All the other measured organs weights are similar, suggesting the reduced fat mass in GPRKO female mice was not due to decreased body growth. Our H&E staining of fat sections revealed that GPRKO female mice had smaller adipocytes as compared to WT female mice fed a HFD. While the reasons for these disparities with respect to GPER modulation of body weight gain from past studies are not clear, which could be due to the different methods generating the transgenic animals as reviewed (71), and the variations of the diet compositions, duration, and environment, but overall they indicate that GPER might play a role in regulating lipid metabolism and controlling adiposity.

While how exactly GPER regulates lipid metabolism is still unclear, it was recently shown that that the effect of GPER on fat mass in HFD-fed female mice was not due to a secondary action by which its deletion altered circulating E2 levels or expression of ERα (154), which is believed to play a major role in mediating estrogenic effects on energy homeostasis (160). Both human and rodent white adipose tissue expresses ERα, ERβ, and GPER, suggesting that E2 signaling could occur through both ERs and GPER. Interestingly, it was reported that GPER and ERα inhibit each other's actions in several types of cells (78, 161, 162). In mice, GPER activation inhibits ERα-dependent uterine growth induced by E2 (78). These data suggest that there could also be a "yin-yang" relationship between GPER and ERα in adipose tissue that balances energy metabolism in response to E2. In that regard, activation of ERα by E2 inhibits adiposity, whereas activation of GPER might promote obesity, an intriguing concept that worth investigation.

EVIDENCE OF E2-INDEPENDENT EFFECTS OF GPER

While data from a large body of literature suggest that GPER appears to mediate E2-triggered several intracellular signaling pathways, this evidence was primarily obtained from *in vitro* studies in which cultured cells were often either overexpressed with GPER or absent of endogenous ERα/β and typically exposed

to well-above physiological doses of E2. As aforementioned, the estimated binding affinities of E2 to GPER (3–6 nM) (30, 31) are considerably higher as compared with its binding affinities for classical ERs (0.1–1 nM) (87). This raises an interesting question as to whether GPER plays a significant role in mediating various E2 effects *in vivo*, given that circulating E2 levels in young female rodents are only about 7.3–734.2 pM (163–165), depending on the stage of estrous cycle. Indeed, conflicting results regarding the GPER-mediated signaling events in response to E2 continue to emerge, which warrant further investigations as to whether GPER plays a physiological role as an GPER *in vivo*. Knockdown of GPER in MCF-7 cells expressing ERs and GPER had no impacts on E2-induced cAMP production (166). Others demonstrated that transient expression of GPER in MCF-7 cells resulted in a reduction of cell growth in the absence of E2 (167). Based on these results, GPER may not signal in response to the stimulation of E2 at physiologically relevant levels. Intriguingly, the existence of membrane ERs (mERs) (168–171), though with a limited amount at about 3–10% of the classical nuclear ERs (29, 166), further complicated the rapid non-genomic signaling events mediated by E2. Interestingly, G-1 was shown to induce the phosphorylation of ERK1/2 in GPER-negative HEK293 cells stably transfected with a novel membrane associated ERα, ERα-36 (172), a variant of human ERα-66 (168). Moreover, knockdown of ERα-36 in MDA-MB-231 and SKBr3 cells suppressed the phosphorylation of ERK1/2 and intracellular calcium mobilization stimulated by G-1, suggesting that G-1 also recognizes ERα-36, and therefore it may not be specific for GPER. The use of ICI, an ERα antagonist but GPER agonist, and GPER antagonist G-15, in the mouse hippocampal cell lines mHippoE-14 and mHippoE-18 demonstrated that acute E2 treatment protected hippocampal cells from glutamate-induced neurotoxicity and the protective action requires both mERα and GPER (173). In ovariectomized female mice, it was shown that infusion of E2 into the dorsal hippocampus activated ERα and ERβ, leading to ERK1/2 signaling and improved object recognition and spatial memory. However, infusion of G-1 but not E2 activated GPER, which triggered a different cell-signaling mechanism to facilitate hippocampal memory in female mice (148). These results suggest that GPER in the dorsal hippocampus might not act as an ER. In GPER overexpressed COS-7 and CHO cells, E2 only showed specific saturated binding to ERα, but not to GPER (132). Consistently, in primary endothelial cells from ERα/β DKO mice, E2 failed to specifically bind to GPER and activate cAMP, ERK1/2, or PI3K signaling as observed in clonal cancer cells (166).

The reason for these disparate results on the role of GPER in E2 signaling is unclear. Many of these studies were obtained using clonal cell-based experiments, in which cells were manipulated with overexpression of GPER, which may result in ectopic expression of GPER in the cells. Even using the cells that endogenously express GPER, cellular experiment results cannot recapitulate E2 functions in whole body. Taken together, it remains to be determined whether GPER functions as a specific E2 receptor that mediates endogenous E2 effects *in vivo*.

CONCLUDING REMARKS

GPER is an atypical GPCR and has been named as a new ER. While ERα/β have been well-investigated regarding their roles in mediating E2 effects in health and disease, the physiological and/or pathological roles of GPER remain to be determined. The pace of research into the functions of GPER has been accelerating over the past decade with the generation of GPER transgenic mice as well as its selective chemical ligands, which are powerful tools to investigate the physiological and/or pathological role(s) of GPER. Although the results from *in vitro* studies suggest that E2 could activate to trigger various intracellular signaling pathways, and data from animal studies do not exclude GPER as an ER in mediating estrogenic responses, convincing evidence that E2 acts through GPER to elicit significant physiological events *in viv*o is still lacking (58, 132, 174). While the major physiological function of GPER is likely not for promoting reproductive tissue development, increasing evidence suggest that GPER plays a role in body weight regulation and metabolism. However, the clear metabolic effects of GPER and the role of E2 plays in this context need further investigation. In addition, whether GPER counteracts ERα in energy metabolism is an intriguing question that needs to be addressed in future research. Finally, future research should also be aimed at understanding GPER biology in humans, which has been seldom investigated.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design of this review paper, drafted the manuscript and revised it critically for important intellectual content, and approval it for publication.

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G Protein-Coupled Estrogen Receptor: Rapid Effects on Hippocampal-Dependent Spatial Memory and Synaptic Plasticity

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In the hippocampus, estrogen regulates gene transcription linked to neuronal growth, neuroprotection, and the maintenance of memory function (1–3). The mechanism is likely to involve genomic regulation through classic estrogen receptor (ER) signaling cascades that influence transcription. Estrogens binding to classic nuclear ERs, alpha (ERα) and beta (ERβ), and have pleotropic effects on development, behavior, and neurophysiological functions, including synaptic plasticity and memory consolidation (4–7). In addition to ERα and ERβ, estrogen can also initiate activation of classical second messenger signaling cascades to influence the activity of G-proteins and a host of kinases, resulting in rapid changes in physiology (8–14). These rapid effects of estrogen are commonly mediated by membrane receptors. In the late 90s, multiple laboratories cloned cDNA/gene for an orphan G-protein-coupled receptor with very low homology with other G-protein-coupled receptors and named it G-protein-coupled receptor 30 (GPR30) (15–20). Later in 2007, the International Union of Basic and Clinical Pharmacology designated GPR30 as G protein-coupled estrogen receptor (GPER) (21); GPER is a seven-transmembrane G-protein-coupled receptor, predominantly expressed on the cell membrane (22). Interestingly, GPER is reported to mediate many of the rapid responses of estradiol in the adult brain, and is widely distributed in the mammalian brain including the plasma membrane of hippocampal neurons (23–31). GPER modulates second messenger signaling cascades involving $G\alpha_s$- and $G\alpha_{i/o}$-associated increase in cyclic adenosine monophosphate and phosphoinositide 3-kinase or Src protein kinase respectively (32, 33). Activation of GPER is also associated with phospholipase C, and the inositol receptor and ryanodine receptor-mediated increase in intracellular calcium (24, 34). This commentary is concentrated specifically on the possible rapid effects of GPER in hippocampal-dependent spatial memory function and synaptic plasticity.

Keywords: estrogen, estrogen receptor, GPER, spatial memory, synaptic plasticity

ROLE OF GPER IN HIPPOCAMPAL-DEPENDENT SPATIAL MEMORY

In hippocampal neurons, GPER immunoreactivity is associated with the plasma membrane and endoplasmic reticulum, along with axon terminals and dendritic spines (22, 24, 29–31, 35–40). It is well established that estrogen can influence synaptic function and improve memory (12, 41–49). G1, a nonsteroidal high-affinity selective GPER agonist, does not bind classical ERs (50), but similar to estrogen, improves cognitive performance, including social recognition, spatial working memory, and long-term spatial memory consolidation (51–59). Results from recent studies by the Frick group, elegantly demonstrate that like 17-beta estradiol (E2), activation of GPER, by direct infusion of G1 into the dorsal hippocampus, can facilitate object recognition memory and hippocampal-dependent spatial memory in ovarectimized female mice. The

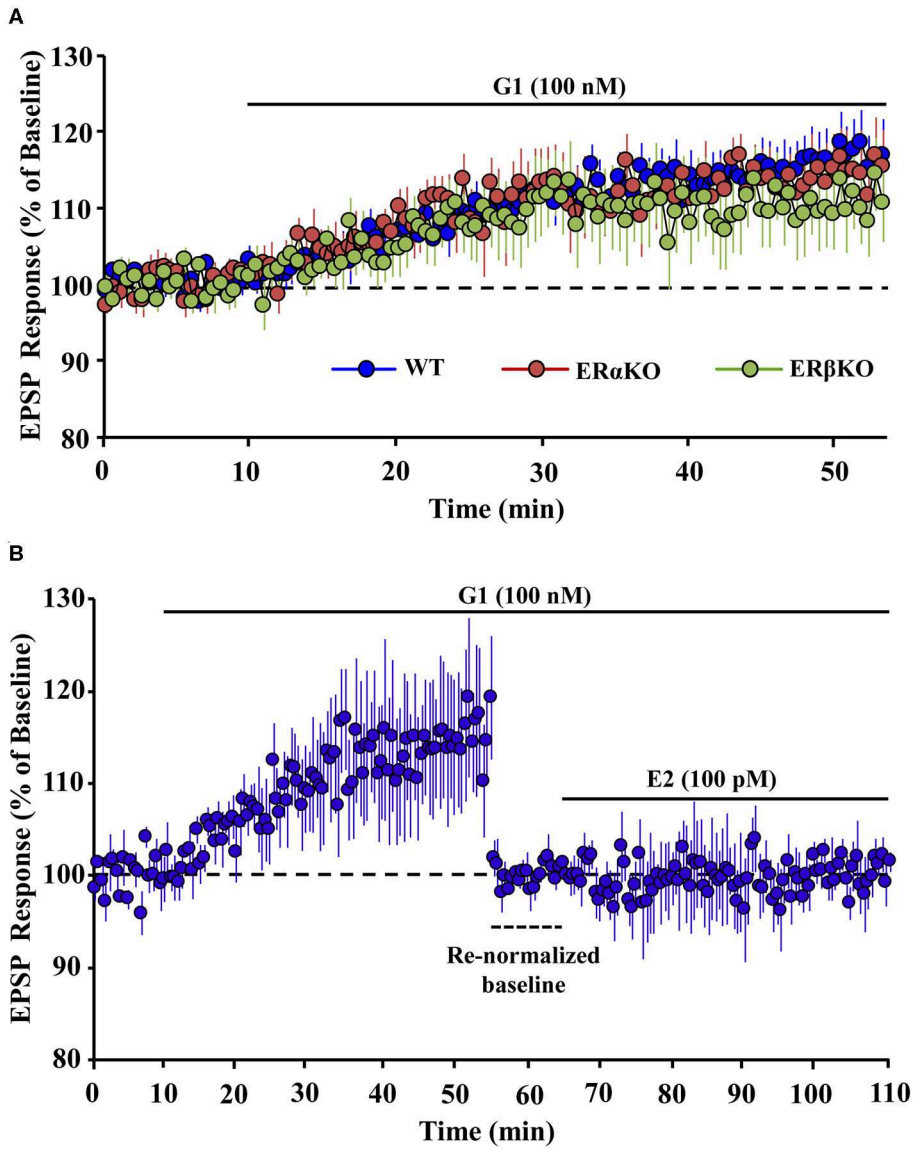

FIGURE 1 | Effect of GPER selective agonist, G1 on hippocampal synaptic responses. **(A)** Time course of the field EPSP measurements on slices obtained from wild type (WT, blue), estrogen receptor (ER) alpha knockout (ERαKO, red), and ER beta knockout (ERβKO, green) mice obtained 10 min before and 45 min after application of G1. **(B)** G1 blocked the 17-beta estradiol (E2)-induced enhanced synaptic responses in hippocampal slices. Time course of field EPSP measurements obtained from hippocampal slices 10 min before and 45 min after G1 application. Bath application of G1 significantly enhanced the synaptic response. Baseline was re-normalized from last 10 min recording (dashed line) following the start of G1 application, and E2 was bath applied in the continued presence of G1. E2 in presence of G1 failed to further enhance synaptic response. Adapted from Kumar et al. (11). Copyright permission granted order # 480097130349.

enhancement of memory was not due to activation of the extracellular signal-regulated kinase signaling normally observed following E2 treatment. Rather, GPER activation was associated with phosphorylation of c-Jun N-terminal, cofilin-mediated actin polymerization, and spinogenesis in region CA1 (55, 57). Overall, these studies provide strong evidence that like E2, activation of GPER can facilitate hippocampal-dependent memory performance.

GPER AND HIPPOCAMPAL SYNAPTIC FUNCTION

In addition to enhancing memory performance, GPER activation also contributes to synaptic plasticity. Activation of GPER enhances synaptic transmission at hippocampal CA3-CA1 synapses (11, 54, 60, 61). We recently demonstrated that GPER is a major component of E2-mediated upregulation in extracellular signal-regulated kinase and the rapid facilitation of synaptic responses at CA3-CA1 hippocampal synapses of ovariectomized mice. In addition, the GPER agonist, G1, induced an increase of excitatory postsynaptic potentials (EPSPs) in hippocampal slices obtained from ovariectomized ER alpha knockout (ERαKO) and ER beta knockout (ERβKO) mice (**Figure 1A**). Confirmation that GPER is a mechanism for rapid E2 effects on synaptic transmission was proven by demonstrating that prior application of G1 blocked the E2-induced enhancement of synaptic responses in hippocampal slices (**Figure 1B**), while bath application of E2 in absence of G1 increases synaptic responses (11). Interestingly, Oberlander and Woolley demonstrated that GPER-induced potentiation of excitatory synaptic responses in CA1 hippocampal pyramidal neurons is restricted to females and involves postsynaptic mechanisms (61). The role of GPER in synaptic plasticity is still evolving (62–65); however, a number of recent studies indicate that activation of GPER contributes to a rapid increase in hippocampal dendritic spinogenesis and spine density (11, 54, 57, 60, 61, 66, 67).

CONCLUDING STATEMENT

In many ways, the effects of E2 are opposite to that of aging (3, 68). Recent findings indicate that similar to E2, GPER participates in the rapid effects of the E2-induced increase in hippocampal synaptic transmission and improved cognition. Thus, it will be interesting for future research to explore changes in GPER expression or function over the life span, and their contribution to impaired cognitive and synaptic function associated with aging and neurodegenerative diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Florida.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Computational Approaches for the Discovery of GPER Targeting Compounds

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Estrogens exert a panel of biological activities mainly through the estrogen receptors α and β, which belong to the nuclear receptor superfamily. Diverse studies have shown that the G protein-coupled estrogen receptor 1 (GPER, previously known as GPR30) also mediates the multifaceted effects of estrogens in numerous pathophysiological events, including neurodegenerative, immune, metabolic, and cardiovascular disorders and the progression of different types of cancer. In particular, GPER is implicated in hormone-sensitive tumors, albeit diverse issues remain to be deeply investigated. As such, this receptor may represent an appealing target for therapeutics in different diseases. The yet unavailable complete GPER crystallographic structure, and its relatively low sequence similarity with the other members of the G protein-coupled receptor (GPCR) family, hamper the possibility to discover compounds able to modulate GPER activity. Consequently, a reliable molecular model of this receptor is required for the design of suitable ligands. To date, convergent approaches involving structure-based drug design and virtual ligand screening have led to the identification of several GPER selective ligands, thus providing important information regarding its mode of action and function. In this survey, we summarize results obtained through computer-aided techniques devoted to the assessment of GPER ligands toward their usefulness in innovative treatments of different diseases.

Keywords: G protein-coupled estrogen receptor 1, estrogen receptors, ligands, drug design, molecular docking, molecular dynamics

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INTRODUCTION

The multifaceted responses to estrogens are principally mediated by the estrogen receptors (ERs) α and β, which act as transcription factors by binding to estrogen response elements (EREs) located in the promoter regions of target genes (1). Recently, a seven-transmembrane G protein-coupled receptor, known as G protein estrogen receptor (GPER), has attracted the attention of several researcher groups working on the identification of the intricate estrogen routes in different biological systems. A panel of experiences has highlighted the involvement of GPER in various pathophysiological processes. For instance, its role in hormone-dependent cancers has been addressed in several studies, providing a better understanding of the related gene landscape

and transduction pathways. In particular, GPER modulates signaling processes leading to the transcription of genes promoting tumor growth *in vitro* and *in vivo*, such as calcium mobilization, cAMP synthesis, the cleavage of matrix metalloproteinases, the transactivation of epidermal growth factor receptor (EGFR) and the activation of PI3K and MAPK transduction pathways (2–11). To date, GPER expression has been correlated with negative cancer features including increased tumor size, distant metastasis and tumor recurrence (12–14). In addition, a bioinformatic analysis of large cohorts of patients has recently demonstrated that GPER expression is correlated with the expression of pro-metastatic genes in ER-negative breast tumors (15). On the basis of the aforementioned findings, this receptor might be considered as a promising therapeutic target for the treatment of diverse types of tumors, including breast cancer. Nevertheless, other studies reached different conclusions (16), therefore indicating that further investigations are required to better appreciate the role exerted by GPER in cancer.

Most estrogens and anti-estrogens are able to bind to GPER and ERs, albeit with a different affinity and even with an opposite action (i.e., agonism vs. antagonism) (10, 17, 18). Considering the interest to identify specific GPER ligands to decipher its unique potential, several successful efforts have been made during the last few years (19–25). In this context, it should be mentioned the intriguing discovery of the indole derivative MIBE, which has the property of binding to and antagonizing the effects of both GPER and ER, thus representing a useful tool toward more comprehensive approaches in estrogen-dependent tumors (22).

The overall structural heterogeneity among agents targeting these receptors constitutes an obstacle to identify agonists or antagonists and to predict their effects. Thus, the design of potent selective GPER ligands and dual ER/GPER inhibitors is still challenging. While the crystallographic structure of the ER ligand-binding domain is available, the detailed structure of GPER remains yet unsolved due to the well-known difficulties in fully characterizing membrane proteins. Nevertheless, a homology model of GPER can be obtained with the help of computational techniques (**Figure 1**), allowing access to relevant structural information. More importantly, virtual ligand screening approaches and structure-based drug design methods can support experiments aiming to identify new GPER ligands. The results obtained by application of computational techniques are herein summarized toward their adoption as starting point for the design and development of novel active agents.

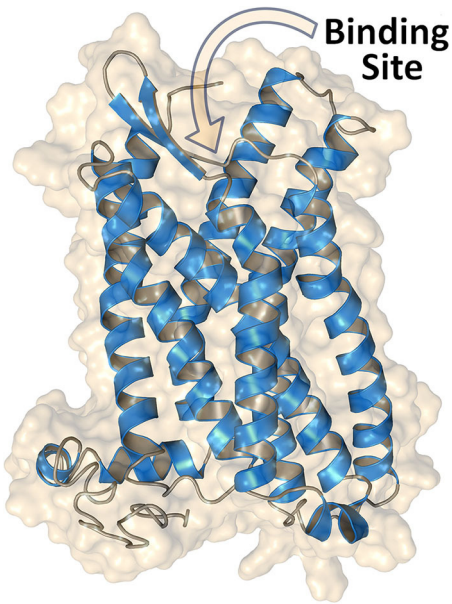

FIGURE 1 | Structure of GPER obtained through homology modeling by using the web server GPCR-I-TASSER (26). The extracellular N-terminal region (residues 1–50) is omitted, and access to the binding site for the ligands is indicated.

THE EARLY AGE OF LIGAND-BASED DESIGN FOR TARGETING GPER

With respect to the computational design of GPER ligands, one of the earliest achievements is indisputably the synthesis of the quinoline G-1 (19). To this aim, a library of 10,000 candidate molecules has been analyzed by combining their structural features in common with the archetypal ligand 17β-estradiol (E2). Three distinct categories of criteria were defined: (i) 2D structural patterns, including both symmetric and asymmetric features, (ii) 3D shape analogies and metrics and (iii) pharmacophore-based motifs, including hydrogen-bond donors/acceptors and the possibility of forming hydrophobic interactions. The resulting computational analysis was completed with an *in vitro* biomolecular screening to validate the occurrence of a competitive ligand binding. The molecule G-1 (for the chemical structure of this compound and all the other mentioned throughout the text, see **Figure 2**) has emerged from this screening funnel as the first GPER-specific agonist able to activate the receptor in cells expressing both GPER and ERs. A similar virtual screening methodology, applied on a larger library with more than 140,000 compounds, led successively to the identification of a high-affinity GPER ligand named G-15, a G-1 analog acting as a selective antagonist (20).

The success of these original ligand-based virtual screenings was facilitated by the limited number of internal degrees of freedom of the studied compounds. These observations referred also to the conformational space of the ligand-binding pocket of the different steroid hormone receptors (27). In the absence of a model for the GPER tertiary structure, the previous works were, therefore, expanded to an "indirect" structure-based approach consisting in the analysis into deeper details of the ERα and ERβ binding sites. In fact, the main concern was to focus on the acetyl moiety of the ER agonist G-1, which is lacking in the antagonist G-15 (21). Molecular docking was then performed to explore the possibility to increase steric clashes within the binding pocket of the ERs through an isopropyl moiety instead of

Abbreviations: AhR, aryl hydrocarbon receptor; E2, 17β-estradiol; E3, 16α,17β-estriol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen response element; GPCR, G protein-coupled receptor; GPER (or GPR30), G protein-coupled estrogen receptor 1; MD, molecular dynamics.

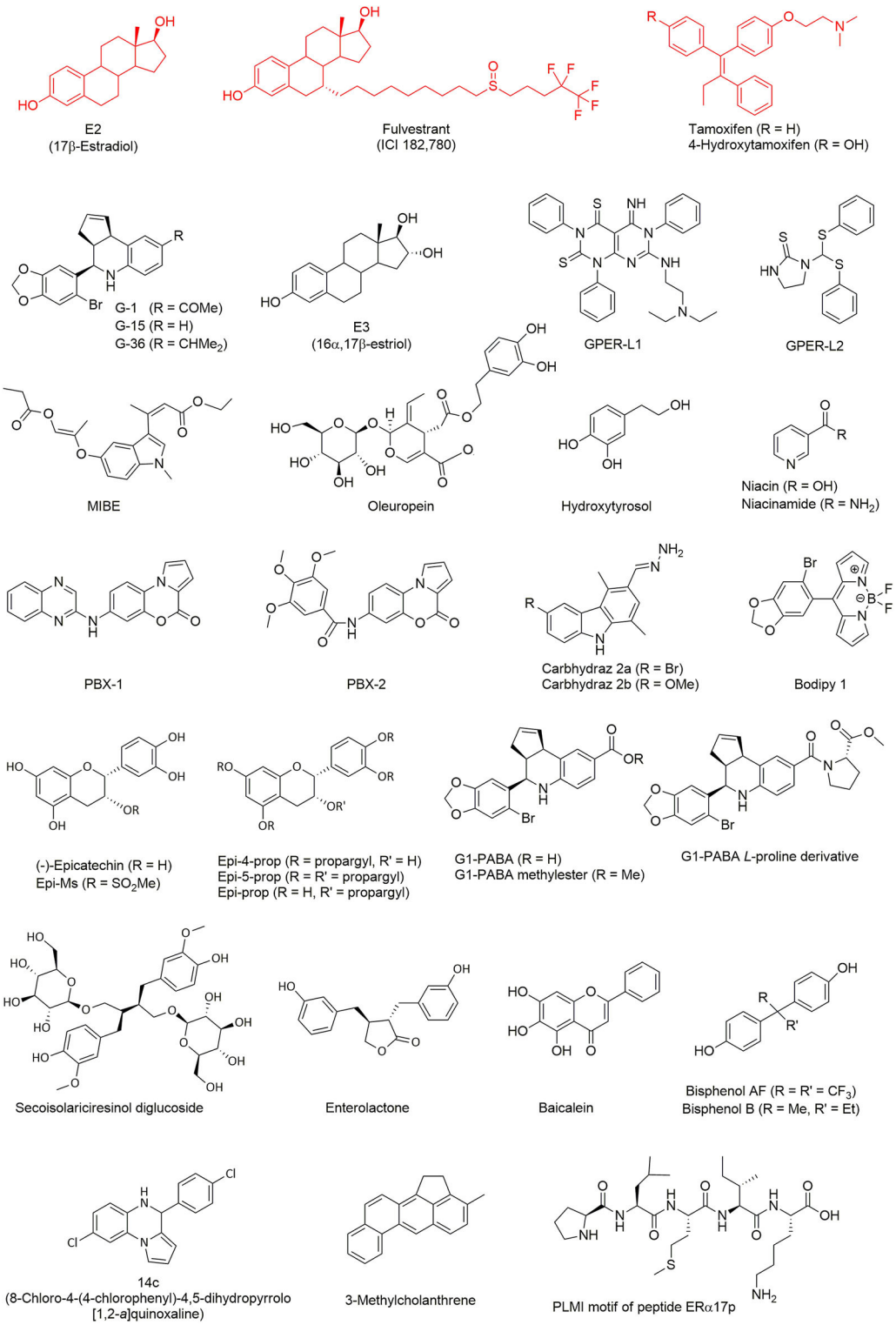

FIGURE 2 | GPER ligands investigated by computational methods. Typical ligands (represented in red), including E2, fulvestrant, and tamoxifen, have been extensively tested in simulation as reference GPER binders. All the other ligands (in black) were discovered or characterized through virtual screening techniques.

the acetyl group. The resulting compound, named G-36, showed an enhanced selectivity with an antagonist effect toward GPER, and a low-affinity cross-reactivity toward ERα.

HOMOLOGY MODELING FOR PREDICTING THE STRUCTURE OF GPER

The possibility of investigating in simple and accurate ways the binding location and the affinity of molecules to GPER is intimately linked with the availability of a correct molecular description of the protein structure. The first crude GPER model was built to predict ligand binding, through computational approaches (28). A more accurate structure of GPER was later used as a support to elucidate a number of "wet-lab" experiments (18, 22, 29). In all cases, a special emphasis was put on providing details about the topology of the putative binding-site of the protein, rather than the whole protein tertiary structure. The 3D structure of GPER was constructed through homology modeling (30, 31), by using the crystal structure of bovine rhodopsin (32) as a template.

Subsequent efforts were devoted on the improvement of the molecular description of the protein structure, with a broader emphasis on the description of the overall protein architecture. Consistent results were achieved by using as a template the X-ray structure of the β_2-adrenergic receptor, which was found to possess a higher degree of homology with GPER, when compared to bovine rhodopsin. The crystal structures of both the active and inactive states of the β_2-adrenergic receptor were available (33, 34), allowing to model different conformations of GPER in complex with agonists or antagonists (35).

Alternatively, GPER has been modeled (36) by using as a template the crystallographic structure of the chemokine receptor CXCR4 (37). It was also possible to identify some structural differences between agonists and antagonists, depending on their ability to form hydrogen bonds with key residues located in specific transmembrane helices. The quality level of this new GPER model was further improved through molecular dynamics (MD) simulations (36, 38, 39), which were helpful to take into account the internal dynamics of the protein, concomitantly to the contribution of the surrounding lipid matrix.

A more comprehensive view of the 3D structure of GPER has been recently obtained by using the web server GPCR-I-TASSER (26, 40), which is a computational suite derived from the parent I-TASSER package (41), one of the most popular on-line resources for protein structure prediction and refinement. A major strength of the algorithm used in GPCR-I-TASSER is that it was specifically devised for the prediction of GPCRs and, as such, it works with a dedicated knowledge-based force field as a guide for an accurate assembly of the receptor structure. A number of works (25, 38, 42, 43) have adopted this computational tool to design high-quality GPER models (see again **Figure 1**). High-quality and tailored software, together with the growing number of experimentally-determined GPCR structures that can be used as GPER templates, are contributing to make homology modeling algorithms more accurate in predicting the receptor structure.

THE BEGINNING OF STRUCTURE-BASED DESIGN OF LIGANDS FOR GPER

In the last decade, one of the major aims of our research group has been the identification of novel GPER ligands supported by computational drug discovery approaches. A first result (18) was the identification of 16α,17β-estriol (E3) as a GPER antagonist (see **Figure 2**). This compound, which corresponds to a final metabolite of E2 (44), is one of the three natural estrogens produced by the human body (i.e., estradiol, estrone, and estriol). It is of note that E3 can also be biosynthesized from estrone sulfate as well as from testosterone and androstenedione (44).

A computational analysis also allowed the discovery of two new molecules (22), named GPER-L1 and GPER-L2, both acting on GPER as selective agonists but unable to bind and activate ERs. These two ligands were selected through a typical process of virtual screening starting from a chemical library including more than 300 molecules. Despite strong differences in their chemical structure, they share some crucial features such as the ability of exposing a phenyl ring into a hydrophobic protein pocket and, thereby, of forming stabilizing π-π stacking interactions. Due to their selectivity, these molecules contribute to increase our understanding of the function of various cancer phenotypes, through different estrogen-targeted receptors.

In sharp contrast, MIBE was identified as a unique case of dual antagonist for both GPER and ERα (29), by using molecular docking. The possibility of targeting simultaneously GPER and ERα is particularly interesting from a pharmacological point of view, because active compounds antagonizing both proteins could be useful in tackling breast carcinomas at the initial stage or during their progression. In a subsequent work (45), it was demonstrated that oleuropein and hydroxytyrosol, two natural phenols, were able to bind GPER. Flexible molecular docking calculations were performed with these two molecules, considering free rotation of seven bulky side chains in the binding site of the receptor, which was especially required to accommodate the relatively large molecular structure of oleuropein. The following experiments demonstrated that both ligands act as GPER inverse agonists in ER-negative and GPER-positive SkBr3 breast cancer cells, as recently found with the peptide ERα17p (25).

A virtual screening campaign on a library of chemical fragments demonstrated the binding of niacin (also known as nicotinic acid, vitamin B3, or vitamin PP) and of its amide form niacinamide (or nicotinamide) to GPER and niacin receptor GPR109A/HCA2 (46). The latter is a subtype of the receptor GPR109 that mainly, although not exclusively, mediates the action of niacin (but not of niacinamide). Sequence alignment revealed a lack of homology between GPER and GPR109A, at least in their ligand-binding sites, thus the anchoring of these two molecules to both GPER and GPR109A was not obvious. Nevertheless, molecular docking suggested some similarities in the binding mode of the pyridine ring of both compounds. The presence of two important arginine residues able to form hydrogen bonds with the carboxylic acid moiety of niacin, allowing GPR109A specificity, were also highlighted. Both niacin

and niacinamide were demonstrated to exert an agonist activity toward GPER in the successive experiments.

Computational techniques further helped to design two novel benzopyrroloxazines (24) acting as selective GPER antagonists. The starting point was the virtual screening of a compound library and the identification of a rigid molecular structure closely resembling the chemical skeleton of other known GPER ligands. This structure was used as a template and two derivatives, named PBX1 and PBX2, were demonstrated to bind to GPER. To this aim, flexible molecular docking was performed by allowing the rotation of the dihedral angles in the side chain of eight residues within the protein-binding site.

In a separate study (23), two novel carbazole derivatives were synthesized. While both of them did not activate the classical ERα, one of them, abbreviated as carbhydraz 2a, showed a favorable affinity for GPER, as shown by docking assays with seven flexible residues. This compound was then shown to display an agonistic response on GPER.

As a final example, a rational design has been completed with the first GPER selective fluorescent organoboron probe (47), which consists in a boron-dipyrromethene difluoride derivative. In this case, the starting molecular template was a bromobenzodioxolyl substituent, which is also present in the structure of G-1 and constitutes a key motif for GPER binding. Using molecular docking, the obtained compound, named Bodipy 1, was predicted to share a binding mode similar to G-1, by interacting with the key protein residue Phe-208 and by forming π-π stacking with its bromobenzodioxole moiety. Bodipy 1 was later demonstrated to compete with niacin in ER-negative and GPER-positive SkBr3 breast cancer cells.

THE CURRENT AREA OF COMPUTATIONAL METHODS FOR STUDYING GPER

The study of GPER through theoretical modeling approaches has lately benefited from a number of improvements, including the use of targeted simulations to capture important aspects of the protein dynamics. Molecular docking on GPER structures extracted from all-atom MD has demonstrated (48) that the natural polyphenol (–)-epicatechin (see **Figure 2**) has the ability to anchor to this receptor with a binding mode similar to the agonist G-1. It is interesting to note that flavonoids sharing structural similarities to estrogens, such as genistein and other phytoestrogens (49–51), not only bind but also activate the classical receptors ERα and ERβ. In contrast, and in spite of its evident structural analogies to these phytochemicals, (–)-epicatechin fails to bind ERα and ERβ. Since (–)-epicatechin can associate within the GPER binding pocket, an important role of this receptor on cardiovascular system protection seems likely. In a subsequent study (52), (–)-epicatechin derivatives were obtained where the phenol and alcohol groups are functionalized with a propargyl or a mesyl group. The resulting compounds, i.e., Epi-4-prop, Epi-5-prop, Epi-prop and Epi-Ms, were investigated by docking methods on the GPER structures obtained through MD. Strikingly, it was observed that the alkyne function of the propargyl was prone to generate additional interactions in the receptor-binding site and to enhance the GPER agonistic activity, when compared to the parent compound. Later, MD simulations showed that Epi-4-prop and Epi-5-prop share with (–)-epicatechin similar interactions at the GPER binding site (53).

A number of additional GPER binders have also been identified using theoretical modeling calculations. It is the case of G1-PABA (54), a compound part of a small series of G1 analogs, in which the acetyl moiety is replaced with a carboxyl group. The same pharmacophore core was further used to obtain a G1-PABA methylester and *L*-proline derivative, with similar structural and energetic binding properties (55). All these newly synthesized compounds were validated *in vitro* in experimental assays using breast cancer cell lines. Another example is secoisolariciresinol diglucoside, a phytoestrogen extracted from flaxseed and able to suppress benign prostatic hyperplasia (56), which was indirectly investigated through molecular docking of its mammalian metabolite enterolactone (57). Similarly, the binding mode to both GPER and ERα of baicalein, a flavonoid derived from the roots of a medicinal herb, has been extensively studied (58). Molecular docking analysis was especially focused on the hydrogen-bond network favoring the ligand binding. The observation that GPER appeared to mediate the estrogenic effects of some bisphenol A analogs was the starting point of another computational study (59), which predicted a favorable binding of bisphenol AF and bisphenol B. Experimental assays have confirmed the agonistic activity of these compounds, supporting the hypothesis of their disrupting action on the GPER-mediated pathway. These observations clearly highlight the lack of selectivity of phytoestrogens (60).

Similarly to G-15, theoretical methods have also been used to identify new compounds (61) with a selective anti-proliferative activity against GPER-expressing breast cancer cells. A virtual screening campaign was carried out on a chemical library of about 1,000 compounds, in search of molecules showing a binding mode close to G-15 and G-36. They were selected on the basis of their binding score and by visual inspection, as well as their ability to form polar contacts with previously identified GPER residues. Four different chemical scaffolds were found. A particularly promising compound, named 14c and based on one of these scaffolds, has been proposed as a starting point for a future hit-to-lead optimization process.

By using combined docking and MD simulations approach, the association of the chemical carcinogen 3-methylcholanthrene with GPER has been recently studied (43). This environmental pollutant, which is generated by incomplete combustion processes including cigarette smoke, was known to bind to both ERα and the aryl hydrocarbon receptor (AhR), stimulating thereby a functional interaction between these two receptors. The results pointed out to a functional crosstalk and a cross-stimulation between GPER and AhR. Computational methods have also been used to investigate the binding to GPER of the peptide ERα17p, which encompasses a part of the hinge region/AF2 domain of the human ERα (25, 62). This peptide acts as a GPER inverse agonist and shows anti-proliferative effects in breast cancer cells and a decrease in the volume of breast

tumors in xenografted mice (63). The N-terminal PLMI motif of this peptide presents some chemical analogies with the GPER antagonist PBX1 and exerts the same anti-proliferative potency as the whole length peptide (25, 62), suggesting strongly that this region corresponds to the active motif. Due to the large number of rotatable bonds in ERα17p, MD simulations were necessary to map the conformational landscape of the receptor-peptide molecular system. The fact that a specific amino acid drives the anchoring of ERα17p opens the way to the possibility of modulating GPER by using peptide-based compounds. It is particularly notable that ERα17p is, to the best of our knowledge, the first peptidic GPER modulator.

CONCLUSIONS

GPER is increasingly recognized as a mediator of different estrogen-dependent pathophysiological responses, such as those that characterize cancer progression. The persistent difficulty in obtaining an experimental structure of the native structure of this membrane receptor, let alone in complex with any endogenous or exogenous ligands, has prompted an abundance of theoretical studies to clarify its conformation and binding properties. In this context, molecular modeling of GPER ligands has demonstrated that targeting this receptor with computational methods is feasible. Accordingly, a number of compounds has been defined toward the development of innovative molecular modulators of GPER action in different biological systems. In particular, the first identified GPER agonist, G-1, is currently undergoing phase 1 clinical trials for its immunomodulatory and antineoplastic properties. In this respect, the findings recapitulated and discussed herein could be useful in order to clarify the potential role of GPER in cancer and other diseases, and the advantages of computational approaches to drive drug discovery for this target.

AUTHOR CONTRIBUTIONS

All the authors have contributed to prepare the manuscript and approved it for publication.

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G Protein-Coupled Estrogen Receptor Immunoreactivity Fluctuates During the Estrous Cycle and Show Sex Differences in the Amygdala and Dorsal Hippocampus

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G protein-coupled estrogen receptor (GPER) in the amygdala and the dorsal hippocampus mediates actions of estradiol on anxiety, social recognition and spatial memory. In addition, GPER participates in the estrogenic regulation of synaptic function in the amygdala and in the process of adult neurogenesis in the dentate gyrus. While the distribution of the canonical estrogen receptors α and β in the amygdala and dorsal hippocampus are well characterized, little is known about the regional distribution of GPER in these brain regions and whether this distribution is affected by sex or the stages of the estrous cycle. In this study we performed a morphometric analysis of GPER immunoreactivity in the posterodorsal medial, anteroventral medial, basolateral, basomedial and central subdivisions of the amygdala and in all the histological layers of CA1 and the dentate gyrus of the dorsal hippocampal formation. The number of GPER immunoreactive cells was estimated in these different structures. GPER immunoreactivity was detected in all the assessed subdivisions of the amygdaloid nucleus and dorsal hippocampal formation. The number of GPER immunoreactive cells was higher in males than in estrus females in the central ($P = 0.001$) and the posterodorsal medial amygdala ($P < 0.05$); higher in males than in diestrus females in the *strata orients* ($P < 0.01$) and *radiatum-lacunosum-moleculare* ($P < 0.05$) of CA1-CA3 and in the molecular layer of the dentate gyrus ($P < 0.01$); higher in diestrus females than in males in the basolateral amygdala ($P < 0.05$); higher in diestrus females than in estrus females in the central ($P < 0.01$), posterodorsal medial ($P < 0.01$) and basolateral amygdala ($P < 0.01$) and higher in estrus females than in diestrus females in the *strata oriens* ($P < 0.05$) and

radiatum-lacunosum-moleculare ($P < 0.05$) of CA1-CA3 and in the molecular layer ($P < 0.05$) and the *hilus* of the dentate gyrus ($P < 0.05$). The findings suggest that estrogenic regulation of the amygdala and hippocampus through GPER may be different in males and in females and may fluctuate during the estrous cycle.

Keywords: amygdala, hippocampus, estrous cycle, limbic system, GPER, estrogens, estrus, diestrus

INTRODUCTION

The hippocampus and the amygdala are two anatomically and functionally interconnected brain regions that participate in the regulation of stress responses (1, 2), fear (3–5), emotions (6–8), learning (9), and memory (8, 10). Both structures are integrated in the limbic system, which is altered in different pathological conditions, such as depression, anxiety, stress and schizophrenia, among others (11–20).

Some of the behaviors regulated by the hippocampus, the amygdala and their associated limbic structures are modulated by estradiol and testosterone (21–24) and are affected by sex (25–28) and by the phases of the estrous cycle (29–33). This hormonal regulation may be mediated by the modification of synaptic activity and plasticity in both the hippocampus (33–38) and the amygdala (29–31, 39, 40) and may represent a direct effect of testosterone and estradiol on these two brain structures, which express both androgen (41–43) and estrogen receptors (43–46).

Expression of classical estrogen receptors (ER)α and ERβ in the hippocampus and amygdala is well documented (44–47). After the discovery of the membrane-associated G protein-coupled estrogen receptor 1 (GPER), several studies have also explored its localization and function in the brain (48). GPER protein has been localized in the developing (49–51) and adult rodent hippocampus (52–59). In addition, GPER mRNA (60–62) and protein (63, 64) have been also detected in the adult rodent amygdala. However, the possible changes in GPER distribution in function of sex and the ovarian cycle in the hippocampus and amygdala have not been explored. Therefore, in this study we have analyzed the possible differences in GPER immunoreactivity between male, diestrus and estrus females in different anatomical subdivisions of the rat hippocampus and amygdala.

MATERIALS AND METHODS

Animals and Experimental Procedure

Wistar albino male and female rats from our in-house colony were kept on a 12:12-h light–dark cycle and received food and water *ad libitum*. Animals were handled in accordance with the guidelines published in the "NIH Guide for the care and use of laboratory animals," the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience, and following the European Union (2010/63/UE) and the Spanish legislation (L6/2013; RD53/2013). Experimental procedures were approved by our Institutional Animal Use and Care Committee (UNED, Madrid). Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

Twenty-four adult rats 2 months old (eight males and 16 females) were separately housed in plastic cages. After 2 weeks of habituation and handling, the monitoring of the estrous cycle in female rats was performed during 7 days by vaginal smears (65, 66). At the day 7, female rats were tested for the last vaginal smear in order to select the animals in estrus or diestrus (diestrus-2). Subsequently, all the animals, male and female, were deeply anesthetized with pentobarbital (Normon Veterinary Division, Madrid, Spain, 50 mg/kg) and perfused through the left cardiac ventricle with 50 ml of saline solution (0.9% NaCl) followed by 250 ml of fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were quickly removed and immersed for 4–6 h at 4°C in the same fixative solution and then rinsed with phosphate buffer. Brains were placed for 72 h in a 30% sucrose solution in PBS, frozen in liquid isopentane at −35°C, and stored in a deep freezer at −80°C until sectioning. Brains were serially cut in the coronal plane at 20 μm thickness with a cryostat, obtaining 5 series of adjacent serial sections. In each series, each section was 100 μm distant from the following one. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the rat brain atlas of Paxinos and Watson (67). Sections were collected in multiwell plates with a cryoprotectant solution and kept at −20°C. Immunohistochemical assay for GPER was performed on different series.

Immunohistochemistry

The presence of GPER was detected by immunohistochemistry performed on free-floating sections according to the following steps. Before the reaction, the sections collected in the cryoprotectant solution were washed overnight at 4°C in PBS 0.1 M, pH 7.3–7.4. The following day, free floating sections were first washed for 30 min at room temperature in PBS 0.1 M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA. Sections were then treated for 10 min with a solution of PBS 0.1 M, pH 7.3–7.4, containing methanol/hydrogen peroxide (PBS/methanol 1:1 with 0.3% hydrogen peroxide) to quench endogenous peroxidase activity. Sections were washed for 30 min at room temperature in PBS 0.1 M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA and then incubated for 48 h at 4°C with a rabbit polyclonal GPER antibody (ABCAM, Cambridge, UK, reference ab39742) diluted 1:250 in 0.1 M PBS, pH 7.3–7.4, containing 0.2% Triton X-100, 0.2% BSA and 3% normal serum goat. A biotinylated goat anti-rabbit secondary antibody (Thermo scientific, Pierce, Rockford, IL, USA) was then used at a dilution of 1:300 for 120 min at room temperature. The antigen–antibody reaction was revealed by incubation with avidin-peroxidase complex (Thermo scientific, Pierce, Rockford,

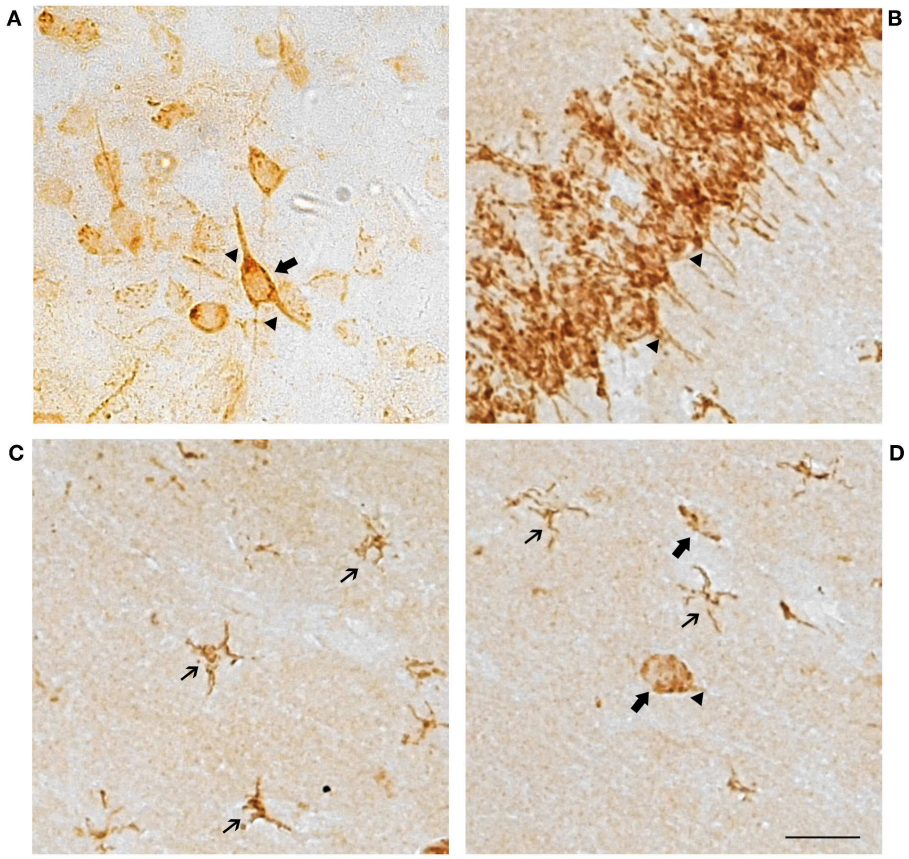

FIGURE 1 | Representative GPER immunostaining showing cell perikaryon and the primary processes labeling in neuronal and glial cells. **(A)** Amygdaloid nucleus and **(B–D)** Hippocampal formation. Scale bar 10 μm. Thin arrows, GPER immunoreactive cells with glial morphology. Large arrows, GPER immunoreactive cells with neuronal morphology. Arrowheads, immunoreactive neuronal processes.

IL, USA) for 90 min. The peroxidase activity was visualized with a solution containing 0.187 mg/mL 3,3- diamino-benzidine (Sigma, Madrid, Spain) in PBS 0.1 M, pH 7.3–7.4. The sections were washed in the same buffer and collected on chromallum coated slides, air dried, cleared in xylene, and cover slipped with Depex (VWR International Eurolab, Barcelona, Spain) for quantitative analysis. One of each five consecutive sections was stained with 0.1% cresyl violet (pH 7.4) to facilitate the identification of the selected structures.

The GPER antibody used in the present study has been previously shown to recognize the full-length receptor protein in lysates of selected brain regions by Western blotting (68–72). Furthermore, immunostaining is abolished in rat brain sections when the GPER antibody is preincubated with the immunizing peptide (73). In agreement with our previous findings, GPER immunostaining was absent in rat brain sections preincubated with the GPER blocking peptide and when the first antibody was omitted.

Morphometric Analysis

The morphometric analysis of GPER immunoreactive cells was performed on coded sections without knowledge of the experimental group. The number of GPER positive cells was assessed in the amygdala and the dorsal hippocampus using two coded sections per animal. Sections selected for analysis corresponded to the following coordinates: bregma −2.8 to −3.14 mm for the amygdaloid nucleus and bregma −2.8 to −3.8 mm for the dorsal hippocampal formation (67). The following regions were considered for the morphometric analysis of GPER immunoreactive cells: (i), the posterodorsal medial (MePD), anteroventral medial (MeAV), basolateral (BLA), basomedial (BMA), and central (CeM) amygdala; (ii), the *stratum oriens* (SO), the *stratum radiatum*, analyzed together with the *stratum lacunosum-moleculare* (SRLM) and the *stratum pyramidale* (SP) in dorsal Ammon's horn and (iii), the *stratum granulosum* (SG), the *stratum moleculare* (SM) and the *hilus* in the dorsal dentate gyrus.

Data presented for each region are the sum of the number of GPER immunolabeled cells in two brain sections per rat. For the amygdala, all cells located within the anatomical borders of each subnuclei were considered for quantification. Cresyl violet stained sections were used as reference for the delimitation of the analyzed structures. Given the anatomical heterogeneity of the hippocampus, counts were limited to the dorsal hippocampus

and performed separately in CA1-CA3 and in the dentate gyrus. Cells were counted in eight fields from CA1-CA3, four fields from the *strata granulosum* and *moleculare* of the dentate gyrus and two fields from the *hilus*. Each field had an average area of 9.63×10^3 μm^2 for the SO; 7.49×10^3 μm^2 for the SP; 23.76×10^3 μm^2 for the SRLM; 6.52×10^3 μm^2 for the SG; 14.54×10^3 μm^2 for the SM and 21.5×10^3 μm^2 for the *hilus*. Selected fields were acquired by a digital camera (Olympus DP25) connected to a Nikon eclipse E600 microscope using x40 and x20 objectives. All GPER positive cells showing a cell nucleus and located within the boundary of the selected anatomical regions were included in the analysis, regardless of differences in cell shape, size and level of immunostaining. As a note of caution, it is important to consider that our morphometric approach is not unbiased from possible differences among the experimental groups in the volume of the anatomical structures analyzed. Thus, it should be considered a semi-quantitative estimation of the number of GPER positive cells.

Statistical Analysis

Data were analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* test, using the SPSS-17.0 software (SPSS Inc, Chicago, USA). A value of $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SEM.

RESULTS

Morphology of GPER Immunoreactive Cells

Cells showed a punctiform staining in the brain sections incubated with the GPER antibody (**Figure 1**). The staining was cytoplasmic, and the cell nucleus was always negative. Numerous cells showed a clear neuronal morphology with cytoplasmic immunostaining in the cell perikaryon and the primary dendritic processes. Dendritic staining was particularly evident in the pyramidal neurons of the hippocampus (**Figure 1B**), but it was also detected in neurons from the other studied regions (**Figure 1A**). In addition to neurons, a population of GPER immunoreactive cells showed a small perikaryon surrounded by tiny cell processes, a morphology that is characteristic of glial cells. These cells with glial morphology were observed in all the studied brain regions and in some of these regions, such as in the *stratum radiatum*, the *stratum lacunosum* and the *stratum moleculare* of the hippocampus, they represented the vast majority of the immunoreactive cells (**Figures 1C,D**).

GPER Positive Cells in the Amygdaloid Nucleus

Representative examples of GPER immunoreactivity in the amygdala of male and female animals are shown in **Figure 2**. Qualitative observation of GPER immunopositive cells in the amygdaloid nucleus revealed some differences in the pattern of staining among the different experimental groups. These differences were confirmed by the morphometric analysis. ANOVA analysis revealed significant differences among experimental groups in the central amygdala (CeM) [$F_{(2,13)} = 23.10$; $P = 0.001$; **Figure 3A**], posterodorsal medial amygdala (MePD) [$F_{(2,14)} = 17.49$; $P = 0.002$; **Figure 3B**] and basolateral medial amygdala (BLA) [$F_{(2,12)} = 25.89$; $P = 0.001$; **Figure 3C**]. The *post-hoc* analysis revealed lower number of GPER immunopositive cells in estrus females that in males in the CeM ($P = 0.001$) and the MePD ($P < 0.05$) (**Figures 3A,B**). In contrast, females in diestrus showed a higher number of GPER immunoreactive cells than males in the BLA ($P < 0.05$) (**Figure 2C**). Moreover, estrus females showed a lower number of GPER immunoreactive cells than diestrus females in the CeM ($P < 0.01$), MePD ($P < 0.01$), and BLA ($P < 0.01$). No significant differences between the experimental groups were found in the basomedial (BMA) [$F_{(2,12)} = 0.828$; $P = 0.38$; **Figure 3D**] and anteroventral medial (MeAV) amygdala [$F_{(2,13)} = 0.76$; $P = 0.41$; **Figure 3E**].

GPER Positive Cells in the Dorsal Hippocampus

Representative examples of GPER immunoreactive cells in the dorsal hippocampal formation are shown in **Figure 4**. ANOVA analysis showed significant differences in the *stratum oriens* (SO) [$F_{(2,10)} = 12.13$; $P = 0.01$; **Figure 5A**] and the *strata radiatum-lacunosum-moleculare* (SRLM) [$F_{(2,10)} = 16.40$; $P = 0.005$; **Figure 5B**]. The *post-hoc* analysis revealed a significantly lower number of GPER immunoreactive cells in diestrus females compared to males in the SO ($P < 0.01$) and the SRLM ($P < 0.05$). Moreover, diestrus females displayed also a lower number of GPER immunopositive cells than estrus female animals in the same regions: SO ($P < 0.05$) and SRLM ($P < 0.05$). In contrast, no significant differences among the experimental groups were detected in the *stratum pyramidale* (SP) [$F_{(2,10)} = 0.08$; $P = 0.78$; **Figure 5C**].

Significant differences in the number of GPER immunoreactive cells among experimental groups were also detected in the dentate gyrus. Thus, ANOVA analysis showed significant differences in the *stratum moleculare* (SM) [$F_{(2,10)} = 12.69$; $P = 0.009$; **Figure 5E**] and the *hilus* [$F_{(2,10)} = 10.89$; $P = 0.013$; **Figure 5F**], but not in the *stratum granulare* (SG) [$F_{(2,10)} = 1.30$; $P = 0.29$ **Figure 5D**]. Diestrus females showed a lower number of GPER immunoreactive cells than males in the SM ($P < 0.01$). In addition, diestrus females showed also a lower number of GPER immunopositive cells than estrus females in both the SM ($P < 0.05$) and the *hilus* ($P < 0.05$) (**Figures 5E,F**).

DISCUSSION

Previous studies have shown that GPER is widely distributed in the brain (50, 53, 55, 56, 60, 73). Indeed, GPER has been shown to be expressed by neurons, astrocytes and oligodendrocytes (56, 57, 59, 74–77) and GPER immunoreactivity has been detected by electron microscopy in both neuronal and glial profiles in the hippocampus (59), which is consistent with the detection of GPER immunoreactivity in cells with either neuronal or glial morphology in our study. Furthermore, we have detected a punctiform pattern of immunoreactivity that is absent in the cell nucleus, in agreement with the reported subcellular localization of GPER, either in the plasma membrane or in the endoplasmic reticulum and Golgi apparatus (52, 54, 57, 78–80).

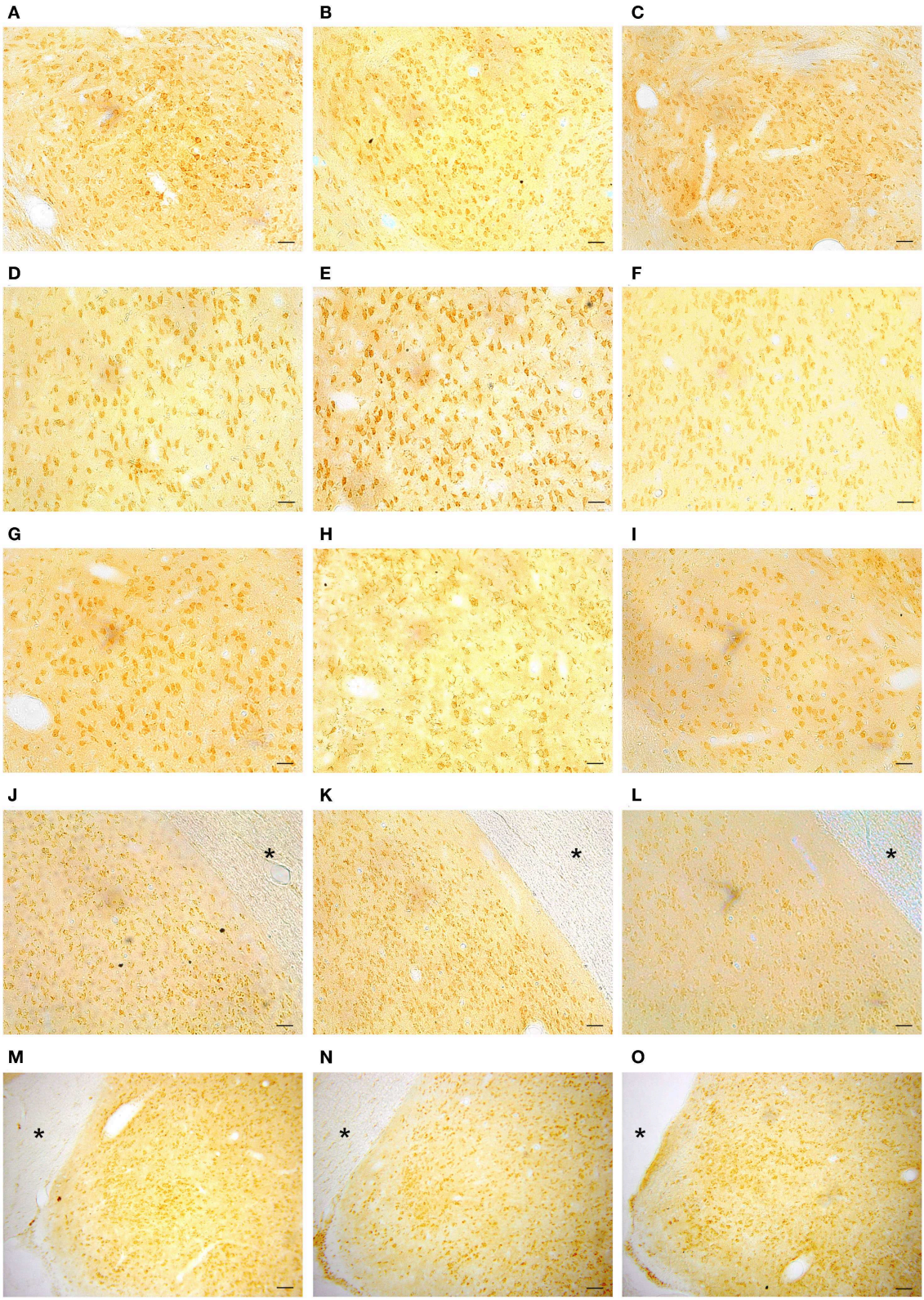

FIGURE 2 | Representative examples of GPER immunohistochemical localization in rat amygdaloid nucleus in male animals (left column; **A,D,G,J,M**) and in females during diestrus (central column; **B,E,H,K,N**) and estrus (right column; **C,F,I,L,O**). **(A–C)** Central amygdala (CeM), **(D–F)** Basolateral amygdala (BLA), **(G–I)** Basomedial amygdala (BMA), **(J–L)** Medial posterodorsal amygdala (MePD), **(M–O)** Medial anteroventral amygdala (MeAV). *Optic tract. Scale bar, 50 μm.

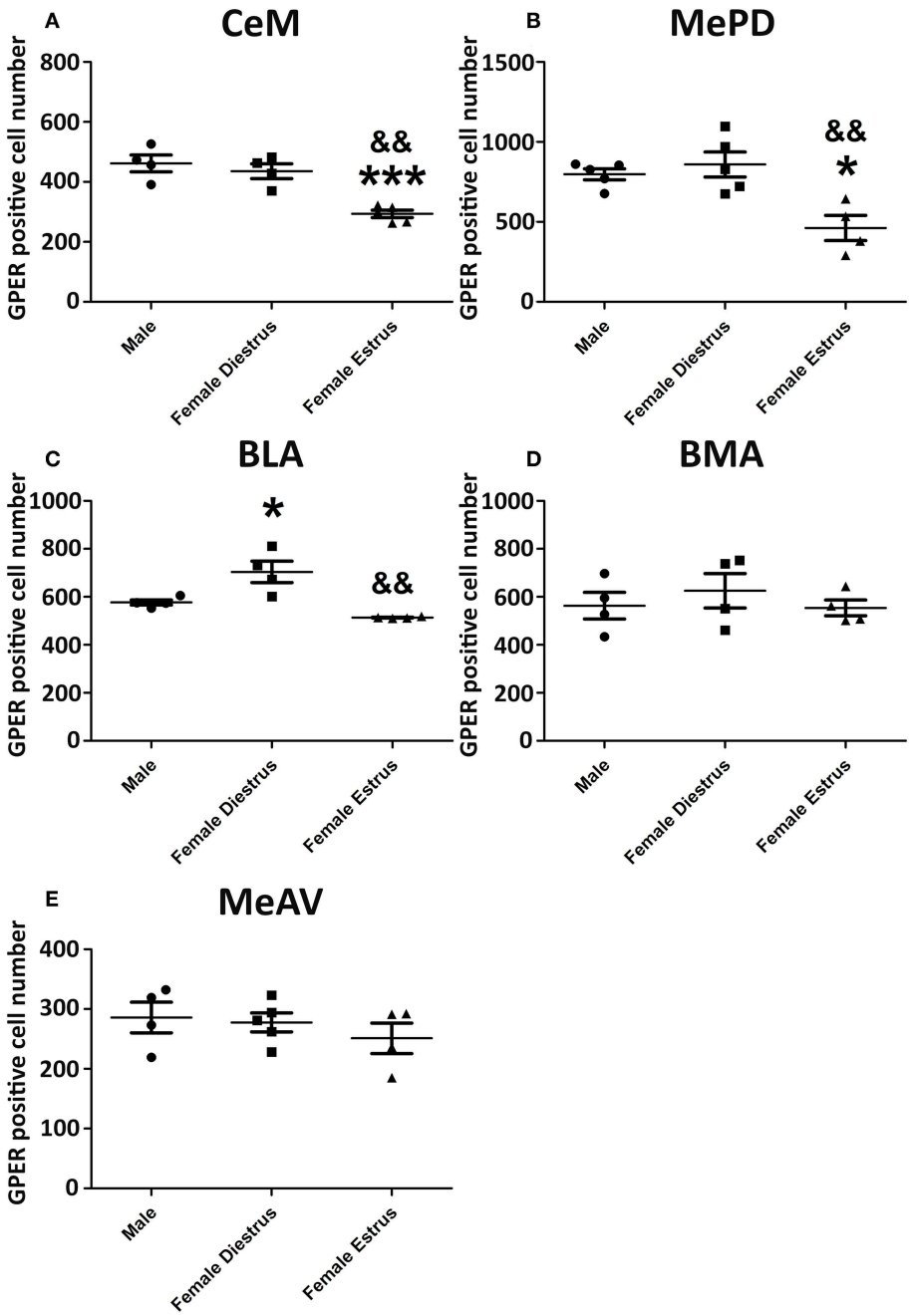

FIGURE 3 | Number of GPER immunoreactive cells in the amygdaloid nucleus of male, diestrus females and estrus female rats. **(A)** Central amygdala. **(B)** Medial posterodorsal amygdala. **(C)** Basolateral amygdala. **(D)** Basomedial amygdala. **(E)** Medial anteroventral amygdala. Data are represented as mean±SEM. *, ***Significant differences (*$p < 0.05$ and ***$p < 0.001$) vs. male values. && Significant differences ($p < 0.01$) vs. females in diestrus.

To explore possible changes in GPER immunoreactivity during the estrous cycle we performed a semi-quantitative analysis of the number of GPER immunoreactive cells. Although our findings need to be confirmed by unbiased stereology, they suggest that the immunoreactive levels of GPER fluctuate during the estrous cycle in the amygdala and the dorsal hippocampus with regional specificity. Thus, significant differences in the number of GPER immunoreactive cells are observed between estrus and diestrus in the central, posterodorsal medial and basolateral amygdala; in the stratum oriens and the *strata radiatum-lacunosum-moleculare* of the Ammon's horn and in the molecular layer and the *hilus* of the dentate gyrus. These fluctuations in the number of GPER immunoreactive cells between estrous cycle stages are associated with transient

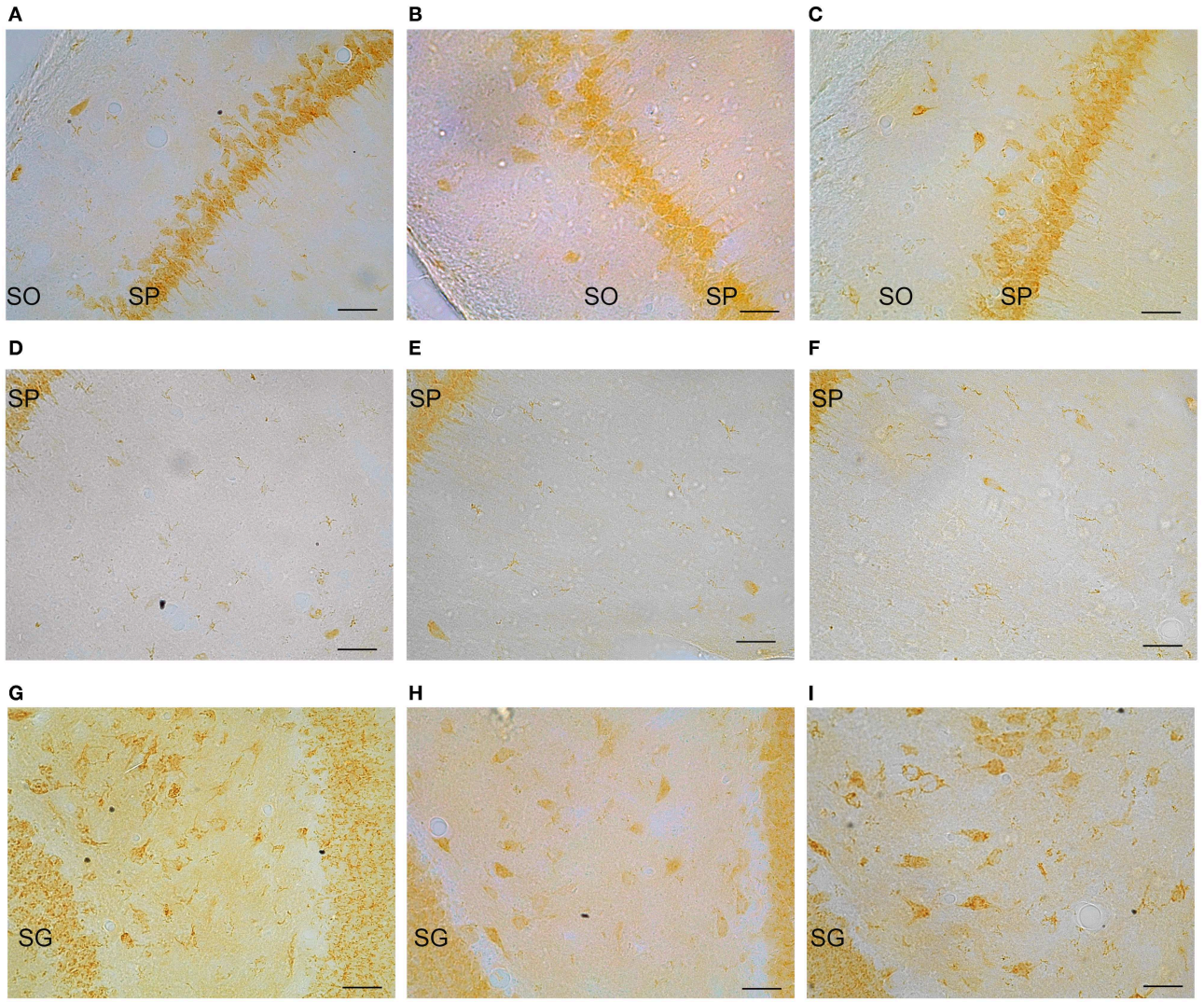

FIGURE 4 | Representative examples of GPER immunohistochemical localization in rat hippocampal formation in male animals (left column; **A,D,G**) and in females during diestrus (central column; **B,E,H**) and estrus (right column; **C,F,I**). **(A–C)** *stratum oriens* (SO) and *stratum pyramidale* (SP) in CA1, **(D–F)** *stratum pyramidale* (SP) and *strata radiatum-lacunosum-moleculare* in CA1. **(G–I)** Dentate gyrus, *stratum granulare* (SG) and *hilus*. Scale bar, 20 μm.

sex differences in GPER immunoreactivity that are also regionally specific.

Our findings extend the results of previous studies showing changes during the estrous cycle in the number of GPER immunoreactive axonal, dendritic and glial profiles in the mouse hippocampal formation (59). Sex differences in GPER expression have been also reported in primary hippocampal neurons (49). Another study has reported increased GPER mRNA levels in the amygdala of male hamster compared to females (60). In addition, differences in the mRNA levels of GPER between different estrous cycle days have been detected in other rat brain regions, such as the nucleus of the solitary tract, the ventrolateral medulla and the periaqueductal gray (81).

One of the limitations of the immunohistochemical analysis is that it cannot discriminate between full length functional receptors and other inactive forms. Therefore, we can only speculate on the possible functional significance of the fluctuation in the number of GPER immunoreactive cells in the amygdala and hippocampus during the estrous cycle and the associated sex differences. Differences in GPER levels may contribute to synaptic changes during the estrous cycle in the posterodorsal medial amygdala, the basolateral amygdala, the central amygdala and Ammon's horn (82–86) and may be also associated with the fluctuation in adult neurogenesis in the dentate gyrus of adult females in response to the cyclic changes in plasma estradiol levels (33). Specifically, GPER has been shown to be involved in the regulation of excitatory and inhibitory transmission in the basolateral amygdala (61, 63, 86) and in the regulation of adult neurogeneis in the hippocampus (58). In addition, previous studies have shown that GPER in

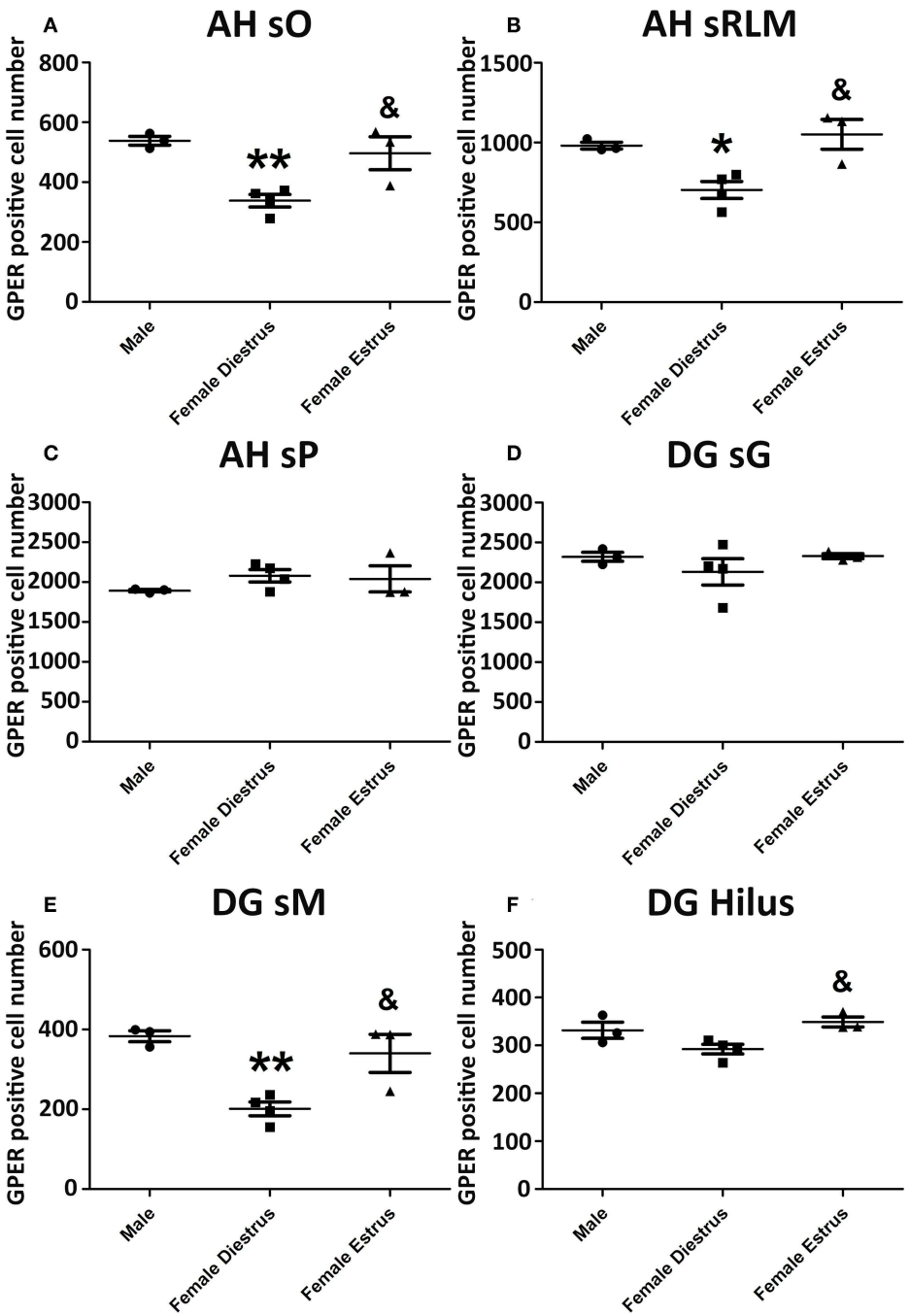

FIGURE 5 | Number of GPER immunoreactive cells in the in hippocampal formation of male, diestrus females and estrus female rats. **(A)** Ammon's horn, *stratum oriens* (SO). **(B)** Ammon's horn, *strata radiatum-lacunosum-moleculare* (SRLM). **(C)** Ammon's horn, *stratum pyramidale* (SP). **(D)** Dentate gyrus, *stratum granulare* (SG). **(E)** Dentate gyrus, *stratum moleculare* (SM). **(F)** *Hilus*. Data are represented as mean±SEM. *, ** Significant differences (*$p < 0.05$; **$p < 0.01$) vs. male values. & Significant difference ($p < 0.05$) vs. females in diestrus.

the basolateral amygdala mediates effects of estradiol on anxiety (64). Furthermore, GPER in the medial amygdala and the dorsal hippocampus participate in the modulation of social recognition by estradiol (23, 24, 87). Moreover, GPER in the dorsal hippocampus also mediates effects of estradiol on object recognition and spatial memory (23, 24, 87–90). Therefore, the observed modifications in GPER immunoreactivity in the amygdala and hippocampus may affect the actions of estradiol on these structures to regulate anxiety, social recognition, object recognition and spatial memory.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Universidad Nacional de Educación a Ditancia (UNED) bioethics comitee in compliance with the Spanish Royal Decree 53/2013 and the European Directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

DG, LG-S, and PC designed and supervised the experiments. JF-G, RL, MM, DG, BB, BC, JS-A, PA-P, MR-A, and HP performed the experiments. RL and JS-A prepared the figures for publication. DG, RL, and LG-S wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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A Cell-Based Method to Detect Agonist and Antagonist Activities of Endocrine-Disrupting Chemicals on GPER

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Endocrine-disrupting chemicals (EDCs) are exogenous compounds that impact endogenous hormonal systems, resulting in adverse health effects. These chemicals can exert their actions by interfering with several pathways. Simple biological systems to determine whether EDCs act positively or negatively on a given receptor are often lacking. Here we describe a low-to-middle throughput method to screen the agonist/antagonist potential of EDCs specifically on the GPER membrane estrogen receptor. Application of this assay to 23 candidate EDCs from different chemical families reveals the existence of six agonists and six antagonists.

Keywords: GPER, endocrine-disrupting chemicals, pharmacology, screening, fibroblasts

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INTRODUCTION

Endocrine-disrupting chemicals (EDCs) can be defined as exogenous compounds that can interfere with hormonal signaling (1). Chemicals classified as EDCs are produced for various industrial purposes (for example as components of pesticides, cosmetic products or plastic components) and belong to different chemical families (for example alkylphenols, parabens or phthalates). EDCs can accumulate in the environment (with varying levels of persistence), diet as well as body fluids and tissues. The adverse health effects elicited by EDCs can be diverse. For instance, as evaluated by epidemiological studies and/or experimental set up, exposure to Bisphenol A (BPA, a prototypical EDC) is correlated with increased cancer risk, obesity and reproductive health defects (2–5). EDCs can impact diverse levels of endocrine signaling, ranging from hormone production to hormone receptor expression and downstream signaling. Mechanistically, their action can be mediated by several receptors onto which they act as agonists or antagonists. For instance, BPA has been reported to dysregulate the activity of several nuclear receptors, such as the estrogen receptors (ERs), androgen receptor (AR) or estrogen-related receptor γ (ERRγ) [(6–11); reviewed in (12)]. EDC receptors are often co-expressed in given cells and tissues, complicating the mechanistic interpretation of the results. As a consequence, determining whether an EDC targets a given receptor leading to the dysregulation of discrete pathway(s), can be a laborious task. There is thus a need for simple systems in which a measurable effect can be directly ascribed to the (dys)regulation of a single receptor/pathway.

G protein-coupled estrogen receptor (GPER, previously known as GPR30 or GPER1) is a membrane-localized receptor with capacities to bind estrogens (13–15) and to crosstalk with the classical nuclear estrogen receptors (16, 17). GPER signaling is involved in various physiological

and pathological processes such as metabolic regulations, diabetes and atherosclerosis, or cancer progression (18–21), suggesting that it could contribute, at least in part, to some of the adverse effect of EDCs. In support to this hypothesis, studies at the cellular level have shown that, in addition to endogenous estrogens, GPER activity can be modulated by several compounds including synthetic selective agonists or antagonists (e.g., G-1 or G-15 and G-36, respectively), selective estrogen receptor modulators (SERMs) and bisphenols (22–25). Altogether, this suggests a capacity of GPER to respond to a broad spectrum of chemicals, including EDCs, many of which remain unknown. This also points to the need of defining a simple test to easily identify agonists/antagonists of this pathway.

Our previous work (26) showed that primary human dermal fibroblasts (hDF), which do not express ER, display a quantifiable morphological change in response to 17β-estradiol (E2), in a strict GPER-dependent manner. This suggested that this cellular phenomenon could be used as a read-out of GPER activation. However, primary cultures originate from different donors. Thus, inter-individual variability in the response as well as a possible exhaustion of the cell batches, may jeopardize reproducibility and efficiency. Using the MRC5 human fibroblast cell line, we here report the establishment of a method for a low-to-middle throughput screen of compounds acting on GPER as agonist or antagonist. We apply this cell-based method to define the capacity (or lack thereof) of 23 EDCs from various chemical families to modulate GPER activity.

MATERIALS AND METHODS

Cells

Cells were cultured in DMEM supplemented with 10% FCS, 10 U/ml penicillin and 10 μg/ml streptomycin (complete medium). For proliferation tests and evaluation of compound toxicity, 2×10^4 MRC5 cells were seeded in 96-well plates and assessed for cell number using CellTiterGlo kit (Promega).

For cell shape studies using the Cytonote lens-free cell imaging device (Iprasense, Montpellier, France), 10,000 MRC5 cells were seeded in 400 μl of complete medium in 4-chambers culture dishes. After 24 h, medium was changed to 600 μl phenol red-free DMEM without serum and cells were further incubated for 48 h. Ten microliter phenol red-free medium containing the tested compound were then added and cell cultures were immediately analyzed for 3 or 4 h in the Cytonote system. ImageJ was used to analyze the reconstituted images of the cell cultures at time point 0, 60, 120, 180, and 240 min after compound addition. Except were indicated, 30 cells per experiment were individually followed at all these time points, at which the ratio long axis to short axis was measured. Suspected antagonists were added 15 min before agonists.

For immunofluorescence experiments, cells (40% confluent) were cultured on glass slides, fixed with 4% paraformaldehyde and then washed with PBS 1x. FITC-phalloidin (P5283, Sigma, 1/750) was then added for 1 h. Nuclei were counterstained with Hoescht staining. Pictures were taken with Zeiss-Axiovert and images were processed and analyzed with the open-source package ImageJ with custom plug-in routines.

Compounds

All compounds used were resuspended in DMSO in 1,000x stock solutions. Characteristics and provenance of EDCs used in this study are shown on **Table S1**. 17β-estradiol (E2) was purchased from Sigma-Aldrich, G-1 from Cayman, G-15 and G-36 from Tocris.

Expression Analyses

For siRNA transfection, 3×10^{-5} cells per ml were seeded in 6-well plate and 25 pmol/ml of siRNA were transfected with INTERFERin (Polyplus Transfection) according to the manufacturer's recommendations.

For Western blot analysis, cells were lysed in NP40 buffer supplemented with Protease Inhibitor Cocktail (Sigma Aldrich). Proteins (50 μg) were resolved on 10% SDS-PAGE, blotted onto PVDF membrane (GE-Healthcare) and probed with specific antibodies after saturation. Primary antibodies used in this study were: hsp90 (API-SPA-830, Enzo Life Sciences, 1/3,000), ERα (sc-8002 F-10 Santa Cruz, 1/1,000), and GPER (sc-48525-R, Santa Cruz, 1/500). Secondary antibodies were: anti-rabbit IgG for ERα and GPER, anti-mouse IgG for hsp90 (W4011 and W402B, respectively; Promega, 1/10,000).

Total RNAs were extracted by the guanidinium thiocyanate/phenol/chloroform method. One microgram of RNA was converted to first strand cDNA using the RevertAid kit (ThermoScientific). Real time PCRs were performed in 96-well plates using the IQ SYBR Green Supermix (BioRad). Data were quantified by ΔΔ-Ct method and normalized to 36b4 expression.

Sequences of the PCR primers used in this study:

36b4: 5′-GTCACTGTGCCAGCCCAGAA-3′ and 5′-TCA ATGGTGCCCCTGGAGAT-3′

GPER: 5′-AGGGACAAGCTGAGGCTGTA-3′ and 5′-GTC TACACGGCACTGCTGAA-3′

Sequences of the siRNA used in this study:

GPER#1: 5′-GGCUGUACAUUGAGCAGAA-3′ and 5′-UUC UGCUCAAUGUACAGCC-3′

GPER#2: 5′-AGCUGAGGCUGUACAUUGA-3′ and 5′-UCA AUGUACAGCCUCAGCU-3′

Statistical Analyses

Distribution of L/s Ratio Over Cells

Using data from the E2 (10^{-7} M) condition obtained on 244 cells, the L/s ratio data distribution was examined using the Shapiro-Wilk normality test. The distribution of L/s ratios observed at each time, as well as the distribution of L/s ratio differences between two exposure times for each cell were found significantly non normal ($p < 10^{-5}$). In addition, homogeneity of variance was tested with the Levene's test. It showed that variance of L/s ratio differences between two times significantly differs ($p < 10^{-8}$). Therefore, L/s ratio was summarized by its median over cells for each condition and only non-parametric statistical tests were used.

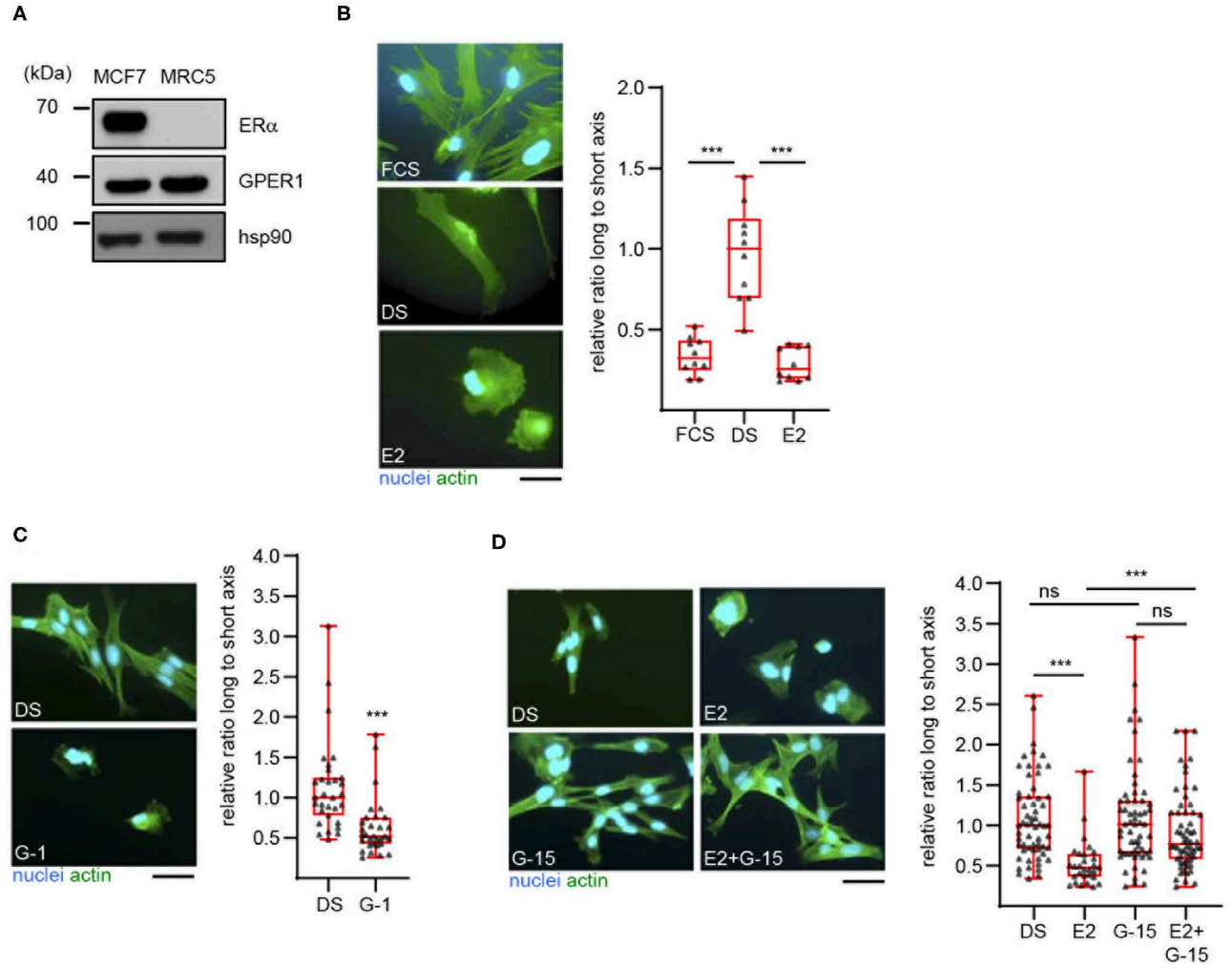


FIGURE 1 | 17β-estradiol induces a morphological change in MRC5 cells in a GPER-dependent manner. **(A)** Expression of the indicated proteins was determined in MCF7 and MRC5 cells by Western blot. Hsp90 was used as a loading control. Significance relative to time 0 (or between times 120 and 240, as indicated) was analyzed using Wilcoxon signed-rank test. ***$p < 0.0005$. **(B)** MRC5 cells were cultured in the presence of untreated (FCS), or desteroidated serum-containing medium supplemented with vehicle (DS) or 10^{-7} M 17β-estradiol (E2). **(C)** MRC5 cells were treated with 10^{-7} M G-1, a GPER agonist. **(D)** Cells were treated with 10^{-8} M E2 and/or 10^{-7} M G-15, a GPER antagonist. Actin and nuclei were stained. Ratio between the long and short cell axes was determined using ImageJ. Data are expressed relative to the median ratio under DS conditions. **(B)** $n = 10$; **(C)** $n = 30$; **(D)** $n = 60$. Significance was determined using Mann-Whitney test. ***$p < 0.0005$; ns: non significant. Scale bar: 50 μm.

L/s Ratio Normalization

For each condition, individual L/s ratios were expressed as relative to the median value at exposure time 0 min.

Comparing L/s Ratios

The comparison of L/s ratios between more than 2 exposure times used the non-parametric Friedman test for repeated measures. *Post-hoc* tests between two times used the Wilcoxon signed-rank test with Bonferroni *p*-value correction. When considering L/s ratios for 2 exposure times, the Wilcoxon signed rank test was used. The comparison of L/s ratios between several conditions or experiments at exposure time 180 used the Kruskal-Wallis test followed by the Mann-Whitney test for comparison between 2 groups. Statistical significance was taken at $p < 0.05$.

RESULTS

To extend our previous findings to a model cell line, we examined the estrogenic response of MRC5 cells, an immortalized human fibroblast cell line. Western blot analysis first showed that these cells indeed express GPER, but not the classical estrogen receptor α (**Figure 1A**). As expected, both receptors were found expressed in the human mammary cell line MCF7. The effect of E2 on the morphology of MRC5 cells was next examined (**Figure 1B**). To this end, cells were exposed to culture medium containing untreated serum (FCS, Fetal Calf Serum) or desteroidated serum (DS) supplemented or not with E2. After fixation, actin was labeled to visualize cell morphology. The longest and shortest cell axes (L and s, respectively) were measured and cell shape was expressed as the ratio (L/s) between these axes. We noted

a more elongated shape (i.e., high L/s ratio) in cells exposed to DS medium, as compared to FCS-exposed cells. Interestingly, E2 supplementation reversed this phenotype. It is unlikely that this phenomenon involves cell proliferation. Indeed, whereas proliferation of MRC5 cells was abolished in DS medium as compared to FCS one, E2 addition did not reverse this effect (**Figure S1A**).

These data suggest that E2 promotes MRC5 cell spreading in a GPER-dependent manner. To prove this dependence, we first searched to inactivate GPER in MRC5 cells, using siRNAs. However, these siRNAs were efficient at the RNA- but not at the protein level (**Figure S1B**), suggesting a high stability of GPER protein and preventing the use of siRNAs in our experiments. We thus turned to a pharmacological approach. We observed that exposure to G-1, a GPER synthetic agonist, efficiently reduces cell elongation (**Figure 1C**). We also used G-15, a GPER synthetic antagonist (**Figure 1D**). By itself, this compound is unable to induce any morphological change in MRC5. However, G-15 efficiently blocks the E2-induced cell spreading, indicating that GPER mediates this estrogenic response. Altogether, MRC5 cells display E2-responses that are similar to primary hDF and could thus be used to measure GPER activation without interference from ERα.

However, the above method measures cell shape at experimental end-point and thus does not provide a dynamic view of cell shape changes at an individual level. To circumvent these limitations, we used a lens-free, live-cell imaging device that allows the monitoring of a large number of cells. Images obtained with this system were then analyzed to determine changes in the L/s ratio of individual cells according to treatment. To set up the experimental conditions, MRC5 cells were first seeded in FCS-containing medium and then switched to serum-free medium (**Figure S2A**). A significant cell elongation was observed 12 h after medium change, a phenomenon that endured for at least 48 h. For all subsequent experiments, treatment was thus applied after 48 h incubation in serum-free medium. Under these conditions, addition of E2 (10^{-7} M) resulted in a significant reduction of L/s ratio, as measured on 244 cells, that was obvious 120 and 240 min after treatment initiation (**Figure 2A**). We next wanted to determine the minimal number of cells to be measured that would allow to reaching statistical significance. To this end, we randomly sampled 100 sets of 10, 30, 40, 50, 60, 80, 100, or 200 cells from our initial 244-cell data set. Medians of L/s ratio were calculated for each extracted sample. As expected, the dispersion of the medians decreases when increasing the sample size (**Figure 2B**). Medians of the 10-cell samples at a given time point are sometimes found overcrossing the 244-cell median at another time point. Thus, the analysis of only 10 cells in a given experiment could lead to false negative results. This effect was not observed for samples comprising 30 cells or more.

Statistical significances of the changes observed in the L/s medians were then determined on the same data sets (**Figure 2C**). Again, data sets comprising 10 cells often failed to reach significance (i.e., $p < 0.05$). In contrast, the use of 30-cell data sets allowed to reaching significance at the global level, i.e., considering all three time points together. This was also the case when comparing time points two-by-two, except for the smaller variations between 120 and 240 min. An additional experiment set with expanded time points showed a continuous reduction of L/s ratio along exposure time (**Figure S2B**). However, the difference between 180 and 240 min after E2 addition, although statistically significant, was much reduced. This indicates that recording cell shape up to 180 min after treatment initiation is fairly sufficient to observe a statistically significant effect. Under these conditions, supplementation with DMSO, the vehicle used for E2 as well as for all hereafter used compounds, did not impact L/s ratio (**Figure S2C**). We next tested the reproducibility of our observation. To this end, we performed three independent experiments and observed similar reduction of L/s ratio upon E2 treatment, whereas DMSO had no effect (**Figure S2D**). Importantly, the L/s values reached after 180 min within each treatment type were not significantly different from one experiment to the other. Altogether, our data show that an E2-induced effect can be reliably evidenced by measuring the L/s ratio of 30 MRC5 cells 180 min after treatment onset.

To further characterize this effect, we investigated the dose-response of L/s ratio to E2 (**Figure 3A**). A decrease of this ratio was observed for E2 concentrations from 10^{-7} to 10^{-10} M, although the latter dose displayed a moderate effect. Applying 10^{-11} or 10^{-12} M did not induce any change in cell shape. As shown above, MRC5 cells express the GPER membrane estrogen receptor, but not the classical ERα nuclear receptor, suggesting that the effect of E2 may be mediated by GPER. Consistently, treating cells with the GPER synthetic agonist G-1 resulted in an effect similar to that obtained with E2 treatment (**Figure 3B**). In contrast, the GPER antagonist G-15, by itself innocuous on L/s ratio, completely blocked the reduction of cell elongation exerted by E2. Co-treatment with G-1 and G-15 resulted in a moderate decrease of L/s ratio. However, further statistical analysis showed that this effect was not significantly different from that of G-15 alone. The effect of E2 was also blocked by another GPER synthetic antagonist, G-36 (**Figure S3**). We conclude that MRC5 cell elongation reflects GPER activation status.

To further validate this hypothesis, we focused on a synthetic compound, bisphenol A (BPA), reported to activate GPER (22, 23, 27). Cell viability tests indicated that exposure to 10^{-5} M BPA did not significantly impact MRC5 cell survival (**Table S2**). We then measured L/s ratio after treatment with various BPA concentrations, with 10^{-6} M as the highest (**Figure 4A**). We observed a time-dependent, dose-dependent reduction of L/s ratio upon BPA exposure. Co-treatment with G-15 partially impaired this effect (**Figure 4B**). As in the case of G-1 and G-15 co-treatment above, statistical analysis showed that the effect exerted by BPA+G-15 did not significantly differ from that obtained with G-15 alone, indicating that BPA induces morphological changes in a GPER-dependent manner. We next studied the effects of three related bisphenol compounds. BPC and BPF dose-dependently reduced the L/s ratio (**Figures 4C,D**), an effect that was blocked by co-treatment with G-15 (**Figure 4E**), indicating that these chemicals activate GPER. In contrast, BPE was inactive at all concentrations tested (**Figure 4F**). We tested the possibility that BPE could behave as a GPER-antagonist,

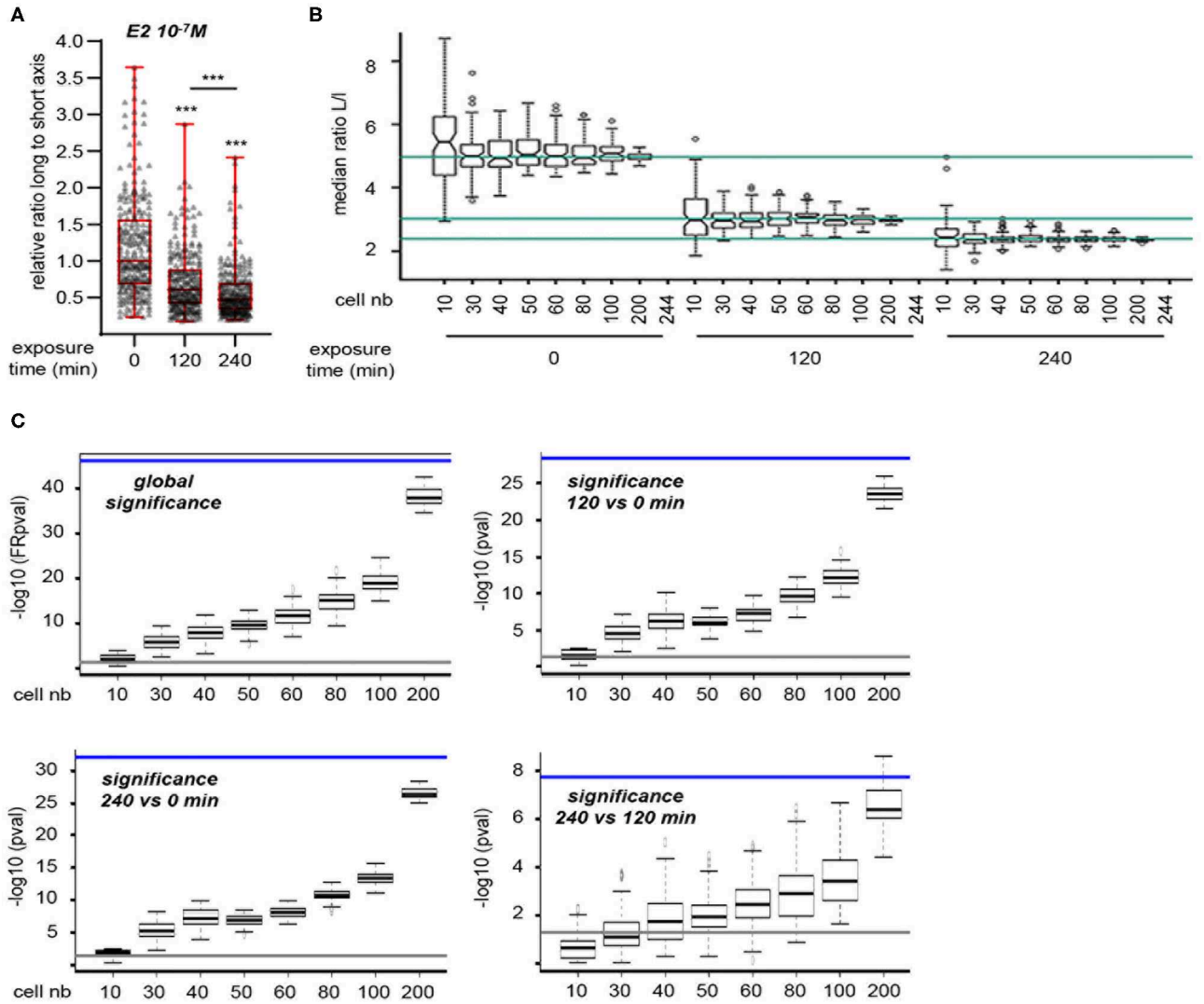
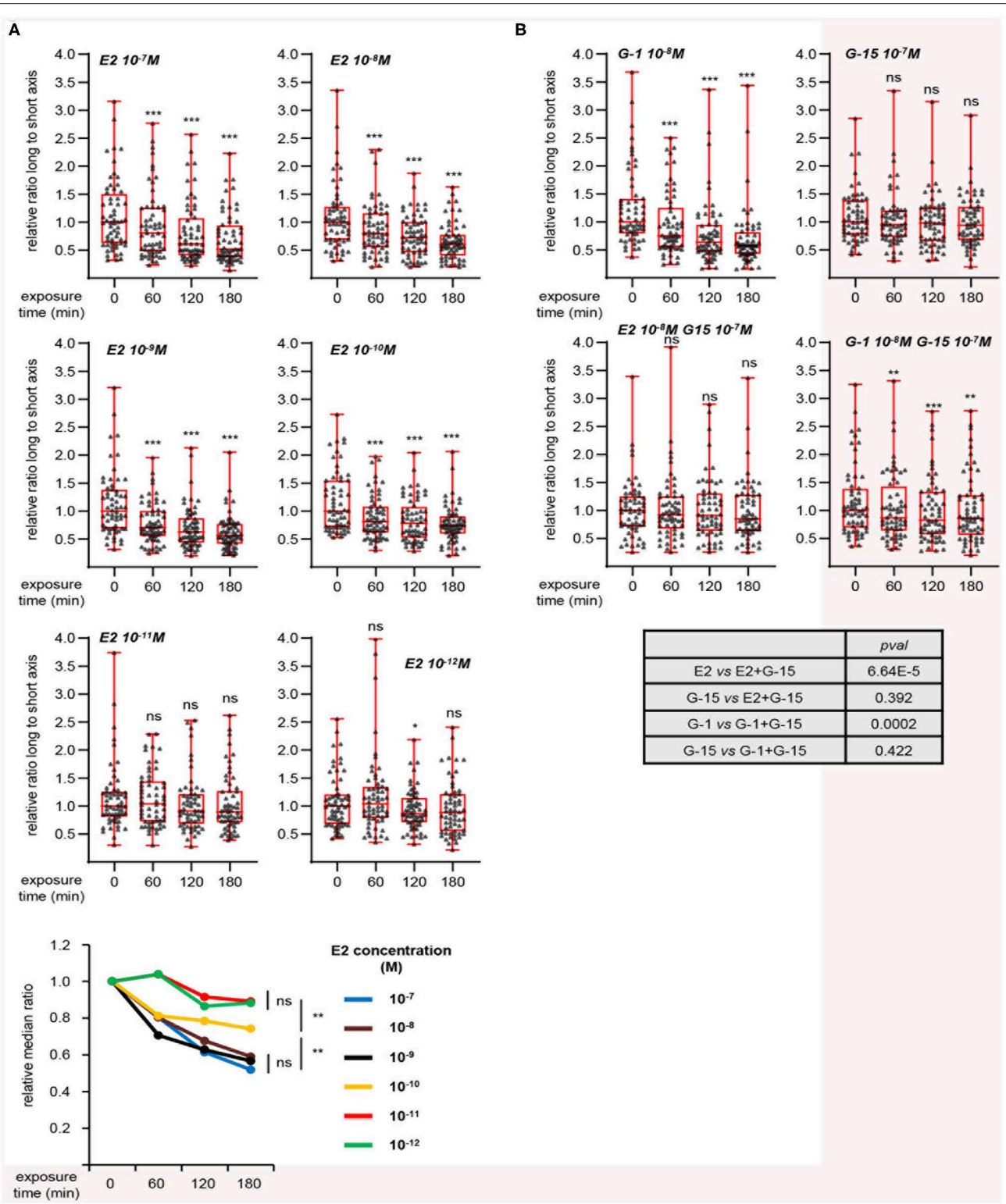


FIGURE 2 | Statistical validation of the approach. **(A)** Cells were exposed to 10^{-7} M E2. 244 cells were individually analyzed for L/s ratio. **(B)** Medians of these ratios at 0, 120, and 240 min are indicated for reference by the turquoise lines (top to bottom, respectively). Hundred sets of 10, 30, 40, 50, 60, 80, 100, or 200 cells were randomly sampled. Medians of L/s ratio were calculated for each extracted sample and are plotted. As expected, the dispersion of the medians decreases when increasing the sample size. Medians of the 10-cell samples at a given time point are sometimes found overcrossing the 244-cell median at another time point. Thus, the analysis of only 10 cells in a given experiment could lead to false negative results. **(C)** Statistical significances between exposure times were determined on the same data set as in **(B)** (comprising the 244 original cells as well as the random samples of varying size). Friedman test was used to determine the global significance (upper left graph), Wilcoxon signed-rank test was used when comparing two time points. Data are expressed as –log10 (*pval*). Significance obtained using the 244 cells original set is shown for reference as a blue line. Gray lines represent the lowest value considered as significant [i.e., –log10 (0.05)]. As expected, values increase when increasing sample size. Thirty-cell sample size always produces significant *pval*, except when comparing time 120 min to time 240 min.

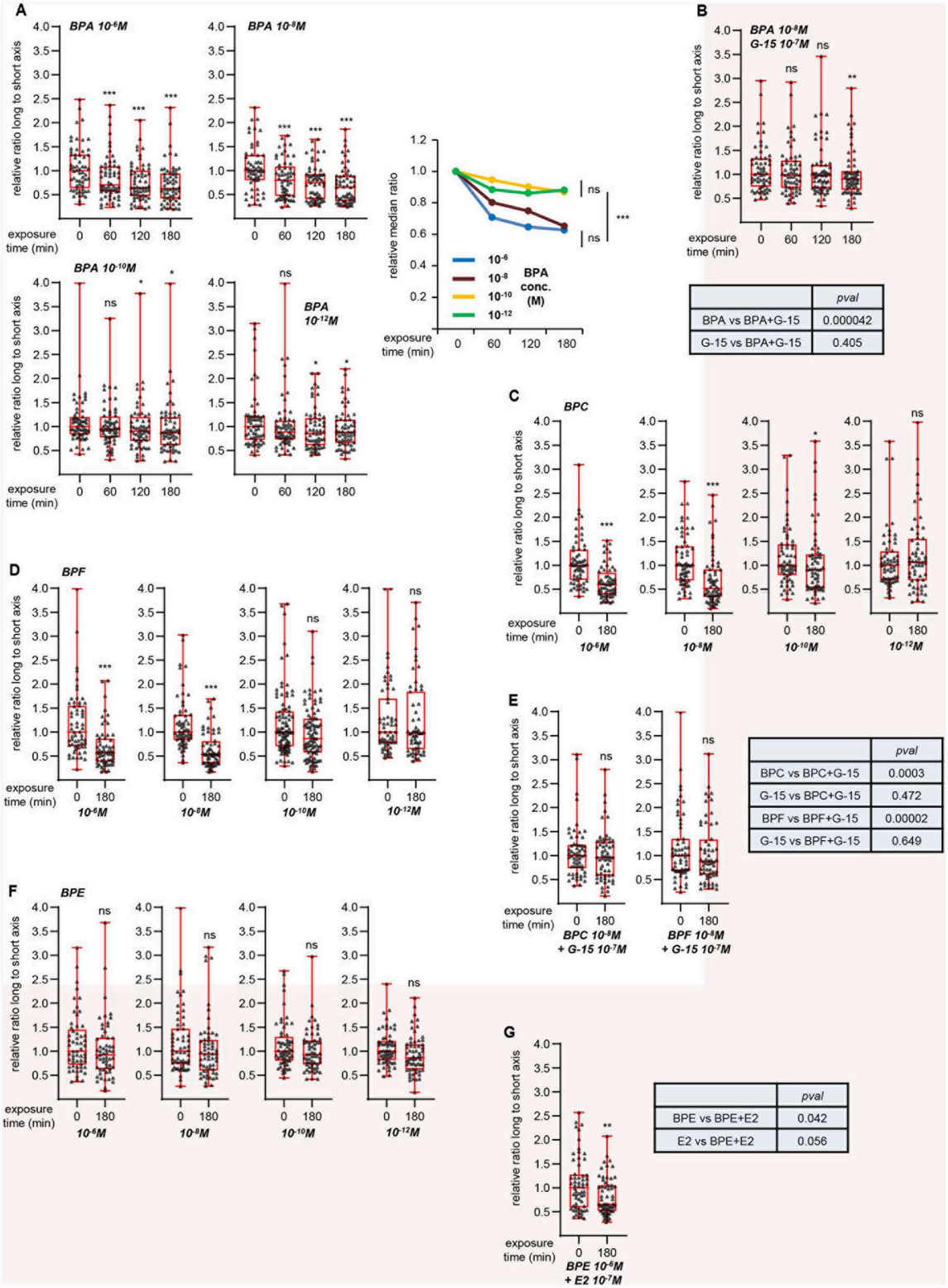
rather than agonist. However, BPE did not inhibit the effect exerted by E2 (**Figure 4G**).

The data above indicate that the dynamic measure of L/s ratio is a read-out for agonist or antagonist effects exerted on GPER. This approach could thus be used as a screening method to determine whether a given compound, including EDCs, targets GPER. The effect of agonists would be blocked by co-treatment with G-15, whereas antagonists would inhibit the action of E2. To validate this possibility, we focused on 19 compounds (see characteristics on **Table S1**), reported to act as EDCs, belonging to different chemical families and with different applications. We first examined their toxicity in MRC5 cells (**Table S2**). We then evaluated these compounds at three different concentrations for their capacity to impact on L/s ratio. For each compound, the maximal concentration that we used was 10-fold less than the highest non-toxic dose. All data are shown on **Figures S4A–S** and summarized on **Table 1**. Four compounds (chlorpyrifos, DEHP, dienochlor and quinoxyfen) induced a dose-dependent reduction of the L/s ratio, to an extent that was comparable to what observed with E2. Co-treatment with G-15 resulted in



	pval
E2 *vs* E2+G-15	6.64E-5
G-15 *vs* E2+G-15	0.392
G-1 *vs* G-1+G-15	0.0002
G-15 *vs* G-1+G-15	0.422

FIGURE 3 | Dynamic measurement of morphological changes induced by E2 in a GPER-dependent manner. **(A)** Cells were treated with the indicated concentration of E2 and individually followed. Images generated with the Cytonote system were analyzed using ImageJ. Ratio long to short axis (L/s) was determined for individual cells followed at the indicated times. Data are expressed relative to the median of L/s ratio at time 0. Graphs represent two pooled experiments, each including 30 cells. Statistical significance relative to time 0 was analyzed using Wilcoxon signed-rank test. Bottom graph summarizes the above data, with the relative medians plotted as a function of time. For this graph, significance at time 180 min was analyzed using Mann-Whitney test. **(B)** Same as above analyzing the effect of G-1 and G-15 (GPER agonist and antagonist, respectively), alone or in combination as indicated. Bottom table displays the significance (estimated by Mann-Whitney tests) of the indicated comparisons. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$; ns: non significant.



	pval
BPA vs BPA+G-15	0.000042
G-15 vs BPA+G-15	0.405

	pval
BPC vs BPC+G-15	0.0003
G-15 vs BPC+G-15	0.472
BPF vs BPF+G-15	0.00002
G-15 vs BPF+G-15	0.649

	pval
BPE vs BPE+E2	0.042
E2 vs BPE+E2	0.056

FIGURE 4 | GPER-dependent morphological changes induced by bisphenols. Same approach as **Figure 3**. **(A)** Effect of the indicated concentrations of bisphenol A (BPA). Right graph summarizes the data, with the relative medians plotted as a function of time. For this graph, significance at time 180 min was analyzed using Mann-Whitney test. **(B)** Effects of G-15 co-treatment on BPA exposure. **(C,D)** Effects of bisphenol C (BPC, **C**) and F (BPF, **D**) at the indicated concentrations. **(E)** Effect of G-15 pretreatment on BPC and BPF. **(F)** Effects of bisphenol E (BPE) at the indicated concentrations. **(G)** Effect of BPE pretreatment on E2 exposure. All graphs represent pools of two independent experiments, each with $n = 30$ cells. Significance relative to time 0 was analyzed using Wilcoxon signed-rank test. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$; ns: non significant. Tables displays the significances (estimated by Mann-Whitney tests) of the indicated comparisons.

TABLE 1 | Effect of EDC exposure on L/s axis in MRC5 cells.

	Effect at max. conc.		Effect with G15			Effect with E2			Comments
	Conc.	Rel. med (% at 180 min)	Rel. med (% at 180 min)	Signif. vs. EDC only	Signif. vs. G15 only	Rel. med (% at 180 min)	Signif. vs. EDC only	Signif. vs. E2 only	
Chlorpyrifos	10^{-6} M	56.52	93.15	0.00003	0.419		nd		Agonist
DEHP	10^{-6} M	61.39	94.84	0.0769	0.8479		nd		Trend to agonist
Dienochlor	10^{-8} M	63.37	80.11	0.0145	0.097		nd		Agonist
Quinoxyfen	10^{-7} M	66.29	92.94	0.0056	0.813		nd		Agonist
4-Tert-Octylphenol	10^{-7} M	81.41		nd		69.37	0.0632	0.0381	Weak antagonist
Fenitrothion	10^{-6} M	82.04		nd		75.54	0.854	0.007	Antagonist
Tau-Fluvalinate	10^{-7} M	84.52		nd		55.12	0.0002	0.466	No effect
Bifenthrin	10^{-6} M	84.57		nd		54.73	7.901E-08	0.459	No effect
Chlorpyrifos-methyl	10^{-6} M	85.46		nd		60.99	0.024	0.3186	No effect
Cypermethrin	10^{-6} M	86.01		nd		88.74	0.532	0.0001	Antagonist
Dieldrin	10^{-6} M	86.24		nd		63.28	0.069	0.217	No effect
Ethylparaben	10^{-6} M	86.88		nd		69.94	0.0054	0.1691	No effect
Azoxystrobin	10^{-6} M	87.18		nd		69.32	0.3779	0.0003	Weak antagonist
Malathion	10^{-8} M	87.34		nd		94.06	0.879	0.00004	Antagonist
Imidacloprid	10^{-7} M	88.22		nd		68.07	0.014	0.195	No effect
Methylparaben	10^{-7} M	89.78		nd		59.60	7.382E-06	1.000	No effect
Penconazole	10^{-6} M	90.98		nd		65.29	0.006	0.268	No effect
Deltamethrin	10^{-6} M	91.31		nd		99.56	0.793	0.0001	Antagonist
Piperonyl-Butoxide	10^{-7} M	107.55		nd		63.78	2.929E-07	0.644	No effect
E2	10^{-7} M	51.9	84.77	0.00006	0.392		na		Agonist
G15	10^{-7} M	94.21		na		84.77	0.00006	0.392	Antagonist

*Cells were exposed to the indicated EDC. L/s ratios were determined for individual cells at 0 and 180 min after EDC addition. Results (representing two experiments, each measuring 30 cells) are expressed as the median of L/s ratios at 180 min relative to 0 min. Where indicated, cells were co-treated with G-15 or E2. Significance was estimated using Mann-Whitney test. Only the effect of the maximal concentration is shown for each EDC (see **Figures S4A–S** for complete data set). Effects observed with E2 or G-15 (complete results on **Figure 3**) are displayed for reference. na, not applicable; nd, not determined.*

effects that were *i*- not significantly different from that of G-15 alone and *ii*- different from those observed when using each compound individually (although significance was not reached when comparing DEHP to DEHP+G-15). We concluded that these four chemicals behaved as GPER agonists.

In contrast, when used alone, the other 14 compounds tested here displayed a more moderate effect (or lack thereof) on L/s ratio. Co-treatment with E2 revealed that some of these chemicals behaved as antagonists. For example, the L/s ratios resulting from malathion + E2 co-treatment were strongly different from what obtained with E2 alone, but not different from what observed with malathion alone. Oppositely, compounds such as penconazole did not block the effect of E2, suggesting that they are inactive on GPER.

DISCUSSION

In this report, we show that E2 induces a dose-dependent, time-dependent morphological change in the MRC5 human fibroblastic cell line. This leads to cell spreading which can be quantified by measuring the ratio between the long and the short cell axes. This effect is analogous to that previously observed in human dermal fibroblasts (hDF) in primary culture (26) as well as in breast cancer cells (28, 29). These actions of E2 do not depend on the classical nuclear estrogen receptors but on GPER, a seven-transmembrane domain estrogen receptor, as well as its downstream effectors ERK1/2. In hDF, this was formally proven by the loss of E2 effects upon shRNA-mediated GPER inactivation. As documented on **Figure S1**, transient genetic inactivation of GPER is inefficient in MRC5 cells. Stably inactivating the receptor (e.g., using a Crispr-Cas9 approach) is difficult to envision given that the selection procedure would likely exceed the low number of possible cell passages in culture. Pharmacological approaches however show that the effects of E2 rely on GPER. Indeed, this phenomenon can be mimicked by supplementation with the synthetic GPER agonist G-1 and the effects of E2 can be blocked by co-treatment with the GPER antagonists G-15 and G-36.

The work presented here points to the possibility of a general method to screen compounds for their capacity to signal through GPER. Cell fixation and staining are not required, enabling a dynamic monitoring of individual cells along treatment. Furthermore, the use of the Cytonote lens-free device allows to visualizing large fields and thus a large number of cells with rapid image acquisition. Our statistical analyses show that

considering as few as 30 cells for 3 h is enough to reach significance. The method used here appears very sensitive and, in some cases, statistical tests can demonstrate that very small changes in the L/s ratio are strongly significant at the population level. However, the relevance of these small variations can be questioned, in particular when no relation between dose and response is observed. For instance, penconazole exerts a 10% reduction of the L/s ratio at 10^{-8} M (with *pval*: 0.008), but not at 10^{-6} or 10^{-10} M (see **Figure S4Q**). Similarly, dieldrin exerts a 12–15% reduction of the relative L/s ratio with $pval < 0.05$ at all three concentrations tested (i.e., without dose-response relations; see **Figure S4K**). Noteworthy, exposure to 10^{-12} M BPA also results in a 12% reduction of the L/s ratio (with $p \sim 0.01$; **Figure 4A**). However, similarly to E2, BPA displays a clear dose-dependent effect with a strong reduction of the L/s ratio at maximal concentration (∼40% at 10^{-6} M). It thus cannot be excluded that small variations in L/s ratio reflect experimental noise rather than relevant signal. With such considerations in mind, it appears sensible to consider only the compounds that induce large variations (i.e., within the range of those observed upon E2 exposure) in L/s ratio as GPER agonists. Altogether, the GPER-specific method described here appears easy to set up, robust and cheap.

As a proof-of-concept, we have performed a low throughput screen in which 23 synthetic compounds were examined for their capacity to modulate GPER activation. To the best of our knowledge, most of these compounds have not been reported for an effect on GPER (or lack thereof). Seven compounds (bisphenols A, C and F, chlorpyrifos, DEHP, dienochlor and quinoxyfen) were found to display agonist activities, according to the above criterion. In support to this view, co-treatment with G-15 abolished the cellular effect elicited by these chemicals. In contrast, 16 compounds appeared inactive on cell morphology when used alone. The capacity of six of these chemicals to block the activities of E2 allows to consider them as GPER-antagonists, whereas the remaining 11 do not appear to exert any effect on GPER. Remarkably, structurally related compounds do not necessarily fall into the same category. Consistent with the current literature, BPA acted as a GPER agonist (22, 23, 27), as did BPC and BPF. In contrast, BPE, which only differs from BPF and BPA by the presence or absence of a methyl group (respectively), was completely inactive on GPER. In this line, we also observed that chlorpyrifos and chlorpyrifos-methyl displayed different behaviors (agonist and inactive, respectively) toward GPER. In contrast, the related compounds cypermethrin and deltamethrin both acted as GPER antagonists. Structural studies will be required to determine the bases of these differences and similarities. Our work identified EDCs that positively or negatively modulate the activities of GPER in normal human fibroblasts. On another hand, activation of GPER in breast cancer-associated fibroblasts (CAFs) promotes cancer progression (30–32). Whether the EDCs identified here also modulate GPER activities in CAFs remains to be investigated, as well as the consequences of these possible regulations.

In summary, we propose our approach as a potential screening method to determine whether a given compound agonizes or antagonizes GPER. Of note, an effect observed here of GPER does not exclude possible actions on other receptors, such as ERα or AR. Furthermore, the present assay is purely cell-based and cannot be used to predict the effects of chemicals *in vivo*.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SP, CF, and VT: performed experiments. CC: performed experiments and analyzed data. J-MV: planned experiments, analyzed data, and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00547/full#supplementary-material

Figure S1 | Analysis of MRC5 cells. **(A)** MRC5 cells were cultured in the presence of untreated (FCS), or desteroidated serum-containing medium supplemented with vehicle (DS) or 10^{-7} M E2. Proliferation is shown relative to day 0. Values are the mean of two independent experiments performed in triplicate with error bars representing SEM. Significance (relative to day 0) was analyzed using Student *t*-test. ***$p < 0.0005$; ns: non significant. Graph on the right zooms the lower part of the left graph. **(B)** Expression of GPER mRNA (left) and protein (right) 72 h after transfection with the indicated siRNA. Left: analysis was performed by real-time PCR. Data are presented relative to siControl-treated samples and are the average of two independent experiments performed in triplicate. Error bars represent SEM. Significance (relative to siC) was analyzed using Student *t*-test. *$p < 0.05$; **$p < 0.005$; ns: non significant. Right: expression of the indicated proteins after transfection with the indicated siRNA. Hsp90 was used as a loading control.

Figure S2 | Validation of the Cytonote approach in MRC5 cells. **(A)** Cells were seeded in untreated serum-containing medium (FCS) then switched to DMEM medium in the absence of serum. Graph represents a single experiment with $n = 30$. **(B)** Cells were treated with 10^{-7} M E2. Graph represents a pool of two independent experiments, each with $n = 30$. **(C)** Cells were treated with 1/1,000 (vol/vol) DMSO. Graph represents a pool of two independent experiments, each with $n = 30$. **(D)** Cells were treated with DMSO (1/1,000 vol/vol) or 10^{-7} M E2. Each graph represent a single experiment with $n = 30$. Lower graph presents the relative median at 180 min (expressed as % from time 0) for each experiment. Global *pval* comparing all DMSO or all E2 treatment was calculated using Kruskal-Wallis test. Data on graphs are expressed relative to the median of the L/s ratio at time 0. Statistical significance was analyzed using Wilcoxon signed-rank test. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ns: non significant.

Figure S3 | Antagonistic activities of G-36. Cells were treated with E2 and/or the GPER antagonist G-36 and analyzed at the indicated time. Statistical significance was determined using Wilcoxon signed-rank test. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ns: non significant. Tables displays the significances (estimated by Mann-Whitney tests) of the indicated comparisons.

Figure S4 | Morphological changes induced by endocrine disrupting compounds on MRC5 cells. Cells were treated with the indicated compounds at the various concentrations. **(A–D)** Compounds, significantly inducing a morphological change in MRC5, were also used in combination with the GPER antagonist G-15. **(E–S)** Compounds that did not significantly induce any morphological change were also used in combination with E2. Data are also summarized on **Table 1**. All graphs represent two pooled independent experiments, each with $n = 30$. Statistical significance was determined using Wilcoxon signed-rank test. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ns: non significant.

Table S1 | Characteristics of the compounds used in this study.

Table S2 | Evaluation of cell viability after exposure to the compounds used in this study. Cell number was estimated after 48 h treatment with the indicated compound and expressed relative (%) to treatment with vehicle (DMSO). Results represent mean of two experiments performed in triplicate and are expressed relative (%) to treatment with vehicle ± s.e.m. Significance was estimated used Student t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; ns: not significant. nd: not determined.

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GPER as a Receptor for Endocrine-Disrupting Chemicals (EDCs)

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Endocrine-disrupting chemicals (EDCs) are exogenous chemicals that interfere with endogenous hormonal systems at various levels, resulting in adverse health effects. EDCs belong to diverse chemical families and can accumulate in the environment, diet and body fluids, with different levels of persistence. Their action can be mediated by several receptors, including members of the nuclear receptor family, such as estrogen and androgen receptors. The G protein-coupled estrogen receptor (GPER), a seven-transmembrane domain receptor, has also attracted attention as a potential target of EDCs. This review summarizes our current knowledge concerning GPER as a mediator of EDCs' effects.

Keywords: GPER, hormone, estrogen, pathophysiology, endocrine-disrupting chemicals (EDCs)

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ENDOCRINE-DISRUPTING CHEMICALS

According to a general definition, endocrine-disrupting chemicals (EDCs) are exogenous compounds that interfere with the endogenous hormonal axes at any level (1). This includes synthesis, metabolism, transport and delivery of hormones, and also perturbation of the expression of hormone receptors as well as with the downstream signals they convey. EDCs comprise compounds that can promote or restrict a hormonal signal (acting as agonists or antagonists, respectively). Under this broad definition, EDCs include natural molecules such as the phytoestrogens (e.g., genistein, which is abundant in soy) that modulate estrogen signaling and also synthetic compounds intended for therapeutic purposes, such as the ones used as adjuvant therapy in breast cancer. Examples of the latter category include inhibitors of aromatase used to reduce the endogenous synthesis of 17β-estradiol (E2) or tamoxifen that act as an antagonist of the estrogen receptor in mammary tumors.

EDCs also comprise chemicals that are produced for various industrial purposes, being used as components of several products (plastics, paints, flame retardants, herbicides, pesticides…), that exert unintended impacts on hormonal signaling. The number and variety (in terms of chemical structure) of molecules that display suspected or validated endocrine disrupting effects increased since years (1). Furthermore, these compounds often display high levels of resistance to natural degradation leading to their accumulation in the environment as well as in body fluids [see (2–4) for examples]. Adverse effects of EDCs have been reported in domains covering all fields related to hormonal signaling, including metabolism, reproduction, induction and progression of hormone-sensitive cancers and neurodevelopment (1).

To investigate the effects of EDCs, it is essential to identify the receptors that mediate their action as well as the downstream cascades they elicit. Given that EDCs largely impact the male and female reproductive axes, it was initially suspected that their effects were largely mediated by the

sex steroid receptors (5). These include the estrogen receptors (ERs) and the androgen receptor (AR), which are members of the nuclear receptor (NR) family and act as transcription factors. In line with this, several EDCs were demonstrated to modulate the activities of these NRs. However, at least in some cases, such as that of the paradigmatic EDC Bisphenol A (BPA), the affinity of these compounds for ERα appeared far lower than that of their natural ligand (6), suggesting the existence of other proteins acting as EDC receptors. Consistently, it was shown that BPA binds to ERRγ, an orphan NR which does not recognize E2, and induces its downstream activities (7, 8). As far as we are aware, the capacity of ERRγ to serve as a receptor for EDCs other than bisphenols has not been published. In contrast, an array of publications suggests that the G protein-coupled receptor (GPER) may serve as a receptor for a vast spectrum of EDCs. The purpose of this review is to (non-exhaustively) summarize what we currently know concerning the relationships between GPER and EDCs.

GPER, AN ALTERNATIVE ESTROGEN RECEPTOR

GPER (initially referred to as GPR30) has been identified as a membrane associated estrogen receptor 15 years ago (9, 10). This seven-transmembrane domain receptor is broadly expressed and has been detected in several sub-cellular localizations, including in internal membrane compartments, such as the endoplasmic reticulum, nucleus and even as a chromatin binding protein under certain circumstances (11). It is expected that different molecular functions could be exerted by GPER, depending on its sub-cellular localization (summarized on **Figure 1**). Indeed, membrane activation of GPER was shown to rapidly promote intracellular calcium mobilization, cAMP production and to induce a phosphorylation cascade in particular involving ERK1/2, PKA, and PI3K (9, 10, 12–14). On another hand, chromatin binding of GPER leads to direct transcriptional activation of target genes (11).

GPER cross-talks with different receptors to convey its downstream effects. For instance, functional interactions with the aryl-hydrocarbon receptor (AhR) or EGF receptors (EGFR) are instrumental for the activation of downstream MAPK activation (12, 15). GPER also functionally interacts with nuclear receptors at various levels. For instance, GPER is required for the effect of aldosterone mediated by the mineralocorticoid receptor (MR) in breast cancer cell lines (16). A more indirect level of cross-talks can be illustrated by the regulation of the circulating level of thyroid hormone which in turn modulates embryonic heart rate in a thyroid hormone receptor-dependent manner (17). Functional interactions between GPER and ER have been abundantly documented, may depend on the cell type considered and may lead to congruent or opposing effects [reviewed in (18)]. For example, in ovarian cancer cells, both GPER- and ER-mediated signals are involved in the activation of ERK1/2 leading to increased c-fos expression and induction of proliferation (19). On another hand, at least in ER-positive breast cancer cells, tamoxifen acts as an ER antagonist, but as a GPER agonist (9). Altogether, this shows that GPER displays a wide array of molecular functions and interactions with other signaling pathways. Given its broad expression spectrum and its described pathophysiological functions, GPER has emerged as a factor of clinical importance [reviewed in (20)].

PATHOPHYSIOLOGICAL FUNCTIONS OF GPER

The functions of GPER have been investigated using *in vivo* and *in vitro* approaches. GPER knocked-out mice [reviewed in (21)] reproduce normally, indicating that GPER is not absolutely required for reproduction. However, pharmacological studies (i.e., using treatments with agonists and antagonists) suggest that GPER intervenes in uterine epithelial proliferation, suggesting a subtle impact on reproductive function that may be compensated for in the absence of the receptor. Other *in vivo* studies have indicated that GPER is involved, amongst others, in glucose and lipid metabolism, bone mass, skin pigmentation, regulation of heart rate, and immune and neural systems [(17, 22–24), reviewed in (25)].

The impact of GPER, as a novel estrogen receptor, on cancers has been extensively analyzed, in particular on hormone-related cancers (e.g., breast, ovary, and endometrium). Several studies report a pro-cancer effect of GPER (26, 27). Indeed, high GPER expression correlates to a poor prognosis in breast and endometrial carcinoma (28, 29). Consistently, GPER activation promotes various traits of cancer progression including cell migration in triple negative breast cancer cells, resistance to hypoxia and proangiogenic response (30–32). GPER is also active in cancer-associated fibroblasts (CAFs) where it favors tumor-promoting activities (33, 34).

In contrast, other studies rather indicate that GPER may exert anti-cancer roles. For instance, high expression of GPER has been reported as a factor of favorable prognosis in triple-negative breast cancers (35). Similarly, low level of GPER protein expression in the cytoplasm is associated with lower levels of disease free survival in breast cancer, even when eliminating potentially confounding factors such as ER/PR/HER2 status (36). Consistently, reports indicate that GPER activation leads to cell cycle arrest, apoptosis and cell death in ER-positive and –negative cell lines (37, 38). Interestingly, an inhibitory effect of GPER has also been noted in cancers that do not depend on estrogen signaling. Indeed, GPER inhibits epithelial-to-mesenchymal transition and cell invasion in prostate and pancreatic cancer cells (39). Furthermore, tamoxifen-mediated GPER activation impairs the conversion of pancreatic stellate cells into myofibroblasts (an equivalent of CAFs in pancreatic tumors), which in turn leads to reduced cancer cell survival (40, 41). Moreover, GPER-deficient mice display increased inflammation in induced liver tumorigenesis resulting in accelerated tumor growth (42).

To date, the roles of GPER in cancer thus appear unclear. However, it is possible to propose non-mutually exclusive hypotheses to solve these apparent contradictions. (i) GPER sub-cellular localization may impact its prognosis value (and

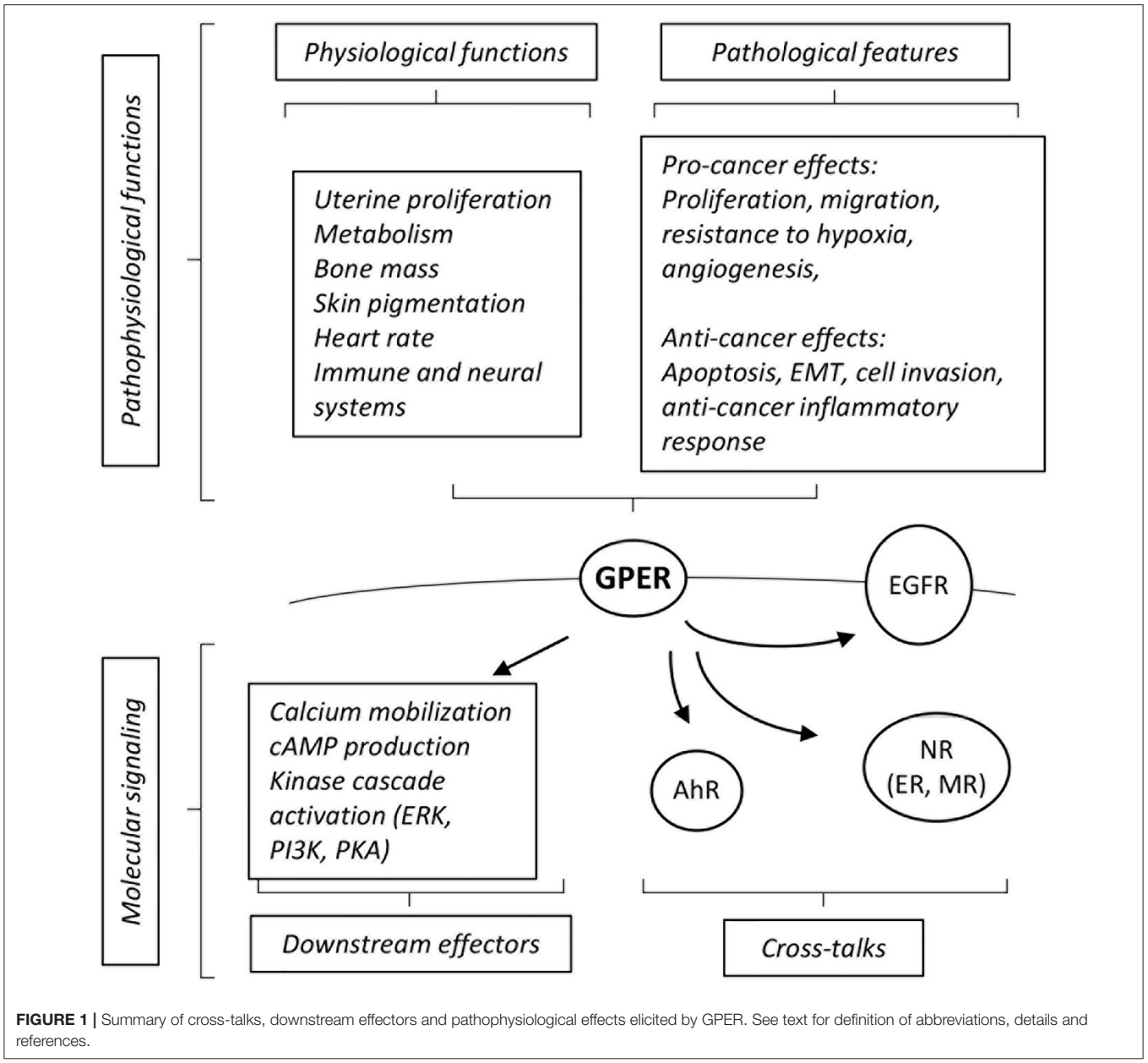

FIGURE 1 | Summary of cross-talks, downstream effectors and pathophysiological effects elicited by GPER. See text for definition of abbreviations, details and references.

its activities). In this respect, in contrast to its detection in the cytoplasm, the low nuclear expression of GPER does not correlate to breast cancer aggressiveness (36). (ii) GPER activities may depend on the tissues in which they are studied. It may indeed be envisioned that, in pancreas and liver, the anti-inflammatory effects displayed by GPER in non-cancer cells may overcome its capacity to promote tumor growth in cancer cells. (iii) GPER may exert different activities on the various steps of cancer progression. In a mouse model of mammary cancer, GPER indeed appears dispensable for cancer initiation but contributes to the establishment of metastasis (43). (iv) GPER may play different roles depending on the expression of cross-talking factor. For example, GPER promotes the growth of ER-negative SKBr3 cells, but reduces that of ER-positive MCF7 cells (44). Furthermore, the stimulating effect of GPER on ovarian cancer cells depends on EGFR (19). More work is obviously required to refine our knowledge on the impact of GPER on cancers.

IDENTIFYING CHEMICAL MODULATORS OF GPER ACTIVITIES

GPER was identified as a functional estrogen receptor in ER-negative cells by a combination of binding and functional studies (i.e., detection of GPER-dependent calcium mobilization or adenylyl cyclase activation) (9, 10), suggesting a shared repertoire between compounds acting on ER and on GPER (summarized on **Figure 2**). ER-binding ligands were thus examined and this led to

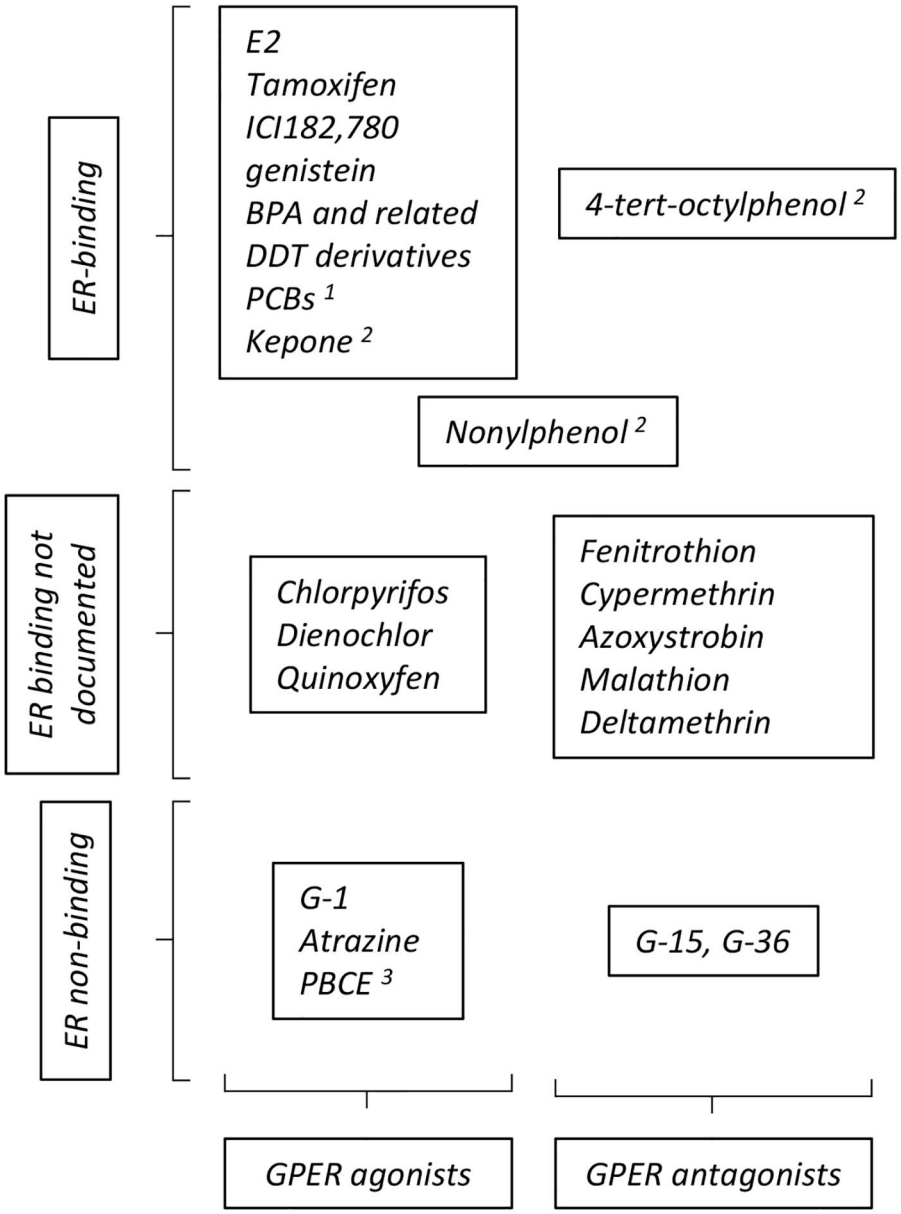

FIGURE 2 | Summary of compounds reported to act as GPER- agonists and antagonists. The particular position occupied by nonylphenol reflects contradictory reports ascribing this compound as GPER- agonist or antagonist. See text for definition of abbreviations, details and reference. Additional references for ER binding: 1: (45); 2: (46); 3: (47).

the surprising finding that tamoxifen and ICI182, 780 (two ER-antagonist used in adjuvant breast cancer therapy) actually acted as GPER-agonists. Furthermore, EDCs, acting as xenoestrogens on ER, including genistein, BPA, and DDT derivatives also impacted GPER, as shown by binding assays coupled to functional signaling assays (48). Although the affinity of these compounds for GPER is less than that of E2, they broadly display similar binding constants as those displayed on ER. However, the repertoires of compounds bound by GPER and ER are not strictly similar. For instance, the potent ER-agonist DES does not bind GPER (10). Moreover, functional screening identified specific synthetic GPER ligands (i.e., not recognizing the nuclear estrogen receptors) that act as agonist (G-1) or antagonists (G-15 and G-36) for GPER [reviewed in (49)]. Altogether, this shows that GPER and ER display both overlapping and distinct repertoires of compound recruitment. Furthermore, molecular modeling and *in silico* docking studies indicated that GPER offers several cavities to accommodate large volume ligands and suggested a broad number of possible binding compounds (50, 51). Indeed, competition assays and measurement of cAMP accumulation

revealed that organochlorides, such as polychlorinated biphenyls (PCBs) and kepone (*aka* chlordecone), act (amongst others) as GPER agonists (48).

The GPER-dependent consequences of EDC exposure in terms of molecular outcomes, as well as at the cellular, phenotypical levels have also been studied. The pesticide atrazine does not transactivate ER but induces GPER-dependent ERK activation in ovarian cancer cells and CAF, leading to increased proliferation and migration (52).

BPA induces proliferation and migration of ER-negative breast cancer cells and CAFs in a GPER-dependent manner (53, 54). Proliferation of mouse spermatogonial and Sertoli cells has also been shown as induced by BPA through GPER (55, 56). Intriguingly, analysis of the dose-response indicated a non-monotonous effect in form of an inverted U-shaped curve. Other bisphenols, used as substitutes for BPA and found in high concentrations (similar to or higher than those of BPA) in the environment and body fluids (57) have also been tested. As compared to BPA, some of these analogs, such as BPAF and BPB, display comparable binding affinities to GPER (as determined by E2 displacement), GPER activation capacities (as assessed by calcium mobilization and cAMP production) and, GPER-dependent induction of cell migration (58). Intriguingly, BPF did not display such activities although other studies indicated that its effects on hormonal axes was comparable to those of BPA [reviewed in (59)]. Other compounds such as polybrominated diphenyl ether (PBCE, used as flame retardant additives) that, as BPA, display a diphenyl core, also display GPER binding with an affinity in the micromolar range (60). These compounds induce cAMP accumulation, calcium mobilization and cell migration in ER-negative breast cancer cells.

Nonylphenol (NP) induces cardiac contractility in a non-monotonic manner (61). The effect at low doses is antagonized by G-15, suggesting that NP acts as a GPER agonist. Such an effect of NP has also been suggested on human ER-negative cells (48) as well as on zebrafish oocyte maturation, where this compound (as well as other alkylphenols, including BPA) blocks oocyte maturation, as does G-1 (62). In contrast, NP has been shown to counteract the action of G-1 as a moderator of asthma symptoms in mouse models (63), suggesting that this compounds acts as a GPER-antagonist. Whether these apparent discrepancies originate from the differences in the pathophysiological situations that are analyzed remains to be established.

CONCLUDING REMARKS

GPER is a promiscuous receptor displaying a broad spectrum of compound recognition, including toward EDCs. There is however a specificity of GPER-recognition within given chemical families, as exemplified by the bisphenol derivatives. It should be noted that most if not all of the studies examining the effects of EDC on GPER have been performed using cell cultures systems and seldom *in vivo*. *In vitro* cell models provide irreplaceable tools for their capacity to be experimentally manipulated. However, comparing the effects of EDCs in wild type and GPER-inactivated animals will greatly increase our understanding of the action of these compounds.

Although several of the compounds impacting on GPER have also been demonstrated to bind ERs, there is a level of selectivity, discriminating these receptors. Various levels of cross-talks have been demonstrated between GPER and other proteins such as ERs, EGFR, or AhR. Whether or not these cross-talks are effective in a given cellular system and may influence the outcome of GPER activation is not always understood. It will thus be of interest to assess the effects of EDCs as GPER modulators under conditions where these cross-talks are controlled.

GPER exerts a large array of pathophysiological functions. A level of overlap between these functions and the perturbations induced by exposure to EDCs is worth noting. Together, this places GPER as a strong candidate to mediate, at least part, of the adverse effects displayed by EDCs. As discussed above, the exact role of GPER in cancer initiation and progression is a matter of debate and may depend on the considered tissue and/or disease stage. How the modulation of GPER activities by EDCs impact cancer features is thus unclear but should be an important field of investigations in the near future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Reciprocality Between Estrogen Biology and Calcium Signaling in the Cardiovascular System

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17β-Estradiol (E_2) is the main estrogenic hormone in the body and exerts many cardiovascular protective effects. Via three receptors known to date, including estrogen receptors α (ERα) and β (ERβ) and the G protein-coupled estrogen receptor 1 (GPER, aka GPR30), E_2 regulates numerous calcium-dependent activities in cardiovascular tissues. Nevertheless, effects of E_2 and its receptors on components of the calcium signaling machinery (CSM), the underlying mechanisms, and the linked functional impact are only beginning to be elucidated. A picture is emerging of the reciprocality between estrogen biology and Ca^{2+} signaling. Therein, E_2 and GPER, via both E_2-dependent and E_2-independent actions, moderate Ca^{2+}-dependent activities; in turn, ERα and GPER are regulated by Ca^{2+} at the receptor level and downstream signaling via a feedforward loop. This article reviews current understanding of the effects of E_2 and its receptors on the cardiovascular CSM and *vice versa* with a focus on mechanisms and combined functional impact. An overview of the main CSM components in cardiovascular tissues will be first provided, followed by a brief review of estrogen receptors and their Ca^{2+}-dependent regulation. The effects of estrogenic agonists to stimulate acute Ca^{2+} signals will then be reviewed. Subsequently, E_2-dependent and E_2-independent effects of GPER on components of the Ca^{2+} signals triggered by other stimuli will be discussed. Finally, a case study will illustrate how the many mechanisms are coordinated to moderate Ca^{2+}-dependent activities in the cardiovascular system.

Keywords: estrogen, G protein—coupled estrogen receptor, calcium, calmodulin, calmodulin-binding proteins, cardiomyocytes, vascular smooth muscle, endothelium

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MAIN COMPONENTS OF THE CALCIUM SIGNALING MACHINERY (CSM) IN CARDIOVASCULAR TISSUES

The CSM herein refers to proteins responsible for the generation or sequestration of intracellular Ca^{2+} signals and their transduction to target activities. In this section, key CSM components in cardiovascular tissues will be briefly described to facilitate review of the relevant effects and mechanisms of estrogenic agonists and receptors.

Intracellular Ca^{2+} Stores, Release, and Uptake Mechanisms

Organelles Functioning as Intracellular Ca^{2+} Stores

The sarcoplasmic/endoplasmic reticulum (SR/ER) is the main Ca^{2+} store in cardiomyocytes, vascular smooth muscle cells (VSMCs) (1, 2), and endothelial cells (ECs), where the ER stores ∼75%

Ca^{2+} and mitochondria house ∼25% (3). The Golgi (4, 5) and lysosomes have more recently been recognized as Ca^{2+} reservoirs (6, 7). Ca^{2+} reaches 5×10^{-4} M in the ER/SR and lysosomes and $1.3–2.5 \times 10^{-4}$ M between the *trans*-Golgi and *cis*-Golgi (5, 8). The medial Golgi also releases Ca^{2+} in response to inositol-triphosphate receptor (IP_3R) and ryanodine receptor (RyR) stimulation (9). Crosstalk between the ER/SR and other organelles affects their Ca^{2+} fluxes (10–14). In neonatal cardiomyocytes, beat-to-beat oscillations in mitochondrial and cytosolic Ca^{2+} occur in parallel (15), and mitochondrial uptake reduces cytosolic Ca^{2+} (16).

Mechanisms of Ca^{2+} Uptake Into Ca^{2+} Stores

SR/ER Ca^{2+}-ATPases (SERCAs) are the key Ca^{2+} uptake mechanisms. For each ATP hydrolyzed, they pump 2 Ca^{2+} ions into the ER/SR in exchange for less than four H^+ ions (17). SERCA2b is ubiquitously expressed. SERCA2a predominates in cardiomyocytes and is essential for cardiac development (18). SERCA3 is the predominant vascular isoform; its deletion causes smooth muscle relaxation abnormality (19, 20). SERCA3 has lower affinity for Ca^{2+} and is only active at high Ca^{2+} levels. Non-phosphorylated phospholamban interacts with SERCA1a, SERCA2a, and SERCA2b and reduces their Ca^{2+} affinity. Phosphorylation at Ser16 and Thr17 removes phospholamban–SERCA interaction, promoting SERCA activity (21, 22). Sarcolipin also binds SERCAs and reduces their Ca^{2+} affinity. Its deletion increases SR Ca^{2+} uptake (23).

The secretory pathway Ca^{2+} pump (SPCA) mediates Ca^{2+} uptake into the Golgi with nanomolar affinity for Ca^{2+}. Unlike the SERCA, Ca^{2+} transport by SPCA is not associated with counter transport of H^+. In the medial Golgi, both SERCA and SPCA participate in Ca^{2+} uptake (9).

Mitochondrial Ca^{2+} uptake is mediated by the voltage-dependent anion channel (VDAC) and the mitochondrial Ca^{2+} uniporter (MCU). VDACs are non-selective anion channels in the open state yet in the "closed" state permit influxes of cations such as K^+, Na^+, and Ca^{2+} into the mitochondria (24). VDAC isoforms participate equally in transporting Ca^{2+} triggered by IP_3-producing agonists; however, VDAC1 selectively transports apoptotic Ca^{2+} signals (25). Myocardial VDAC2 regulates rhythmicity by influencing the spatial and temporal properties of cytoplasmic Ca^{2+} signals (26). The MCU constitutes a low-affinity yet selective Ca^{2+} channel pore as part of a mitochondrial Ca^{2+} uptake protein complex (MICU) and the essential MCU regulator (27, 28).

Mechanisms of Ca^{2+} Release From Ca^{2+} Stores

In IP_3Rs, IP_3 binds with $IP_3R2 > IP_3R1 > IP_3R3$ affinity order (29) and cooperatively switches IP_3R tetramers to an open conformation to form clusters and release Ca^{2+} (30, 31). IP_3Rs regulate Ca^{2+} release from the ER/SR, Golgi apparatus, and nucleus (32). ER/SR Ca^{2+} release depletes ER Ca^{2+} and triggers store-operated Ca^{2+} entry (SOCE). IP_3R2 predominates in the cardiomyocytes (33). In failing hearts, IP_3R-mediated Ca^{2+} transients are enhanced, and mitochondrial Ca^{2+} uptake is reduced, which facilitates contraction and spontaneous action potentials that increase arrhythmogenicity (34). In VSMCs, all IP_3Rs are expressed and are important for agonist-induced contraction (35). Endothelial IP_3R1 is predominant in the brain (36), whereas IP_3R2 and IP_3R3 are abundant in the aorta and pulmonary arteries (37, 38).

RyRs (RyR1–RyR3) are the main SR Ca^{2+} release channels (39). *Regulation by cytosolic* Ca^{2+}: In cardiomyocytes, RyR2 predominates (40) and is closed, activated, and inhibited, respectively, at $Ca^{2+} < 10^{-7}$ M, $\sim 10^{-7}$-10^{-5} M, and $> 10^{-3}$ M (41). Entry via voltage-dependent Ca^{2+} channels (VDCCs) stimulates Ca^{2+}-induced Ca^{2+} release (CICR) via RyR2, contributing to myocardial contraction. In VSMCs, RyR2 predominates in the aorta and pulmonary and cerebral arteries, while RyR3 is the only isoform in basilar arteries (42–44). CICR also contributes to VSMC contraction, but not as critically as in cardiomyocytes; indeed, skinned smooth muscle fiber bundles can contract at Ca^{2+} levels that do not activate RyRs (45). In ECs, RyR2 is on the ER and mitochondria (46); however, RyR agonists only cause a slow Ca^{2+} release that corresponds to a reduction in the IP_3-sensitive Ca^{2+} pool (47, 48). *Regulation by SR* Ca^{2+} is important in cardiomyocytes. SR Ca^{2+} overload triggers spontaneous RyR2-mediated Ca^{2+} release, a phenomenon called store overload-induced Ca^{2+} release (SOICR) (49, 50). SOICR can cause delayed afterdepolarizations leading to tachycardias and is abolished by an E4872A mutation in the RyR2 gate (51).

Ca^{2+} Entry

Store-Operated Ca^{2+} Entry (SOCE)

SOCE is a ubiquitous mechanism where Ca^{2+} store depletion triggers Ca^{2+} influx (52, 53). Proposed in the 1980s, SOCE was confirmed in the mid-2000s with the discoveries of the stromal interaction molecule 1 (STIM1) (54–56) and Orai Ca^{2+} channels (57–59). STIM1 resides mainly on the ER/SR membrane and has a luminal EF hand that houses a Ca^{2+}-binding loop (60). In Ca^{2+}-full ER/SR, the loop is in a closed conformation. Upon ER/SR Ca^{2+} depletion, Ca^{2+} leaving the loop promotes STIM1 oligomerization to interact with Orai1 channels and

Abbreviations: AF domain, transcriptional activation function domain; CaM, calmodulin; Ca^{2+}-CaM, Ca^{2+}-bound calmodulin; cAMP, cyclic adenosine monophosphate; CICR, Ca^{2+}-induced Ca^{2+} release; CRAC, Ca^{2+} release-activated channels; CSM, Ca^{2+} signaling machinery; E_2, 17β-estradiol; ECs, endothelial cells; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ERβ, estrogen receptor β; ERα, estrogen receptor α; ERK1/2, extracellular signal-related kinases 1 and 2; FRET, fluorescence resonance energy transfer; GPER, G protein-coupled estrogen receptor 1; GPR30, G protein-coupled estrogen receptor 1; HEK293 cells, human embryonic kidney 293 cells; $I_{Ca,L}$, L-type Ca^{2+} channel current; IP_3Rs, inositol-trisphosphate receptors; LTCC, L-type Ca^{2+} channels; LV, left ventricle; MAPK, mitogen-activated protein kinases; mCRC, mitochondrial Ca^{2+} retention capacity; MCU, mitochondrial Ca^{2+} uniporter; MEK1, MAP (mitogen-activated protein) kinase/ERK (extracellular signal-regulated kinase) kinase 1; mPTP, mitochondrial permeability transition pore; NCX, Na^+-Ca^{2+} exchanger; OVX, ovariectomy/ovariectomized; PDZ, PSD-95/Dlg/ZO; PKC, protein kinase C; PLCβ, phospholipase C-β; PMCA, plasma membrane Ca^{2+}-ATPase; PSD-95, post-synaptic density protein 95; RMP, resting membrane potential; RyRs, ryanodine receptors; SCPA, secretory pathway Ca^{2+} pump; SERCA, sarcoplasmic/endoplasmic reticulum-ATPase; SMD, submembrane domains of G protein-coupled receptors; SOCE, store-operated Ca^{2+} entry; SOICR, store overload-induced Ca^{2+} release; SR/ER, sarco-endoplasmic reticulum; STIM1, stromal interaction molecule 1; VDAC, voltage-dependent anion channel; VDCC, voltage-dependent Ca^{2+} channels; VDCE, voltage-dependent Ca^{2+} entry; VSMCs, vascular smooth muscle cells.

trigger Ca^{2+} entry (61–63). STIM1 also interacts with L-type Ca^{2+} channels (LTCCs) (64), maintains ER/SR structure (65–67), and is upregulated in atherosclerosis and hypertension (68–71). Myocardial SOCE is normally not prominent; however, STIM1 and SOCE are increased in heart failure (67, 72–76). In VSMCs, SOCE contributes significantly to contraction; α_1AR-mediated contraction is reduced ∼30% in SM-specific STIM1$^{-/-}$ animals (77). In ECs, SOCE is the major Ca^{2+} entry and is required for many critical functions such as endothelial nitric oxide synthase (eNOS) activity and proliferation (78–82).

Voltage-Dependent Ca^{2+} Entry (VDCE)

Functional voltage-dependent Ca^{2+} channels (VDCCs) are the hallmark of tissue excitability and are present in cardiomyocytes and VSMCs, but not ECs. In cardiomyocytes, LTCCs are located mostly in transverse T tubules in apposition to RyR2s (83). Ca^{2+} entry via LTCCs triggers CICR via RyR2. In VSMCs, LTCCs also play a critical role in Ca^{2+} entry and contraction (84). The LTCC complex (85) consists of α_1, α_2, β, δ, and γ subunits. Four LTCC members are named according to their α1 pore-forming subunits: $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$ (86). $Ca_v1.2$ is predominant in cardiac and smooth muscles.

Ca^{2+} Extrusion via the Plasma Membrane/Sarcolemma

The plasma membrane Ca^{2+}-ATPases (PMCAs) prevail for Ca^{2+} extrusion in non-excitable tissues while the Na^+-Ca^{2+} exchanger (NCX) is more important in excitable cells. SERCA2a, NCX, and PMCA sequester, respectively, ∼70, 28, and 2% of cytosolic Ca^{2+} in cardiomyocytes (83) and 25, 25, and 50% in ECs (87).

Plasma Membrane Ca^{2+}-ATPase

PMCAs extrude one Ca^{2+} ion for each ATP used and function as Ca^{2+}-H^+ exchangers (88–90). PMCAs are regulated by a Ca^{2+}-dependent interaction with calmodulin (CaM). At low Ca^{2+}, a C-terminal autoinhibitory domain binds to two cytosolic loops and inhibits pump activity. Increased Ca^{2+} promotes CaM–PMCA interaction, which removes inhibition and activates Ca^{2+} efflux (91, 92). PSD-95 promotes expression and distribution of PMCA4b via PDZ binding (93). PMCAs are inhibited by C-terminal tyrosine phosphorylation (94). Myocardial PMCAs play a little role under physiological conditions. However, expressions of PMCA1 and PMCA4 are reduced by up to 70 and 50%, respectively, in end-stage heart failure (95), and cardiac-specific overexpression of PMCA4b improved myocardial functions in ischemia–reperfusion injury and heart failure (96). PMCAs concentrate in the caveolae of VSMCs and ECs (97, 98). PMCA1 suppresses VSMC proliferation (99, 100), while PMCA4 mediates cell cycle (101, 102). In ECs, PMCA1b, and PMCA4b are predominant (87, 103, 104).

Na^+-Ca^{2+} Exchanger

The NCX may function in two modes. In the *forward mode*, myocardial NCX1 balances LTCC-mediated Ca^{2+} entry and RyR-mediated Ca^{2+} release during cardiac excitation, extruding ∼25% of the Ca^{2+} needed to activate myofilaments (105). NCX1 also predominates in VSMCs (106, 107). In ECs, NCX accounts for ∼25% of Ca^{2+} removal (87). Endothelial NCX and PMCA dynamically adjust their Ca^{2+} extrusion rates to maintain sufficient efflux (104). In the *reverse mode*, upon myocardial depolarization, Na^+ entry causes the NCX to transiently operate in this mode, promoting Ca^{2+} entry. This is much less efficient in triggering SR Ca^{2+} release compared to LTCC-mediated Ca^{2+} entry (108, 109). However, it primes the dyad to increase LTCC-mediated CICR (110). In VSMCs, reverse-mode NCX1 facilitates Ca^{2+} entry and mediates contraction, vascular tone, and blood pressure (111, 112). The reverse mode is not significant in ECs.

Sex Differences in Ca^{2+} Signaling Proteins

Higher mRNA levels of $Ca_v1.2$, RyR, and NCX, but not of phospholamban and SERCA2, have been observed in female than in male rat hearts (113). However, caffeine-induced Ca^{2+} release is lower in cardiomyocytes from female hearts (114). $Ca_v1.2$ mRNA is higher in coronary smooth muscle from male than from female pigs (115). In smooth muscle cells (SMCs), expressions of ERα and ERβ, but not G protein-coupled estrogen receptor 1 (GPER), are higher in female than in male rats (116). These differences and the lower $Ca_v1.2$ expression (115) may be responsible for less contraction of VSMCs from females (116). No studies have examined sex differences in Ca^{2+} handling proteins in ECs.

Transduction of Ca^{2+} Signals—The Essential Role of Calmodulin (CaM)

While some Ca^{2+}-dependent proteins are activated directly by Ca^{2+}, many are activated by a complex between Ca^{2+} and CaM. CaM has two lobes linked by a flexible helix and can interact with ∼300 target proteins (117, 118). Ca^{2+}-free CaM binds or serves as structural subunits of ∼15 proteins (119). However, each CaM lobe has two Ca^{2+}-binding sites, and cooperative Ca^{2+} binding induces conformations that allow CaM to interact with many proteins, aided by the flexibility of the central helix (120, 121). Thus, CaM is the ubiquitous Ca^{2+} signal transducer. Activities of Ca^{2+}/CaM-binding proteins depend on the Ca^{2+} signals, CaM availability, and properties of the interaction between Ca^{2+}-CaM and the target proteins. Many of these factors are subject to estrogenic moderation.

Despite being required for activation of many Ca^{2+}-dependent proteins, up to 50% of cellular CaM is engaged in inseparable interactions, leaving much less available for dynamic target binding (122). This generates an environment of limited CaM (123), as has been demonstrated in ECs (124), VSMCs (125), and cardiomyocytes (126). Consequently, competition for CaM generates a unique crosstalk among CaM-dependent proteins (124, 127), and factors that alter CaM level are predicted to have pervasive functional impact. It is noteworthy that virtually all CSM components interact with CaM and, in the context of reciprocality between estrogenic and Ca^{2+} signaling pathways, that ERα and GPER are both regulated by direct interactions with Ca^{2+}-CaM.

ESTROGEN RECEPTORS AND THEIR CALCIUM-DEPENDENT REGULATION

Estrogen Receptor α (ERα)

ERα (128–130) is a nuclear receptor that, upon E2 binding ($K_d \sim 10^{-10}$ M), assumes an active conformation to bind estrogen-responsive elements (EREs) in the promoters of target genes, modulating their transcription (131). Its N-terminus has a transcriptional activation function (AF-1) domain, a DNA-binding domain, and a hinge region; the C-terminus houses the ligand-binding domain and a second AF-2 domain. ERα is robustly expressed in the heart (132), VSMCs, and ECs (133–136).

ERα activities are strongly regulated by the Ca^{2+}-dependent interaction with CaM. ERα binds CaM in a Ca^{2+}-dependent fashion with a K_d of 1.6×10^{-10} M and an $EC_{50}(Ca^{2+})$ value of $\sim 3 \times 10^{-7}$ M (137). When ERα from Wistar rats' uteri is used, CaM decreases ERα-E_2 binding but increases liganded ERα-ERE interaction (138, 139). A comparison of the CaM-bound/CaM-unbound ERα ratio in the cytosolic (unliganded) and nuclear (liganded) ERα pools isolated from MCF-7 cells suggests that E_2 binding induces a conformation that favors ERα-CaM interaction (138). The CaM-binding domain was initially predicted to be a.a. 298–310 (137) but was later determined to be a.a. 298–317, with a.a. 248–317 required for maximal interaction (140). Further studies revealed that a.a. 287–311 is required to interact with both CaM lobes (141). CaM binding promotes ERα homodimerization that is critical for transcription activity (140, 142). With two lobes, each CaM binds two ERα molecules and thus stabilizes ERα dimerization (143). Notably, analogs of ERα17p (a.a. 295–311) that are unable to bind CaM downregulates ERα, stimulates ERα-dependent transcription, and enhances proliferation of MCF-7 cells, as does the wild-type ERα17p, indicating that this domain may also be involved in CaM-independent posttranslational regulation of ERα (144).

Estrogen Receptor β (ERβ)

ERβ has ∼96% and 55–58% sequence homology with ERα in the DNA- and ligand-binding domains, respectively (145, 146). ERβ binds E_2 with a K_d of $\sim 4–6 \times 10^{-10}$ M. ERβ forms homodimers but more preferentially forms heterodimers with ERα, which bind E_2 with a K_d of $\sim 2 \times 10^{-9}$ M and are transcriptionally active (147). ERβ is abundantly expressed in the vasculature (133–136). However, its expression and direct actions in the heart are controversial; cardiac manifestations in $ERβ^{-/-}$ animals have been attributed to indirect effects from vascular changes (148). ERβ is not regulated by Ca^{2+} or CaM (149).

GPER

GPER (150), aka GPR30, was cloned from various tissues in the 1990s (151–156). GPR30 is required for estrogenic activation of extracellular signal-related kinase (ERK)1/2 via transactivation of the epidermal growth factor receptor (EGFR) and release of the heparan-bound epidermal growth factor (EGF) (157, 158). It was shown to bind E_2 in 2005 (159, 160), and the designation GPER was adopted by the International Union of Basic and Clinical Pharmacology in 2007 (161). A host of steroidal and non-steroidal agents and specific GPER agonists can activate GPER (150). GPER couples with $Gα_s$ or $Gα_{i/o}$. Supporting $Gα_s$ coupling are data that (1) most membrane-bound [^{35}S]GTPγ-S from cells overexpressing GPER and treated with E_2 coimmunoprecipitate with $Gα_s$ (159), (2) GPER is present in $Gα_s$-pull-down fraction from GPER-expressing cells, and (3) E_2 promotes GPER-dependent cyclic adenosine monophosphate (cAMP) production (162). Supporting GPER-$Gα_{i/o}$ association are results that pertussis toxin prevents (1) E_2-induced, GPER-mediated ERK1/2 phosphorylation in cells transfected with GPER (134, 157); (2) upregulation of c-fos in ERα/ERβ-negative, GPER-positive SKBr3 cells (163); and (3) E_2-induced Ca^{2+} signals in ECs (164).

GPER is robustly expressed in cardiovascular tissues (133–136). In ECs, GPER mRNA is increased 8-fold by shear stress (154). GPER is localized on the ER/SR membrane (160) and responds to cell-permeable ligands (165). However, it also resides on the plasma membrane (166) and requires its C-terminal PDZ-binding motif to do so (167). The plasmalemmal GPER pool seems to constitutively undergo clathrin-dependent endocytosis and accumulate in the trans-Golgi network for ubiquitination in the proteasome without recycling to the plasma membrane, a process unaffected by agonist stimulation (168). Despite its predominant expression in the ER/SR, the sequence that drives GPER localization here has not been identified.

GPER is directly regulated by Ca^{2+}-CaM complexes. In VSMCs and ECs, GPER coimmunoprecipitates with CaM in a constitutive association that is promoted by treatment with E_2, G-1, or receptor-independent stimulation of Ca^{2+} entry (169, 170). GPER is the first G protein-coupled receptor (GPCR) shown to possess four CaM-binding sites on its respective four submembrane domains (SMDs) (169). Fluorescence resonance energy transfer (FRET) biosensors based on SMDs of GPER bind CaM with K_d from 0.4 to 136×10^{-6} M and affinity ranking SMD2 > SMD4 > SMD3 > SMD1. These interactions are Ca^{2+} dependent, with an EC_{50} (Ca^{2+}) of 1.3×10^{-7}-5×10^{-6} M, values within the physiological Ca^{2+} range (169). Due to technical challenges with purifying full-length GPCRs, the K_{CaM} for GPER as a holoreceptor is not available. The presence of four CaM-binding sites makes this task even more challenging and, in some way, not useful functionally. *Functionally*, mutations that reduce CaM binding but that do not perturb GPER-$G_{βγ}$ preassociation drastically prevent GPER-mediated ERK1/2 phosphorylation (170).

STIMULATION OF CALCIUM SIGNALS BY ESTROGEN AND GPER AGONISTS

Observations

In rat hearts, E_2 (10^{-12}-10^{-8} M) triggers $^{45}Ca^{2+}$ uptake that is inhibited by LTCC antagonists (171). In VSMCs, GPER agonist G-1 triggers a slow-rising Ca^{2+} signal that is $<2 \times 10^{-7}$ M (172). In MCF-7 cells, E_2 (10^{-7} M) induces Ca^{2+} store release and entry, yet only the former is required to activate mitogen-activated protein kinase (MAPK) (173). Interestingly, the ERα/ERβ antagonist ICI182,780 (10^{-6} M) also triggered

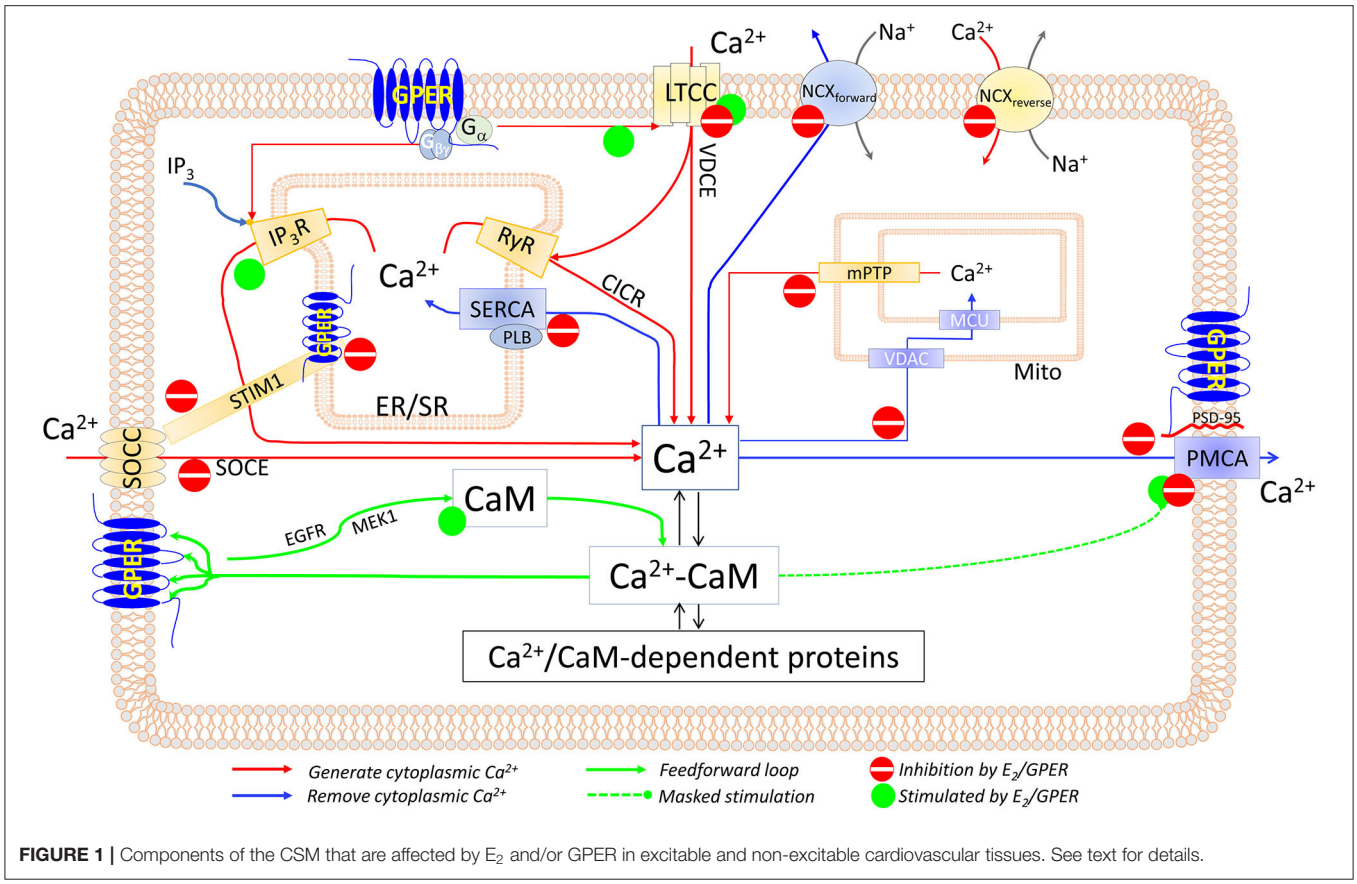

FIGURE 1 | Components of the CSM that are affected by E_2 and/or GPER in excitable and non-excitable cardiovascular tissues. See text for details.

Ca^{2+} signals in these cells. In ECs, E_2 (10^{-10}-10^{-9} M) triggers Ca^{2+} store release and entry, effects not affected by ERα/ERβ inhibitor tamoxifen (164, 174). The data with ICI182,780 and tamoxifen implicate a receptor other than ERα or ERβ in mediating the Ca^{2+} signal. Both reagents were later shown to be GPER agonists, triggering ERK1/2 phosphorylation only in cells expressing GPER (157, 159). Later studies confirmed Ca^{2+} signals stimulated by E_2, GPER agonist G-1, and ICI182,780 in cells expressing GPER endogenously and absence of this effect in GPER$^{-/-}$ cells (160, 175, 176).

Mechanisms (Figure 1)

Direct E_2-$Ca_v1.2$ Interaction

E_2 (10^{-11}-10^{-9} M) potentiates $I_{Ca,L}$ in neurons and HEK293 cells overexpressing the α1C subunit; nifedipine displaces membrane E_2 binding; and E_2's effect is reduced by a dihydropyridine-insensitive LTCC mutant, indicating that E_2 binds to the dihydropyridine-binding site (177). Intriguingly, E_2 and the dihydropyridines exert opposite effects on $I_{Ca,L}$.

Direct, Membrane-Delimited Activation of Ca^{2+} Channels by Gα Subunits

GPCR stimulation can trigger Ca^{2+} signals independently of the second messenger (178–180). GPER couples with $G\alpha_s$ and $G\alpha_{i/o}$, which can interact with LTCC (178, 181, 182) and trigger Ca^{2+} entry.

Release of $G_{\beta\gamma}$ Subunit Upon GPER-Associated $G\alpha_i$ Stimulation

$G_{\beta\gamma}$ stimulates PLCβ (183–185) and activates IP_3R1 (186), both of which trigger Ca^{2+} store depletion and SOCE. Consistently, E_2-induced Ca^{2+} store release and entry in ECs are completely inhibited by pertussis toxin and PLCβ inhibitor U73122 (164). Also, HEK293 cells only produce a Ca^{2+} response to E_2 when expressing HA-tagged GPER (162). Since (1) Ca^{2+} entry channels are located on the membrane and (2) $G_{\beta\gamma}$ activates IP_3Rs by interacting with the IP_3-binding sites (186) on IP_3Rs' cytosolic domains, both the membrane-delimited/Gα-mediated and $G_{\beta\gamma}$-mediated mechanisms should only be operable by the plasmalemmal GPER pool. A distinguishing feature is that the former mechanism would not trigger SR/ER Ca^{2+} release in the absence of extracellular Ca^{2+}, whereas the latter would. Based on this feature, data fitting the former are available from renal tubular cells (176); and data fitting the latter, from vascular ECs (164).

Functional Impact

Do Ca^{2+} signals stimulated by estrogenic agonists activate Ca^{2+}-dependent activities? When reported, the concentration of a Ca^{2+} signal allows for prediction of proteins that may or may not be affected by it. For example, E_2 induces ER Ca^{2+} release signals of ~2×10^{-7} M and activates MAPK (173), because this Ca^{2+} level is sufficient for MAPK activity (187); indeed, Ca^{2+} chelation

abolishes E_2's effect (173). Considering that GPER mediates the effect of E_2 to trigger Ca^{2+} signals that activate MAPK, GPER activity can promote many downstream effects (163, 170, 188). In ECs, E_2 (10^{-9}-10^{-6} M) stimulates very small Ca^{2+} signals ($<10^{-7}$ M) (174). One can predict that only proteins with very high Ca^{2+} sensitivity, for example, phosphorylated eNOS (170, 189, 190), would be activated by these signals. Whether a Ca^{2+} signal can produce a predicted effect also depends on other factors. For example, the Ca^{2+} signal of $\sim 2 \times 10^{-7}$ M triggered by G-1 in VSMCs (172) would be sufficient to activate myosin light-chain kinase (MLCK) and cause vasoconstriction, based on MLCK's properties (191). However, G-1 causes vasodilation (172, 192–194), likely by activating eNOS (170, 193, 195–198), inhibiting VSMC Ca^{2+} (199), and stimulating SMC K^+ efflux (200).

CALCIUM ENTRY INHIBITION BY ESTROGENIC AGONISTS AND ESTROGEN RECEPTORS

To a large extent, estrogenic regulation of Ca^{2+} signaling involves effects of estrogenic agonists and receptors on the Ca^{2+} signals triggered by other stimuli, via both E_2-dependent and E_2-independent mechanisms.

Store-Operated Ca^{2+} Entry (Figure 1)

In VSMCs, E_2 (10^{-8}-10^{-5} M) inhibits norepinephrine- and phenylephrine-induced arterial constriction in the presence of extracellular Ca^{2+} but not that induced in Ca^{2+}-free medium (201, 202). These effects may be attributed to inhibition of both VDCE and SOCE, as α_1 adrenoceptor agonists can activate both (77). GPER-mediated inhibition of SOCE has been shown in ECs, where G-1 (10^{-8}-10^{-6} M) suppresses SOCE induced by thapsigargin or bradykinin (203). Interestingly, the observations that in the absence of any treatment with agonists, thapsigargin-induced SOCE is increased by 80% in GPER-knockdown ECs and is reduced by 40% in GPER-overexpressing HEK293 cells implicate *E_2-independent mechanisms* (203).

How E_2/GPER suppresses SOCE seems to involve STIM1. G-1 treatment prevents thapsigargin-induced STIM1 puncta, indicating inhibition of STIM1's association with the Ca^{2+} channel; and Ser575/608/621Ala mutations of STIM1 reduce the inhibitory effect of G-1 (203). Consistently, E_2 inhibits Ser575 STIM1 phosphorylation in bronchial epithelial cells, thus suppressing STIM1 mobility and SOCE (204). Our initial data also indicate that dynamic physical interaction between them contributes importantly to GPER's inhibition of SOCE (205).

Voltage-Dependent Ca^{2+} Entry (Figure 1)

Electrically induced Ca^{2+} signals are increased in cardiomyocytes from ovariectomized (OVX) animals (206–208). Many lines of evidence indicate that GPER mediates the inhibitory effect of E_2 on $I_{Ca,L}$. These include inhibitory effects of E_2 ($1–3 \times 10^{-5}$ M) and combined ERα/ERβ antagonists/GPER agonists (ICI182,780, tamoxifen, or raloxifene) on $I_{Ca,L}$ in cardiomyocytes from both WT and ERα$^{-/-}$/ERβ$^{-/-}$ animals, as reviewed in (132). Similarly, in VSMCs, E_2 inhibits electrically induced $I_{Ca,L}$ (209, 210), and ERα/ERβ antagonists/GPER agonists tamoxifen and ICI164,384 inhibit high-K^+-induced contraction (202). GPER agonist G-1 (10^{-6} M) inhibits nifedipine-sensitive Ca^{2+} spikes in LTCC-expressing A7R5 SMCs, an effect prevented by GPER antagonist G-15 (10^{-6} M) (199); these concentrations are specific for GPER (175, 211). Consistently, ERα knockout does not affect E_2's inhibition of KCl-induced $^{45}Ca^{2+}$ uptake in VSMCs and vasorelaxation (212).

How E_2 inhibits electrically induced VDCE is still unknown. Hypothetically, at high levels, E_2 binding to the dihydropyridine-binding site on LTCC (177) may instead inhibit $I_{Ca,L}$. As for prevention of β adrenoceptor (βAR)-mediated potentiation of VDCE, recent evidence suggests that GPER may be an intrinsic component of β_1AR activation. Thus, G-1 inhibits isoproterenol-induced increases in left ventricle (LV) pressure, heart rate, ectopic contractions, $I_{Ca,L}$, LTCC phosphorylation, and total myocardial Ca^{2+} signal, while the GPER inhibitor G-36 promotes ISO-induced Ca^{2+} signal and LTCC phosphorylation (213). Speculatively, GPER may do so in part by interacting with β_1AR or with A kinase-anchoring protein 5, thus inhibiting cAMP production (167). These may represent some *E_2-independent* effects of GPER. Studies in GPER-knockout tissues are needed to further clarify the mechanisms.

ESTROGENIC REGULATION OF CYTOPLASMIC CALCIUM REMOVAL MECHANISMS

SERCA Activity

Few studies, mostly in cardiac tissues, have examined the effects of E_2 on *SERCA activity*, with somewhat conflicting results. E_2 ($1–30 \times 10^{-6}$ M) does not affect the V_{max} of SR vesicle Ca^{2+} uptake in canine LV tissue (214). However, ovariectomy reduces the V_{max} but increases the Ca^{2+} sensitivity for SR Ca^{2+} uptake of rat LV homogenates or SR-enriched membrane fractions; *mechanistically*, these effects appear to be associated with reduced Thr17 phosphorylation of phospholamban and are restored by treatment with either E_2 or progesterone (215) (**Figure 1**). How E_2 and progesterone promote Thr17 phosphorylation of phospholamban is unknown, perhaps by inhibiting CaM kinase II (216), the enzyme that phosphorylates phospholamban (21). The effect of E_2 on SERCA activity in VSMCs has not been examined.

NCX Activity

As with SERCA activity, few studies have measured the effects of E_2 on NCX activity. Na^+-dependent $^{45}Ca^{2+}$ uptake in rat LV myocytes is increased by ~3-fold after 60 days of ovariectomy, which is restored by replenishment with E_2 (1.5 mg/60 days) (208). During myocardial ischemia, intracellular Na^+ concentration is higher in male than in female cardiomyocytes and is associated with increased Ca^{2+} concentration as a result of increased NCX activity (217). These studies are consistent with an inhibitory effect of E_2 on NCX activity in both the forward and reverse modes (**Figure 1**). However, the mechanisms of this inhibition are unclear.

Mitochondrial Ca^{2+} Uptake

In the heart, diethylstilbestrol (0.9–1.8 × 10^{-3} M) inhibits mitochondrial $^{45}Ca^{2+}$ uptake (218). Mitochondrial Ca^{2+} retention capacity (mCRC), a combination of mitochondrial Ca^{2+} uptake, total mitochondrial Ca^{2+}-binding sites, and mitochondrial Ca^{2+} release mechanisms, is a determinant of the protective role of the mitochondria during cytoplasmic Ca^{2+} overload. E_2 (4 × 10^{-8} M) increases myocardial mCRC following ischemia–reperfusion, an effect abolished by genetic deletion of GPER but not of ERα or ERβ; *mechanistically*, this effect seems to involve PKC-dependent, MAPK-dependent phosphorylation of glycogen synthase kinase (GSK)-3β, leading to inhibition of the mitochondrial permeability transition pore (219). Consistently, E_2 (10^{-8} M) inhibits high Ca^{2+}-induced cytochrome c release from myocardial mitochondria (220). In ECs, 48-h E_2 (10^{-8} M) treatment inhibits mitochondrial Ca^{2+} uptake, an effect abolished by the ERα/ERβ antagonist ICI182,780 (10^{-8} M) (221). The mechanisms whereby E_2 inhibits mitochondrial Ca^{2+} uptake are still unknown (**Figure 1**).

PMCA Activity

Recent data show that GPER inhibits PMCA activity via both E_2-dependent and E_2-independent mechanisms (**Figure 1**). *E_2-dependent mechanisms* are evidenced by the effects of G-1 (10^{-8}-10^{-6} M) and E_2 (1–5 × 10^{-9} M) to inhibit PMCA-mediated efflux in primary ECs without affecting PMCA expression levels and to promote PMCA phosphorylation at Tyr1176 (135, 170), which is known to inhibit pump activity (94). Notably, this phosphorylation masks the stimulatory effect of enhancing the PMCA–CaM interaction produced by 48-h E_2 treatment (170). *E_2-independent mechanisms* are indicated by the findings that (1) GPER constitutively interacts with PMCA4b via the anchoring action of PSD-95 at their C-terminal PDZ-binding motifs; (2) overexpression of GPER decreases PMCA activity; (3) GPER knockdown promotes PMCA activity; and (4) PSD-95 knockdown or truncation of the PDZ-binding motif on GPER releases GPER–PMCA association and promotes PMCA activity (135). *Functionally*, these mechanisms collectively prolong agonist-induced Ca^{2+} signal and enhance eNOS activity in ECs (135, 170, 203). Consistent with suppressed Ca^{2+} efflux, the Ca^{2+} signals stimulated by E_2 and the GPER agonist G-1 in cells overexpressing GPER reported by various laboratories display much more prolonged plateau phases compared to Ca^{2+} signals in cells not overexpressing GPER or those stimulated by other agonists such as ATP or bradykinin (160, 162, 164, 175). GPER–PMCA4b interaction seems to be mutually influential, such that knockdown of PMCA decreases GPER-mediated ERK1/2 phosphorylation, while GPER knockdown does the opposite on PMCA activity (135).

ESTROGENIC REGULATION OF CALCIUM SIGNAL TRANSDUCTION—THE CALMODULIN NETWORK

Since CaM is the universal Ca^{2+} signal transducer for numerous proteins (117, 118), is insufficiently expressed for its targets (122, 125, 126), and is a source of competition among target proteins (124, 127), factors that regulate its expression and target interactions are predicted to have a pervasive impact. The effects of E_2 on the CaM network have been examined in some detail in vascular ECs in recent studies (135, 169, 170). E_2 treatment (1–5 × 10^{-9} M, 48 h) upregulates total CaM by around 7-fold and free Ca^{2+}-CaM by ∼15-fold in primary ECs. Data obtained using specific estrogen receptor agonists, gene silencing, and receptor overexpression indicate that GPER, but not ERα or ERβ, mediates this effect. Thus, the GPER agonist G-1 (10^{-9}-10^{-7} M), but not the ERα agonist propyl pyrazole triol (PPT) (3 × 10^{-10}-2 × 10^{-7} M) or the ERβ agonist diarylpropionitrile (DPN) (10^{-10}-5 × 10^{-8} M), increases CaM expression; GPER knockdown reduces the effect of E_2 to upregulate CaM; and E_2 upregulates CaM in SKBR3 cells that express only GPER and not ERα or ERβ (170). Consistently, the ERα/ERβ antagonist/GPER agonist ICI182,780 dose-dependently upregulates CaM. *Mechanistically*, GPER exerts this action via the activities of EGFR and MAPK/ERK kinase 1 (MEK1). *Functionally*, E_2 upregulates CaM and promotes the PMCA–CaM interaction; however, the predicted stimulatory effect on Ca^{2+} extrusion is masked by E_2-induced inhibitory phosphorylation at Tyr1176 of PMCA (170); additionally, GPER exerts E_2-dependent and E_2-independent effects to inhibit PMCA (135). These collective actions prolong Ca^{2+} signals, promote Ca^{2+}-CaM complex formation, and increase Ca^{2+}-CaM associations with low- to high-affinity CaM network members, represented by GPER itself, ERα, and eNOS (170). Considering that CaM binding stabilizes ERα homodimers, these effects are expected to promote other genomic actions of E_2 as well. Thus, a feedforward mechanism exists in which GPER mediates E_2's effects to increase CaM and inhibits Ca^{2+} efflux, prolonging cytoplasmic Ca^{2+} signals, and the resultant increases in Ca^{2+}-CaM complexes in turn promote the activities of GPER itself and other CaM network members (170) (**Figure 1**).

ESTROGENIC MODERATION OF CALCIUM-DEPENDENT ACTIVITIES

How do the various mechanisms discussed so far come together in regulating cardiovascular functions? An immediate challenge is how to reconcile the effects of estrogenic agonists to both trigger acute Ca^{2+} signals by themselves and inhibit otherwise stimulated Ca^{2+} signals. The Ca^{2+} signals triggered by estrogenic agonists in primary cardiovascular cells are generally of very low amplitude. Furthermore, as in experiments testing their effects on Ca^{2+} signals otherwise triggered, estrogenic agonists are present *in situ* with other stimuli whose Ca^{2+} signals they inhibit. Thus, for *mechanisms that generate cytoplasmic Ca^{2+} signals*, E_2 and GPER exert ultimate inhibitory effects. For *cytoplasmic Ca^{2+} removal mechanisms*, estrogenic agonists and GPER also are inhibitory. For *Ca^{2+} signal transduction*, E_2, via a feedforward at GPER, increases CaM expression and enhances linkage in the CaM-binding proteome.

All things considered, E_2 and GPER, via both E_2-dependent and E_2-independent mechanisms, act to *moderate*

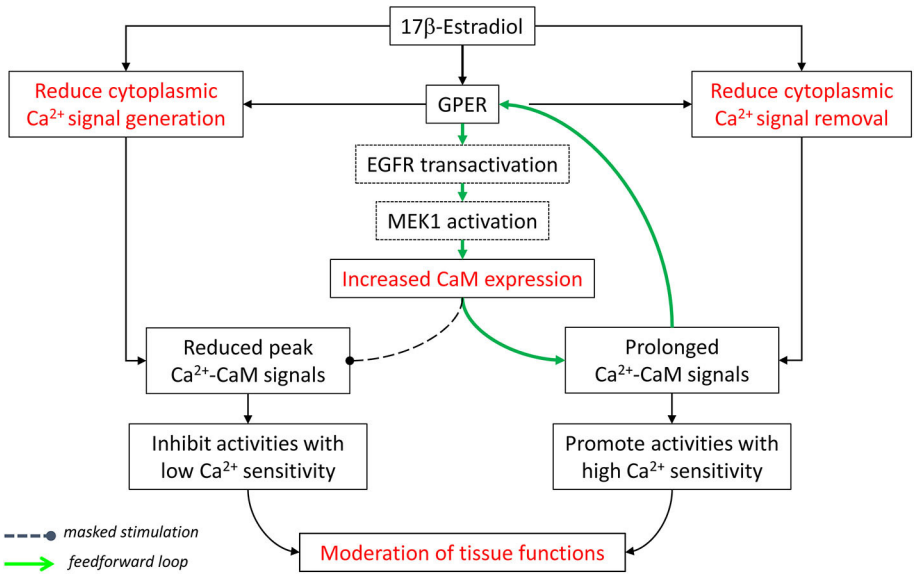

FIGURE 2 | Moderation of cardiovascular functions by E_2 and GPER via effects on Ca^{2+} signal generation, Ca^{2+} signal removal, and Ca^{2+} signal transduction. See text for details. Modified with permission from the author's previous publication (170).

Ca^{2+}-dependent activities in the cardiovascular system. They "clamp" cytoplasmic Ca^{2+} signals by lowering peaks (inhibition of signal generation) and raising troughs (inhibition of signal removal), collectively confining tissues in a narrower yet more sustained operating range of Ca^{2+}. Also, GPER-mediated increases in CaM expression and CaM network linkage improve Ca^{2+} signal transduction efficiency. Considering the Ca^{2+} sensitivity of Ca^{2+}-dependent proteins in this context, one can predict that those with low Ca^{2+} sensitivity (requiring high Ca^{2+} for activation) are more likely to be affected by the inhibition of Ca^{2+} signal generation. On the other hand, proteins with high Ca^{2+} sensitivity (requiring low Ca^{2+} for activation) are more likely to be promoted by the inhibition of Ca^{2+} removal and less affected by the suppression of Ca^{2+} signal generation (**Figure 2**).

This notion has been demonstrated experimentally via the case of eNOS, a Ca^{2+}-dependent CaM-binding protein (222) with sub-nanomolar affinity for CaM (127). CaM interaction and subsequent activation of wild-type eNOS have high Ca^{2+} sensitivities, with respective $EC_{50}(Ca^{2+})$ values $\sim 1.8 \times 10^{-7}$ and 4×10^{-7} M (190). eNOS is also regulated by multisite phosphorylation (223). Notably, its bi-phosphorylation at Ser617 and Ser1179 promotes NO production by increasing the Ca^{2+} sensitivity for both CaM binding and enzyme activation, reducing their respective EC_{50} (Ca^{2+}) values to $\sim 0.7 \times 10^{-7}$ and 1.3×10^{-7} M, thus rendering the synthase active at resting cytoplasmic Ca^{2+} (189). E_2 and GPER (1) prolong endothelial cytoplasmic Ca^{2+} signal by inhibiting Ca^{2+} efflux (135, 170), (2) promote eNOS phosphorylation at Ser617 and Ser1179 (170, 198), (3) increase CaM expression and eNOS–CaM interaction (170), and (4) suppress endothelial SOCE (203). When we incorporate these effects into a verified sequential "CaM binding eNOS activation" model (189, 190), eNOS activity and NO accumulation are shown to substantially increase across the time course of bradykinin-induced Ca^{2+} signal in ECs by treatment with G-1 (203). Importantly, major contributions to this outcome include the increases in CaM binding, phosphorylation, Ca^{2+} sensitivity, and duration of Ca^{2+} signals due to Ca^{2+} efflux inhibition, but little or no effect of the inhibition of SOCE (203), due obviously to the synthase's high Ca^{2+} sensitivity (**Figure 3**). Thus, via multifaceted actions on components of the CSM, E_2 and GPER moderate Ca^{2+}-dependent activities by differentially affecting the continuum of Ca^{2+}-dependent proteins based on their Ca^{2+} sensitivities for Ca^{2+} or Ca^{2+}-CaM complexes.

Considering the two Ca^{2+}-dependent estrogen receptors—ERα and GPER—how does the presence of one influence the effects of the other on Ca^{2+} signaling? A complex relationship is predicted to exist in which ERα transcriptional activities affect the expression of certain Ca^{2+} signaling proteins but are themselves influenced by the amplitudes and dynamics of Ca^{2+} signals limited by GPER activation and the availability of CaM that is promoted by GPER action (170). In turn, as CaM is limited in cells (122, 124, 126, 127), the high affinity binding of CaM by ERα and GPER further limits CaM availability and will influence CaM-dependent regulation of each other at the receptor level, a predictable outcome of the functional crosstalk via competition for limited CaM (124, 127). These relationships may represent but a small aspect of the reciprocality between estrogen and Ca^{2+} signaling.

CONCLUSION AND FUTURE PERSPECTIVES

Reciprocality between estrogen signaling and Ca^{2+}-dependent activities is becoming evident. *Considering the impact of estrogen*

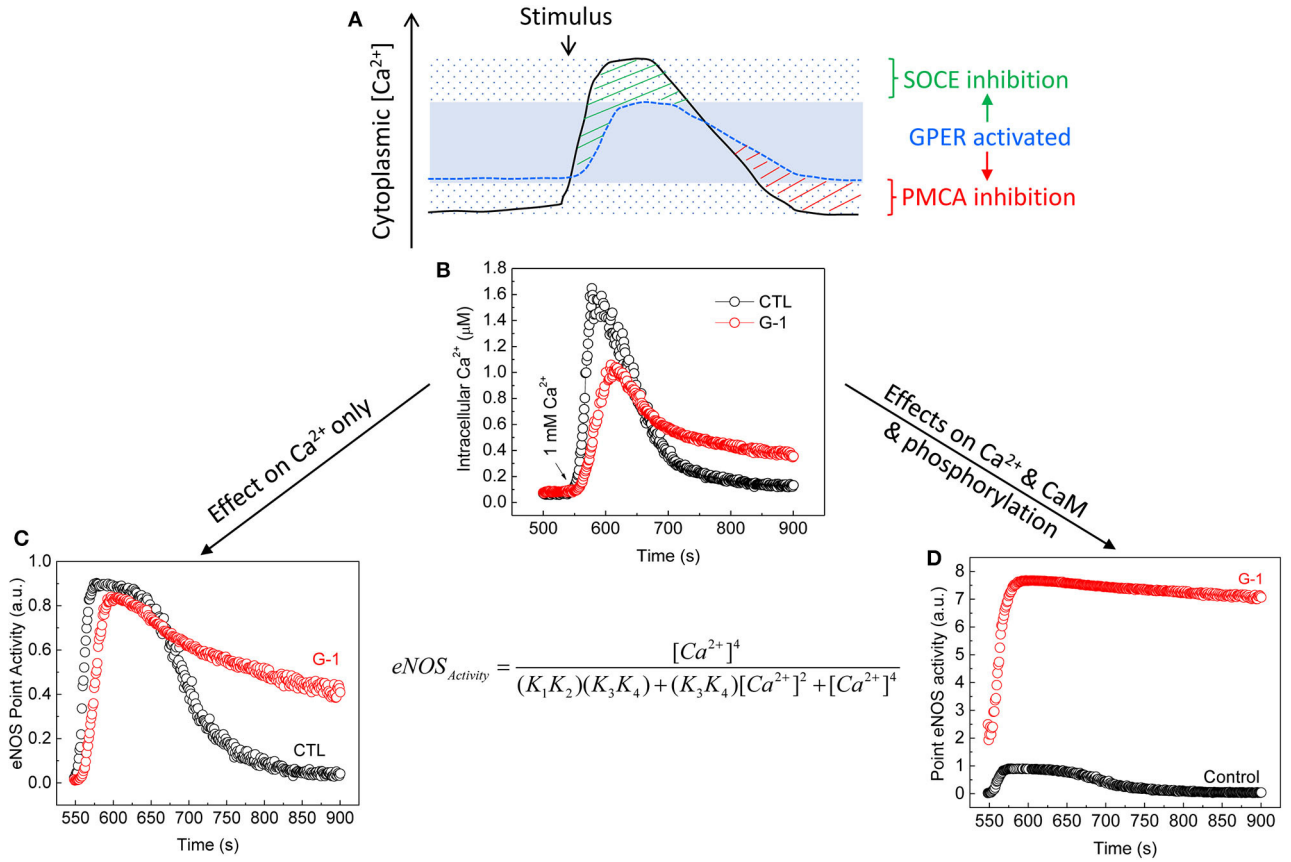

FIGURE 3 | Moderation of Ca^{2+}-dependent eNOS activity by GPER activation. **(A)** Cytoplasmic Ca^{2+} clamping by GPER activation in ECs (203). The solid line represents Ca^{2+} signals produced in response to agonist stimulation in the absence of GPER activation. The sparsely dotted area represents the range of cytoplasmic Ca^{2+} signals, in which peak and trough are seen due to maximal effects of Ca^{2+} entry and Ca^{2+} efflux. The stippled blue line represents Ca^{2+} signals produced in the presence of GPER and its activation. These signals are clamped in a narrower range (the blue area) due to inhibitory effects on both SOCE [green stripes (203)] and PMCA4b-mediated Ca^{2+} efflux [red stripes (135, 170)]. **(B)** Average time courses of cytoplasmic Ca^{2+} signals measured in primary ECs treated with bradykinin in the absence of extracellular Ca^{2+} followed by treatment with vehicle or G-1; total Ca^{2+} signals were triggered by re-addition of extracellular Ca^{2+} [arrow (203)]. **(C)** Calculated eNOS point activity corresponding to each Ca^{2+} value in **(B)** considering only changes in Ca^{2+} due to GPER activation using a verified sequential eNOS–CaM binding eNOS activation model [equation, where (K_1, K_2) and (K_3, K_4) are derived products of the binding constants of Ca^{2+} at the Ca^{2+}-binding sites on the N and C lobes of CaM in binding to CaM and interaction of Ca^{2+}-CaM and eNOS (189, 190). **(D)** Calculated eNOS point activity corresponding to each Ca^{2+} value measured in **(B)**, factoring in changes in Ca^{2+}, CaM binding, and eNOS phosphorylation (170, 203). See details in text and (170, 203). Reproduced with permission from the author's previous publication (203).

and its receptors on Ca^{2+} signaling, E_2, and in many cases, GPER exert inhibitory effects on many components of the CSM in cardiovascular tissues, from Ca^{2+} store release and uptake (214, 215, 221) and Ca^{2+} entry (199, 201–210, 212, 213) to cytosolic Ca^{2+} removal mechanisms (135, 170, 208, 217–221). *Considering the impact of Ca^{2+} signaling on estrogen biology*, both ERα and GPER are strongly regulated by direct Ca^{2+}-dependent interactions with CaM. These interactions serve to stabilize receptor dimerization and enhance subsequent transcriptional activities [the case of ERα (137, 138, 142, 143)] or promote receptor-mediated downstream signaling [the case of GPER (169, 170)]. Also, E_2-induced MAPK activation has long been known to be dependent on the Ca^{2+} signal produced (173). Reciprocality between estrogen biology and Ca^{2+} signaling is further evidenced by the demonstration of *a feedforward mechanism*, in which E_2, via GPER activation, upregulates total cellular CaM expression and free intracellular Ca^{2+}-CaM concentration, which promotes functions of GPER and ERα and other classes of Ca^{2+}-CaM-dependent proteins (170). The combination of these various actions is predicted to affect Ca^{2+}-dependent functions depending on the affinity and Ca^{2+} sensitivities of the proteins involved, as exemplified by the case of eNOS (**Figures 2**, **3**) (170, 203).

The moderating effects that estrogenic agonists and receptors exert on the CSM can explain many of their cardiovascular effects, such as preventing excessive cardiac contraction during sympathetic stress, limiting adverse outcomes related to Ca^{2+} overload, and reducing vascular tone. Nevertheless, the effects of E_2 and estrogen receptors on many CSM components have not been examined. Additionally, many questions remain regarding

mechanisms of the observed effects that estrogenic agonist and receptors produce on the CSM. For example, how do E_2 and GPER inhibit $I_{Ca,L}$? What are the mechanisms that position GPER as an intrinsic component of β_1AR signaling in the myocardium? What are the mechanisms whereby E_2 inhibits the activities of SERCA and NCX? What are the mechanisms whereby E_2 inhibits mitochondrial Ca^{2+} uptake? Further studies are needed to answer these questions. Through many examples, however, it is clear that GPER produces both E_2-dependent and E_2-independent effects on the CSM. While the search is ongoing for approaches to apply specific estrogen receptor agonists to the prevention of cardiovascular disease, the therapeutic potential of E_2-independent effects of GPER and other estrogen receptors is as yet an unexplored territory.

AUTHOR CONTRIBUTIONS

Q-KT conceived the ideas, generated the figures, and wrote the manuscript.

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Continuous Exposure of Breast Cancer Cells to Tamoxifen Upregulates GPER-1 and Increases Cell Proliferation

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GPER-1 is a novel membrane sited G protein-coupled estrogen receptor. Clinical studies have shown that patients suffering an estrogen receptor α (ERα)/GPER-1 positive, breast cancer have a lower survival rate than those who have developed ERα-positive/GPER-1 negative tumors. Moreover, absence of GPER-1 improves the prognosis of patients treated with tamoxifen, the most used selective estrogen receptor modulator to treat ERα-positive breast cancer. MCF-7 breast cancer cells were continuously treated with 1,000 nM tamoxifen for 7 days to investigate its effect on GPER-1 protein expression, cell proliferation and intracellular $[Ca^{2+}]i$ mobilization, a key signaling pathway. Breast cancer cells continuously treated with tamoxifen, exhibited a robust $[Ca^{2+}]i$ mobilization after stimulation with 1,000 nM tamoxifen, a response that was blunted by preincubation of cells with G15, a commercial GPER-1 antagonist. Continuously treated cells also displayed a high $[Ca^{2+}]i$ mobilization in response to a commercial GPER-1 agonist (G1) and to estrogen, in a magnitude that doubled the response observed in untreated cells and was almost completely abolished by G15. Proliferation of cells continuously treated with tamoxifen and stimulated with 2,000 nM tamoxifen, was also higher than that observed in untreated cells in a degree that was approximately 90% attributable to GPER-1. Finally, prolonged tamoxifen treatment did not increase ERα expression, but did overexpress the kinin B1 receptor, another GPCR, which we have previously shown is highly expressed in breast tumors and increases proliferation of breast cancer cells. Although we cannot fully extrapolate the results obtained *in vitro* to the patients, our results shed some light on the occurrence of drug resistance in breast cancer patients who are ERα/GPER-1 positive, have been treated with tamoxifen and display low survival rate. Overexpression of kinin B1 receptor may explain the increased proliferative response observed in breast tumors under continuous treatment with tamoxifen.

Keywords: GPER-1, GPR30, G1 agonist, calcium signaling, tamoxifen resistance, kinin B1 receptor, breast cancer, cell proliferation

INTRODUCTION

Breast cancer is the most common type of cancer that produces high mortality in women, worldwide. In general, breast cancer is classified as estrogen receptor alpha (ERα) positive or negative. ERα-positive tumors comprise approximately 70% of all breast tumors and depend on estrogen to develop and grow (1, 2). It has been estimated that a large number of the responses mediated by 17β-estradiol, a kind of estrogen, occur through its binding to ERα, triggering a "genomic response" that initiates the transcription of genes associated to cell proliferation, survival and migration (1, 3). Nevertheless, estrogen also promotes a "rapid cellular response" (4), which includes an increase in intracellular calcium and activation of ERK1/2 mitogen-activated protein kinases (MAPKs), a signaling pathway that is considered crucial for cell proliferation (5, 6). Therefore, the efforts made so far to reduce breast cancer progress aim to suppress the synthesis of endogenous estrogen or to block ERα, through the use of selective estrogen modulators (SERMs), among which tamoxifen stands out (3). However, the molecular heterogeneity of breast cancer, together with the existence of more aggressive forms of the disease and resistance to conventional drug therapy, suggest that other players may be involved in the pathogenesis and progress of this neoplasia.

G protein-coupled estrogen receptor-1 (GPER-1 or GPR30) is a G protein-coupled receptor (GPCR) sited in the cell membrane that triggers a broad range of biological activities in response to stimulation by endogenous estrogens or dietary phytoestrogens (2, 7). Its gene is located on chromosome 7p22.3 and encodes a protein of 375 amino acids with a theoretical molecular mass of 41 kDa that is ubiquitously expressed in a large number of tissues (8–11). GPER-1 is highly expressed in the nervous and adipose tissues, liver and in the circulatory and immune systems among others. Its activation by 17β-estradiol has been corroborated by the use of labeled estradiol, and its synthetic agonist (G1) complemented with its pharmacological antagonist (G15) in normal and cancerous tissues and in various cell lines that do not express ERα (12, 13). GPER-1 mRNA has been detected in several breast cancer cell lines and its expression has been associated with the increased proliferation rate exhibited by these cells. GPER-1 signaling involves cAMP production and Ca^{2+} mobilization most likely through protein Gαs (13) and Src activation through Gβγ (14) and the subsequent shedding of heparin-binding EGF-like growth factor (HB-EGF) and transactivation of epidermal growth factor receptor (EGFR). GPER-1 induces also the activation of phospholipase C and cFos and various kinases such as ERK1/2 MAPK, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) (6, 15–17). Evidence suggests that many of the responses attributed to ERα can be mediated, at least in part, by GPER-1. In fact, several of the beneficial responses produced by estrogens are absent in GPER-1 knockout mice (18, 19).

It has been shown that approximately 60% of all breast tumors are GPER-1-positive. In addition, expression of GPER-1 correlated with over-expression of HER-2, EGFR (HER-1), and lymph node status. Surprisingly, GPER-1 was negatively correlated with relapse-free survival in patients that were treated with tamoxifen compared to those receiving aromatase inhibitors (20–23). Surprisingly, independent studies have shown that tamoxifen and 4-OH tamoxifen (the main tamoxifen metabolite), two ERα antagonists, act as GPER-1 agonists (17, 22, 24). Furthermore, GPER-1 expression seems to be a favorable factor for relapse-free survival, but only in patients that did not receive tamoxifen; consequently, loss of GPER-1 improves the prognosis in patients treated with tamoxifen indicating that GPER-1 might be related to tamoxifen resistance in breast cancer (25). Activation of GPER-1 by 4-OH tamoxifen also increases the expression of connective tissue growth factor (CTGF), which may be related to a more aggressive behavior of some breast tumors (26).

In general, it is estimated that resistance mechanisms are related to mutations that arise within the intermediates that are part of the signaling pathways triggered by estradiol or its metabolites, promoting the survival and proliferation of tumor cells (27). Isolated models like those using tamoxifen-resistant MCF-7 cells (a cellular model that imitates therapeutic conditions), stimulated with estradiol point to an overexpression of GPER-1 (20). These observations showed that tamoxifen could act as non-specific GPER-1 agonist increasing breast cancer cells proliferation and migration. Moreover, it has recently been reported that patients with GPER-1-positive breast tumors, after four to six months of treatment with tamoxifen, not only generated resistance to therapy, but also suffered an increase in the size of tumor mass (28).

The current experiments were designed to examine the protein levels of GPER-1 in ERα-positive breast cancer cells that were continuously treated with tamoxifen for a period of 7 days and to investigate the mobilization of intracellular Ca^{2+} and cell proliferation that follows their stimulation with tamoxifen or GPER-1 agonists. We also investigated the protein levels of classical ERα and kinin B1 receptor (B1R), another GPCR associated to breast cancer progression (6, 29).

MATERIALS AND METHODS

Cell Culture

MCF-7 cells, an estrogen-sensitive or ERα-positive/GPER-1-positive breast cancer cell line was used for all experiments. The MCF-7 cell line was obtained from the American Type Culture Collection (Manassas, VA USA). Cells were grown in modified Eagle's Dulbecco (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and penicillin-streptomycin (10,000 U/ml sodium penicillin G and 10,000 μg/ml streptomycin sulfate; GIBCO BRL, Life Technologies) and 250 μg/ml fungizone. Cells were cultured at 37°C in a humidified incubator under 5% CO_2 and 95% air (6, 29).

Prolonged Exposure of Breast Cancer Cells to Tamoxifen

MCF-7 cells were grown and expanded for 7 days as mentioned above, in the presence of 1,000 nM tamoxifen (Sigma-Aldrich, USA). Medium containing tamoxifen was replaced every 48 h. After 7 days of exposure to tamoxifen, cells were frozen in cell

culture freezing medium containing dimethyl sulfoxide (GIBCO BRL, Life Technologies) and stored until used. When required, these cells were grown again in a medium containing 1,000 nM tamoxifen (24). In parallel experiments, control cells that were not treated with the drug were grown as described above. Once a confluence of 80% was reached, both tamoxifen-treated and untreated cells were maintained for 24 h in culture medium without phenol red, and FBS. Once synchronized, tamoxifen-treated and untreated control cells were stimulated with 1,000 nM tamoxifen for 24, 48, and/or 72 h.

Western Blotting

Cells were homogenized with cold RIPA buffer (5 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 10 µg/ml aprotinin, 1 mM phenylmethane-sulphonyl fluoride, 1 µg/ml leupeptin, and 10 µg/ml pepstatin). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P membranes (Millipore, Billerica, MA USA). Membranes were incubated with primary antibodies for 2 h and then with the corresponding peroxidase-labeled secondary antibody for 30 min. Peroxidase activity was visualized using a commercial chemiluminescence kit (Pierce, Rockford, USA). Anti-GPER-1 is a rabbit polyclonal antibody directed to the C-terminus of the human receptor (ab39742; Abcam, UK). Furthermore, antibodies raised against ERα (PA5-16440; Invitrogen, USA) and the kinin B1R (6) were used. The antibodies used for the first immunodetection procedure were stripped off as previously described (6) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Millipore) was then detected as control of protein loading.

Measurement of the $[Ca^{2+}]_i$

Cells were grown and synchronized in medium without phenol red, FBS and antibiotics for 48 h. Then, they were gently trypsinized, washed with PBS and incubated for 30 min at 37°C at a concentration of 5×10^6 cells/ml, in the darkness with 5 mM Indo-1 AM, a ratiometric fluorescent probe (Life Technologies). Next, cells were washed with PBS, resuspended in 25 mM Hepes buffer pH 7.4 containing (125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM NaH_2PO4, 0.1% bovine serum albumin and 0.1% glucose) (6, 30). During the assay, cells were stimulated with 100 nM ATP, which was used as a positive control. Additional controls were performed by using the anionic detergent triton X-100 and the calcium-chelating agent, EGTA (**Figure 3C**). Following stimulation with ATP, cells were stimulated with a range of concentrations of 17β-estradiol (0.1, 1 and 10 nM) (Sigma-Aldrich, Germany) or G1, the synthetic GPER-1 agonist (1, 10 and 100 nM) (Tocris, USA). Additionally, cells were pretreated for 5 min with an excess (1,000 nM) of the GPER-1 antagonist, G15 (Tocris, USA) prior stimulation with 17β-estradiol or G1. Both, cells under prolonged treatment with tamoxifen (tamoxifen-treated cells) and untreated cells were stimulated with 10 nM 17β-estradiol, 100 nM G1 or 1,000 nM tamoxifen. Tamoxifen was dissolved in ethanol and the other drugs were solubilized in dimethyl sulfoxide (Sigma-Aldrich, Germany).

Measurements were carried out in a spectrofluorometer LS55 (Perkin Elmer, Wellesley, MA USA) using 2×10^5 cells/ml in a thermostated cuvette that was under continuous agitation. Measurements were performed using an excitation wavelength of 330 nm, and emissions of 405 nm and 480 nm were recorded. Data were expressed using the ratiometric relationship between the absorbance at 405 and 480 nm (30).

Cell Proliferation Assay

Cells were seeded on 96-well plates and cultured at 70% subconfluence. After synchronization for 48 h in phenol red-free DMEM without FBS (6), cells were stimulated with a range of concentrations of tamoxifen (50, 100, 500, 1,000, and 2,000 nM). Comparative experiments were performed on cells stimulated, under identical conditions, but following pretreatment with the GPER-1 antagonist G15. After 24 h under stimulation, cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 2 h. Incorporation of BrdU was determined by a colorimetric immunoassay according to manufacturer's protocol (Roche, Germany). At least three independent experiments were carried out for each concentration point and each point was performed in triplicates. Positive control experiments were carried out stimulating cells with 10% FBS.

Statistical Analysis

Statistical evaluation between experimental groups was done with ANOVA analysis followed by post-test pairwise comparisons using Tukey's test. Values were expressed as mean ± SEM and significance was considered acceptable at the 5% level ($P < 0.05$).

RESULTS

Prolonged Tamoxifen Treatment Induces GPER-1 Overexpression and Increases Breast Cancer Cells Proliferation Rate

It has been shown that use of tamoxifen on patients with initial GPER-1 positive breast tumors increases GPER-1 protein expression and markedly reduces patient survival (20). Here we have corroborated that GPER-1 is overexpressed in breast cancer cells exposed for 7 days to 1,000 nM tamoxifen (**Figure 1A**), a concentration similar to that found in breast tissue of patients treated with this drug (24). When untreated cells and those under continuous treatment with tamoxifen were challenged with 1,000 nM tamoxifen for 24, 48, or 72 h, a significant increase in the expression of GPER-1 was observed in the cells under continuous treatment with the drug (**Figure 1A**). Furthermore, the proliferation rate, assessed as BrdU incorporation, in the cells that were under prolonged treatment with tamoxifen was significantly higher than those that were not under treatment (**Figure 1B**). A concentration-dependent response was clearly observed when the cells under prolonged treatment were stimulated with tamoxifen ranging 50 to 2,000 nM (**Figure 1B**). By comparison, cells that were not under continuous treatment with tamoxifen showed an increase in

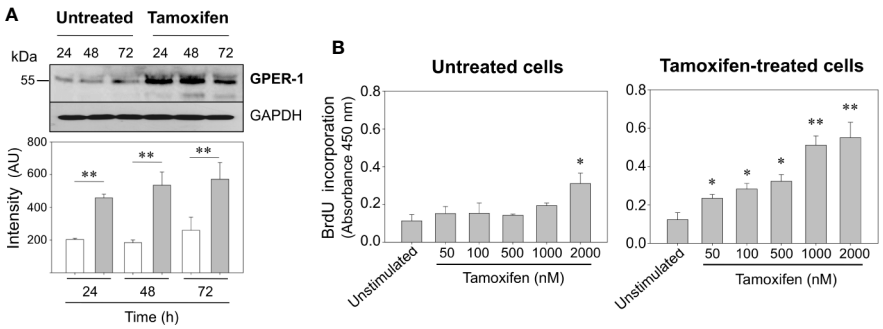

FIGURE 1 | Continuous tamoxifen treatment induces GPER-1 overexpression and an increase in BrdU incorporation in response to stimulation with tamoxifen. **(A)** MCF-7 cells that were under treatment with tamoxifen for 7 days together with their respective untreated controls were cultured, synchronized and then stimulated with 1,000 nM tamoxifen for 24, 48 and 72 h. Cells were homogenized and proteins separated by SDS-PAGE, transferred onto Immobilon-P membranes and immunoblotted using a specific antibody for GPER-1. The antibody was stripped off and the same membrane incubated with anti-GAPDH antibody as loading control. Data are representative of two independent experiments (n = 2). **P < 0.001. **(B)** Untreated cells or continuously exposed to tamoxifen were grown on 96-well plates, synchronized and stimulated with various concentrations of tamoxifen for 24 h. BrdU incorporation was determined by a colorimetric cell proliferation immunoassay and measuring absorbance at 450 nm. Results are shown as mean ± SEM (n = 3) *P < 0.05; **P < 0.001 between stimulated and unstimulated cells in both tamoxifen-treated and untreated cells.

BrdU incorporation only when they were stimulated with 2,000 nM tamoxifen (**Figure 1B**).

Prolonged Tamoxifen Treatment Increases Intracellular Calcium Signaling in Breast Cancer Cells Challenged With Tamoxifen

To examine whether continuous tamoxifen exposure modifies intracellular calcium signaling, cells were synchronized before labeling with the Indo-1 AM probe. Following a pulse with 1,000 nM tamoxifen, a significant $[Ca^{2+}]_i$ mobilization was generated in cells under prolonged tamoxifen treatment when compared with those that were not under prolonged treatment; in the latter, $[Ca^{2+}]_i$ mobilization was almost negligible (**Figure 2**). Cell integrity was assessed by stimulation of the same cells with a pulse of 100 nM ATP (**Figure 2**, top inbox). This response was comparable to that obtained in cells that were not under prolonged treatment with tamoxifen and were also stimulated with 100 nM ATP (not shown). Further controls of the technique included addition of triton X-100 and EGTA. Interestingly, preincubation of cells continuously treated with tamoxifen, for 5 min with 1,000 nM G15 (GPER-1 antagonist) significantly reduced the $[Ca^{2+}]_i$ mobilization triggered by tamoxifen in these cells, suggesting that an important fraction of the response to tamoxifen may be due to GPER-1 activation (**Figure 2**, bottom inbox).

Breast Cancer Cells Continuously Exposed to Tamoxifen Display Higher $[Ca^{2+}]_i$ Mobilization Than Untreated Cells When Stimulated With Estrogen or G1

To examine the influence of ERα and GPER-1 in calcium signaling of breast cancer cells, several approaches were carried out. These experiments showed that cells continuously treated with tamoxifen exhibited higher $[Ca^{2+}]_i$ mobilization than untreated cells when they were stimulated with 10 nM 17β-estradiol or 100 nM G1, a specific GPER-1 agonist (**Figures 3A, B**). The increase in $[Ca^{2+}]_i$ mobilization in response to 17β-estradiol was approximately 50% greater than that produced by the cells which had not been under prolonged treatment with tamoxifen; by comparison the increase produced by G1 was approximately 25% higher than that observed in untreated cells (**Figures 3A, B**). Preincubation of continuously treated cells with 1,000 nM G15, a GPER-1 antagonist, blunted the response triggered by G1 (**Figure 3C**). Interestingly, the same antagonist significantly reduced the response generated by 17β-estradiol. Additional controls were performed by using the anionic detergent triton X-100 and the calcium-chelating agent EGTA (**Figure 3C**). These results suggest that the increase in $[Ca^{2+}]_i$ mobilization, triggered by G1 or 17β-estradiol in tamoxifen-treated cells, is mediated mainly by GPER-1.

Prolonged Tamoxifen Treatment Increases the Proliferation Rate of Breast Cancer Cells in Response to Estrogen, G1, and Tamoxifen

To examine whether prolonged exposition of breast cancer cells to tamoxifen increases the cycling activity of these cells, a proliferation assay based on the incorporation of BrdU, an analogue of thymidine was performed. An increase in the incorporation of BrdU was observed in untreated and continuously treated cells stimulated for 24 h with 10 nM 17β-estradiol and 100 nM G1 (**Figure 4**). This increase was also significant when cells were stimulated with 2,000 nM tamoxifen (**Figure 4**). Interestingly, the increase in BrdU incorporation observed following stimulation with tamoxifen was inhibited by pretreatment of both continuously treated and untreated cells with G15, the GPER-1 antagonist (**Figures 4A, B**).

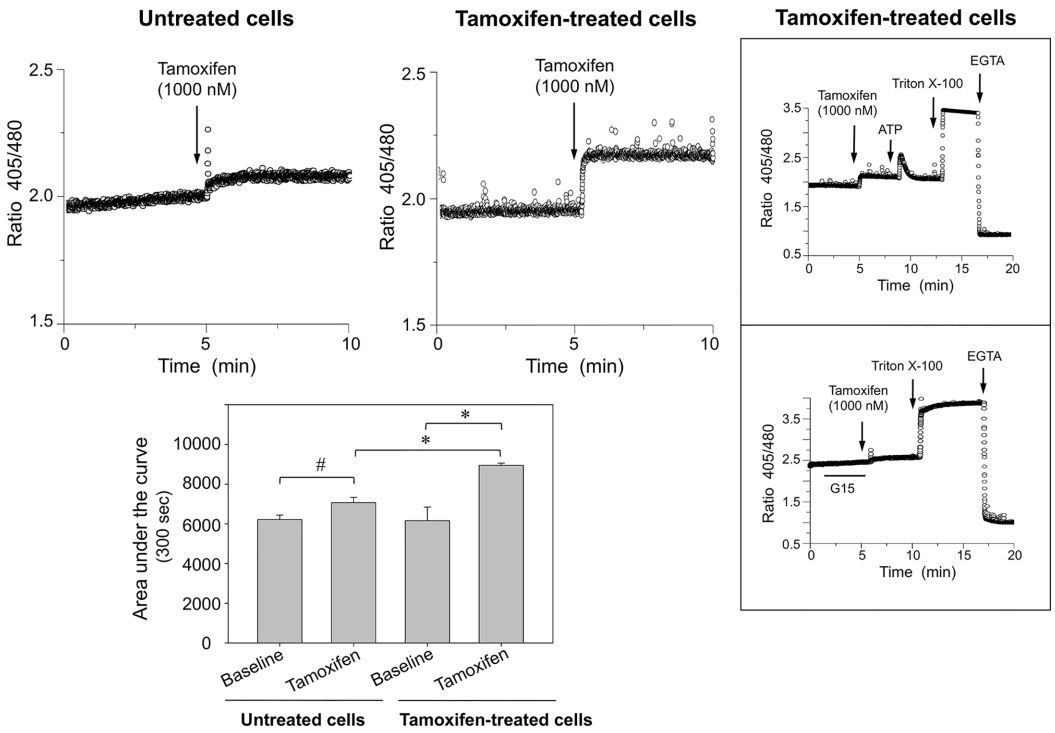

FIGURE 2 | Breast cancer cells continuously exposed to tamoxifen display higher $[Ca^{2+}]_i$ mobilization than untreated controls following stimulation with tamoxifen. MCF-7 cells that were under prolonged treatment with tamoxifen for 7 days, together with their respective untreated controls, were cultured, detached, and loaded with Indo-AM calcium probe and stimulated with 1,000 nM tamoxifen. The area under the curve was estimated to assess the magnitude of the response in both conditions. Results are shown as mean ± SEM (n = 3); *P < 0.05; #Not significant. *Insert*: The response elicited by tamoxifen was inhibited by preincubation of cells for 5 min with 1,000 nM G15, a GPER-1 antagonist. Additional controls using ATP, triton X-100 and EGTA are also shown.

Prolonged Tamoxifen Treatment Overexpresses the Kinin B1 Receptor but Not ERα in Breast Cancer Cells

Finally, we addressed two crucial questions: the first one was whether the treatment of MCF-7 cells with 1,000 nM tamoxifen for 7 days modified the expression levels of the classical estrogen receptor, ERα. As expected, protein expression levels of ERα did not change in tamoxifen-treated breast cancer cells respect to the untreated cells (**Figure 5A**). The second question was to examine the expression levels of another GPCR already known to favor the malignant phenotype of ERα positive breast cancer cells. Our previous studies have shown that stimulation of the kinin B1R results in an increase in cell proliferation, chemotaxis and release of matrix metalloproteases 2 and 9 from breast cancer cells (6, 29). Unexpectedly, kinin B1R protein expression was dramatically increased in the continuously treated cells that were additionally stimulated with 2,000 nM tamoxifen for 24, 48, and 72 h (**Figure 5B**), an effect that reinforces our first observations that kinin B1R stimulation increases the proliferation of breast cancer cells even at higher levels than estrogen (6). Our results suggest that ERα would not be directly involved in pharmacological resistance.

DISCUSSION

Estrogens, predominantly 17β-estradiol and its classical receptor, ERα, contribute to the development and progression of breast cancer. Drugs that block estrogen production or signaling by binding to ERα have been successfully used for many years. Such therapy includes SERMs (e.g., tamoxifen, raloxifene), antagonists of ERα (e.g., fulvestrant) and aromatase inhibitors, including reversible non-steroidal agents (e.g., letrozole, anastrozole), among others. Tamoxifen is, so far, one of the most commonly antiestrogenic drugs used for breast cancer treatment (31, 32). Endocrine therapy, based on the use of tamoxifen, has predominantly antiestrogenic effects in the breast and is aimed to block ERα in estrogen-sensitive breast cancer. Nevertheless, breast cancer patients may acquire resistance to antiestrogenic drugs complicating treatment. On the other hand, the existence of more complex and undiscovered signaling pathways beyond estrogen receptors appears to control cancer progression (33, 34). Thereby, the use of tamoxifen on breast cancer patients with initial GPER-1 positive tumors increased GPER-1 protein expression and markedly reduced survival (20).

The MCF-7 breast cancer cell line has emerged as one of the most widely used tool to scrutinize the effects of estrogen, SERMs

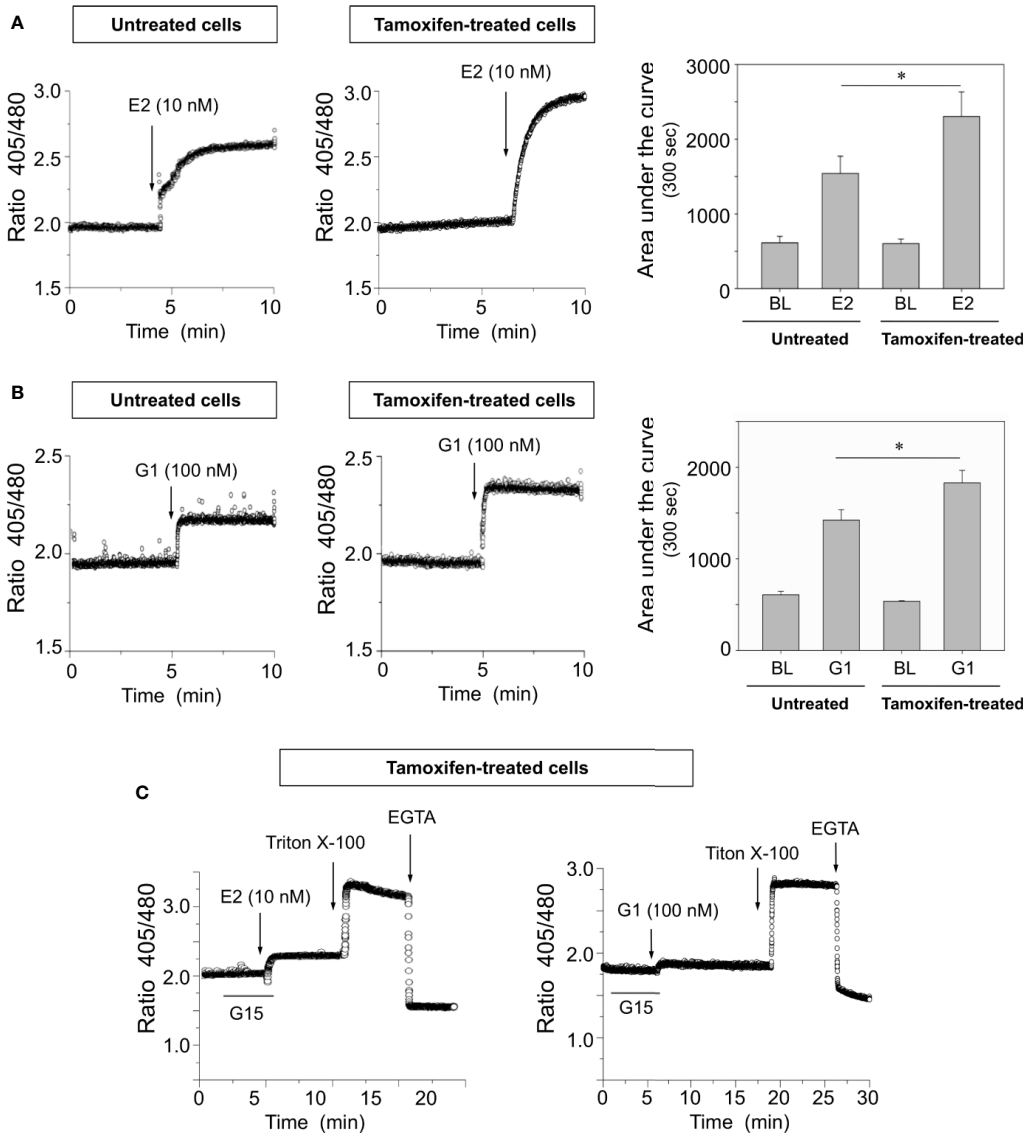

FIGURE 3 | Breast cancer cells continuously exposed to tamoxifen exhibit higher $[Ca^{2+}]_i$ mobilization than untreated controls when are stimulated with 17β-estradiol or G1. Continuously treated and untreated MCF-7 cells were cultured, detached and loaded with Indo-AM calcium probe before stimulation with 10 nM 17β-estradiol (E2) **(A)** or 100 nM G1 **(B)**, a specific synthetic agonist of GPER-1. The area under the curve was calculated and graphed to assess the magnitude of the response in each condition. BL, baseline. **(C)** Preincubation for 5 min with 1,000 nM G15 significantly decreased $[Ca^{2+}]_i$ mobilization in tamoxifen-treated cells; additional controls using triton X-100 and EGTA are also depicted. Results are shown as mean ± SEM (n = 3) *P < 0.05; between tamoxifen-treated cells and untreated controls.

and ERα antagonists. Although we cannot fully extrapolate the results obtained *in vitro* to the patients, this cell line is a good example of those mammary tumors made up of cells that express both ERα and GPER-1. Similarly, MCF-7 cells have been used to investigate the resistance to antiestrogenic drugs such as tamoxifen. Clearly, these studies should be expanded to other ERα positive breast cancer cell lines such as T47D and ZR-75-1 cells or better yet to 3D cultures using different cell lines or tumor cells directly obtained from patients with ERα positive breast tumors. Our experiments showed that MCF-7 cells exposed for 24 h to various concentrations of tamoxifen increased BrdU incorporation (DNA synthesis) when tamoxifen was present at 2,000 nM. A similar observation had been reported in the late eighties by Wakeling et al. (35), using also MCF-7 cells. Furthermore, Reddel and Sutherland (36) found that 10 nM tamoxifen had a proliferative effect on T47D breast cancer cells, a cell line which like MCF-7 cells expresses both ERα and GPER-1. Moreover, almost 50 years ago, tamoxifen had already been blamed to increase the growth of some types of breast cancer (37, 38). Interestingly, tamoxifen and 4-OH tamoxifen (the main metabolite of tamoxifen), two compounds that antagonize estrogen binding to ERα, are GPER-1 agonists (8, 17, 39). In a

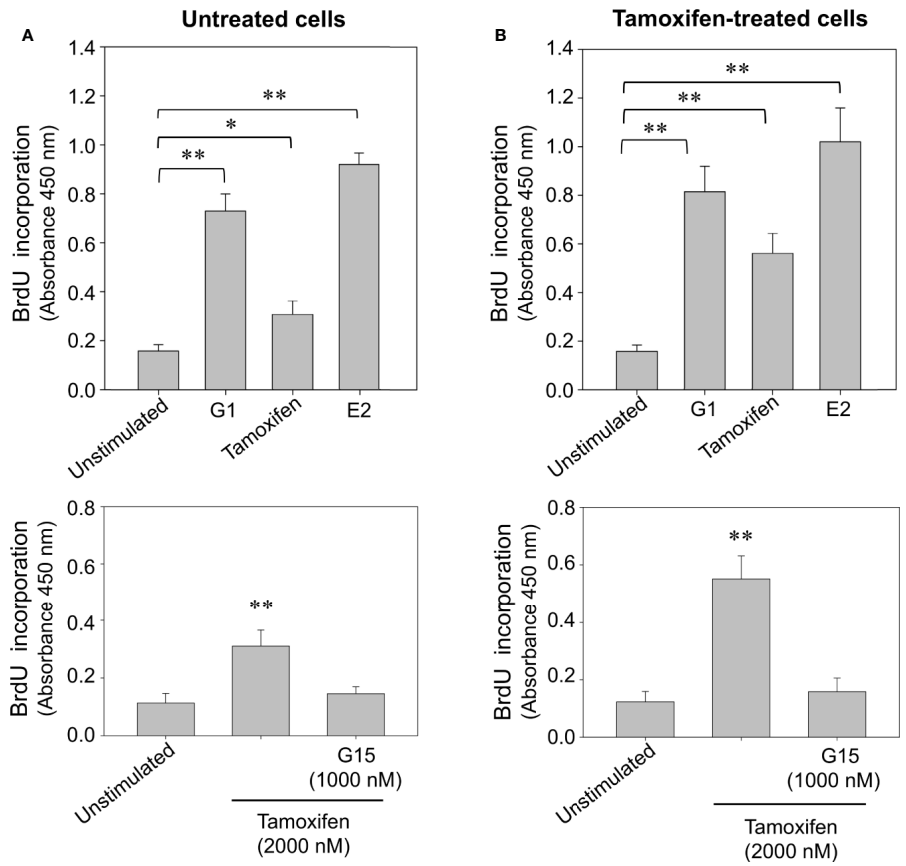

FIGURE 4 | Continuous tamoxifen treatment induces an increase in BrdU incorporation in breast cancer cells stimulated with tamoxifen. Untreated cells **(A)** or continuously exposed to tamoxifen **(B)** were grown on 96-well plates, synchronized and stimulated with 2,000 nM tamoxifen, 10 nM 17β-estradiol (E2) or 100 nM G1 for 24 h. BrdU incorporation was determined by a cell proliferation immunoassay and by measuring absorbance at 450 nm. The effect produced by tamoxifen on BrdU incorporation was significantly reduced by preincubation of cells with 1,000 nM G15 in both, untreated cells and cells continuously exposed to the drug. Results are shown as mean ± SEM (n = 3) *P < 0.05; **P < 0.001 versus unstimulated cells or preincubated with G15.

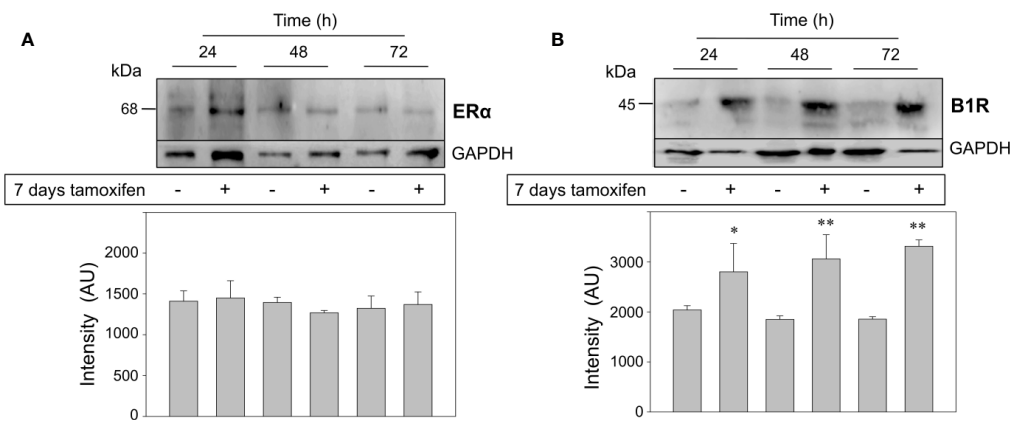

FIGURE 5 | Breast cancer cells continuously treated with tamoxifen overexpress the kinin B1R, but not ERα. MCF-7 cells that were under treatment with tamoxifen for 7 days together with their respective untreated controls were stimulated with 1,000 nM tamoxifen by 24, 48, and 72 h. Cell proteins were separated by SDS-PAGE, transferred onto Immobilon-P and immunoblotted with antibodies for detection of ERα **(A)** and kinin B1R **(B)**. Antibodies were stripped off and the same membranes were incubated with an antibody directed to GAPDH as control for protein loading. Data are representative of two independent experiments (n = 2). *P < 0.05; **P < 0.001 between tamoxifen-treated and untreated cells.

series of seminal experiments, Thomas et al. (13) were the first to describe a competitive binding (K_i values in the 10^{-7} M range) between estrogen and tamoxifen for GPER-1 expressed by SKBr3 breast cancer cells (ERα and ERβ negative, GPER-1 positive) or expressed by HEK cells transfected with GPER-1. Moreover, tamoxifen binding to GPER-1 resulted in activation of a stimulatory G protein and increase in adenylyl cyclase activity and cAMP levels. Subsequent experiments have shown that agonistic activity of tamoxifen or 4OH-tamoxifen triggers signaling pathways such as PI3K, ERK1/2 MAPK, and EGFR transactivation (14). The EGFR/ERK1/2 signaling cascade upregulates the expression of Egr-1 that in turn participates in the transcription of CTGF and cyclin D1, two genes that regulate breast cancer growth (16, 40). Similarly, agonistic activity of tamoxifen increases the proliferation of endometrial cancer cells by activating the GPER-1/EGFR/ERK1/2/CyclinD1 route, data that is in agreement with the observation that endometrial cancer patients under tamoxifen treatment exhibit a worse prognosis (41).

Previous reports have shown an increased translocation of GPER-1 to the cell surface of MCF-7 breast cancer cells that were continuously exposed to 10 nM tamoxifen for 6 months and stimulated with 17β-estradiol (20). Furthermore, other studies indicate that concentrations of tamoxifen and 4-OH tamoxifen reached in breast tissue of patients with ERα-positive breast cancer are significantly higher (up to about 100 times) than those present in plasma (42). Our results show that treatment of MCF-7 cells with 1,000 nM tamoxifen for 7 days produces a significant increase of GPER-1 protein expression. It is important to point out that this concentration is similar to that found in breast tissue of breast cancer patients treated with the drug (24). Other studies have shown that after 12 months of treatment with 10^{-7} M tamoxifen, this drug no longer inhibits the proliferative effect of estrogen on MCF-7 cells; during this period of time, tamoxifen did not increase cell proliferation (43). Furthermore, long-term exposure to tamoxifen has been shown to increase aromatase expression and activity, effects that depend on GPER-1 activity (44). Our results indicate that a short period of 7 days under continuous treatment with 1,000 nM tamoxifen induces overexpression of GPER-1, making breast cancer cells more sensitive to tamoxifen, which following GPER-1 activation triggers DNA synthesis, an effect that can be blocked by a specific GPER-1 antagonist. Therefore, overexpression and activity of GPER-1 appear as crucial steps for tamoxifen resistance since tamoxifen could increase cell proliferation directly by stimulating GPER-1 or indirectly by rising estrogen levels as result of an increase in the activity and expression of aromatase.

GPER-1 overexpression could be associated to carcinogenesis and to molecular strategies developed by tumor cells to escape tamoxifen treatment. GPER-1 overexpression has been observed in invasive ductal carcinomas of the breast when compared to adjacent healthy tissue (23) and in inflammatory breast cancer, a more aggressive form of this neoplasia (45). Signaling through GPER-1 has been shown to trigger the expression of IL-1β and IL-1R1 in cancer-associated fibroblasts and breast cancer cells, respectively. Thus, coupling of IL1β secretion by cancer-associated fibroblasts to the expression of IL-1R1 by cancer cells, promotes a positive regulation of protumoral genes such as those for COX-2 and ATP-binding cassette super-family G member 2 (46). Yu et al., 2020 (47) reported that ERα positive metastatic tissue shows increased levels of GPER-1 and ATP-binding cassette super-family G member 2 genes, which have been involved with multiresistance to different types of chemotherapy. Additionally, treatment of tamoxifen-resistant MCF-7 cells with G1 or with Fulvestrant (ICI 182,780) significantly increased GPER-1 expression, when compared to non-resistant MCF-7 cells (47). Pharmacological therapies can also alter intracellular signaling cascades such as $[Ca^{2+}]_i$ signaling, a key pathway in which calcium itself acts as second messenger or may participate in signal transduction to open ion channels. However, few studies have addressed the release of intracellular calcium triggered by tamoxifen in breast cancer cells. Our experiments showed that after prolonged treatment with tamoxifen, breast cancer cells stimulated with 1,000 nM tamoxifen mobilized $[Ca^{2+}]_i$ whereas untreated cells did not generate such a response. This response is a result of GPER-1 overexpression attributed to the prolonged treatment of breast cancer cells with the drug. In fact, preincubation of tamoxifen-treated cells with 1,000 nM G15 reduced $[Ca^{2+}]_i$ to basal levels. Interestingly, $[Ca^{2+}]_i$ was also increased when continuously treated cells were stimulated with 10 nM 17β-estradiol, an effect that was greatly reduced following preincubation of cells with the GPER-1 antagonist suggesting that GPER-1 may also be involved in this response. GPER-1 overexpression was further manifested when continuously treated cells were stimulated with 100 nM G1, a synthetic GPER-1 agonist that also increased $[Ca^{2+}]_i$ in these cells.

Relevance of $[Ca^{2+}]_i$ mobilization in breast cancer cells has recently been adressed by Ji et al. (48) who showed that Cav1.3 (a subunit of the L-type calcium channel) is widely expressed in breast cancer tissue and is upregulated by estrogen. Notably, silencing of GPER-1 inhibited the positive regulation of Cav1.3 induced by estrogen, reversing the increase in intracellular calcium release and proliferation of breast cancer cells. Our experiments indicate that breast cancer cells continuously treated with tamoxifen exhibited a concentration-dependent increase in BrdU incorporation after stimulation with various concentrations of tamoxifen. As expected, preincubation of cells with 1,000 nM G15 reduced the BrdU incorporation induced by tamoxifen. Although the mechanisms of resistance in estrogen-sensitive breast cancer are probably multifactorial, our evidence indicates that at least part of the phenomenon may be due to overexpression and activation of GPER-1. This observation may be of great relevance for breast cancer patients that suffer from breast tumors that co-express GPER-1 and ERα and undergo tamoxifen treatment.

GPCRs are preponderant for tumor development, progression and generation of drug resistance (32). Therefore, we explored the possibility of molecular interactions between GPER-1 and the kinin B1R, another member of the GPCR family, which is strongly expressed in ERα positive breast cancer cells. We have previously shown that kinin B1R favors the malignant phenotype of breast cancer cells (MCF-7, T47D, and ZR-75-1 cells) because its stimulation by B1R agonists induces cell proliferation and secretion of metalloproteinases-2 and -9, and of kallikrein-related peptidase 6, among other effects (6, 29, 49). Coincidently, kinin B1R agonists are at higher levels in serum of patients with breast cancer than in healthy subjects (50), an observation that matched the

presence of kinin B1R binding sites detected by our group in fibroadenomas, ductal carcinomas *in situ* and in invasive ductal carcinomas (6). Furthermore, the use of inhibitors has shown that metalloproteinases secretion and proliferation of breast cancer cells relies on EGFR transactivation and activation of the EGFR/ERK1/2 MAPK cascade (6, 28).

Interestingly, we observed B1R overexpression in breast cancer cells that were under exposure to tamoxifen for a 7-day period. Our data suggest that kinin B1R overexpression is an early event and that together with GPER-1 it may be part of a cross-talk network in estrogen-sensitive breast cancer cells to enhance cell proliferation and/or metastasis by activating signaling mechanisms, which are independent of ERα. Analysis of data from a subset of breast cancer patients has shown that GPER-1 expression has also been positively correlated with overexpression of EGFR (21). Since, a cooperative effect or association between GPER-1 and kinin B1R in breast cancer has not been explored yet; a next set of experiments should be focused on the role of both receptors in breast cancer patients. In addition, the GPER-1/EGFR signaling axis mediates the expression of cell cycle regulatory genes in cancer-associated fibroblasts derived from breast cancer patients, favoring tumor progression (40). It has recently been shown that stimulation with tamoxifen, activates GPER-1, improving breast cancer stem cells viability and stemness and BAD phosphorylation, event that seems to be an alternative survival mechanism for these cells (51).

Together our findings suggest that GPER-1 plays a key role in the mechanisms of tamoxifen resistance of estrogen-sensitive breast cancer cells, extending the limits of understanding of the effects generated by tamoxifen in these cells.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LM contributed to the design and execution of the experiments, writing, discussion, and revision of the article. PE and CF contributed to the experimental design, discussion, writing, and revision of the article. FB, AO, and IR contributed to the experimental procedures.

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MINI REVIEW
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Does GPER1 Play a Role in Sexual Dimorphism?

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Estrogens are critical in driving sex-typical social behaviours that are ethologically relevant in mammals. This is due to both production of local estrogens and signaling by these ligands, particularly in an interconnected set of nuclei called the social behavioural network (SBN). The SBN is a sexually dimorphic network studied predominantly in rodents that is thought to underlie the display of social behaviour in mammals. Signalling by the predominant endogenous estrogen, 17β-estradiol, can be either *via* the classical genomic or non-classical rapid pathway. In the classical genomic pathway, 17β-estradiol binds the intracellular estrogen receptors (ER) α and β which act as ligand-dependent transcription factors to regulate transcription. In the non-genomic pathway, 17β-estradiol binds a putative plasma membrane ER (mER) such as GPR30/GPER1 to rapidly signal *via* kinases or calcium flux. Though GPER1's role in sexual dimorphism has been explored to a greater extent in cardiovascular physiology, less is known about its role in the brain. In the last decade, activation of GPER1 has been shown to be important for lordosis and social cognition in females. In this review we will focus on several mechanisms that may contribute to sexually dimorphic behaviors including the colocalization of these estrogen receptors in the SBN, interplay between the signaling pathways activated by these different estrogen receptors, and the role of these receptors in development and the maintenance of the SBN, all of which remain underexplored.

Keywords: social behavior network, estrogen receptor isoforms, sex differences in brain, neuroestrogens, aromatase, mood, behavior

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INTRODUCTION

The steroid hormone 17β-estradiol (E_2) is the most physiologically relevant estrogen, with a myriad of effects that is dependent on signaling from a receptor. The classical genomic mode of estrogen signaling is *via* nuclear estrogen receptors (ER) α and β, which translocate to the nucleus upon ligand binding to act as transcription factors, regulating transcription over hours to days (1). Nongenomic signaling is a second mode of estrogen signaling which employs membrane-limited forms of ERα and ERβ, as well as the novel G protein-coupled estrogen receptor (GPER)1, to activate second messenger pathways resulting in rapid outputs within seconds to minutes. In the brain, E_2 acts *via* both signaling mechanisms to facilitate spinogenesis and dendrite growth (2, 3), cell survival (4), and neuroprotection (5). All these processes contribute to the sexual differentiation of the brain, a process that is restricted to critical periods of development in conserved nuclei of the brain referred to as the social behavior network [SBN; (6)]. After development, the SBN remains responsive to E_2 acting *via* the ERs,

integrating information about external and internal stimuli to drive sexually dimorphic expression of behaviors including reproductive behaviors, aggression and anxiety, and to some extent neuroprotection. In this review, we detail the contribution of the various ERs to the formation of the sexually dimorphic SBN and to the local production of estrogens, with areas of future exploration highlighted.

THE SOCIAL BEHAVIOR NETWORK

The social behavior network (SBN) is a conserved set of hypothalamic and limbic nuclei that contribute to the expression of sex-typical social behaviors (6) *via* sexually dimorphic nuclei (SDN). These are structures within the SBN that differ in volume, cell type, and receptor expression between sexes. The neuroanatomical connections, and the contribution of each SBN nuclei to social behavior has been reviewed in detail in (7).

E_2 in the critical developmental period organizes the SBN (**Figure 1**) *via* molecular mechanisms that include neurogenesis (10, 11), programmed cell death (12), and synaptogenesis (13) and pruning (14). Following reproductive maturation, E_2 then activates the SBN. Which ERs regulate these processes? In the female hippocampus, both ERα and GPER1 increase spinogenesis *via* ERK and JNK pathways (15) to consolidate spatial memories. Moreover, GPER1 activation leads to rapid increases in hippocampal spine density and promotes social cognition (16, 17). In neocortical cultures, GPER1 activation increases apoptosis mediated by the endocrine disrupter benzoquinone (18) while GPER1 activation can increase the migration of stem cells in the subventricular zone (19). Presumably, these processes are required for GPER1 modulation of sex-typical behaviors such as lordosis and social cognition. For details of the GPER1 including pharmacology, subcellular distribution and signaling, its role in behavior including its modulation of ERα function, the reader is directed to both (9, 20).

Preoptic Area of the Hypothalamus

The sexually dimorphic nucleus of the preoptic area (SDN-POA) has a larger volume in the male due to increased cell density (21). The perinatal androgen surge at E18 and subsequent aromatization to E_2 protects dopaminergic cells in the male SDN-POA from apoptosis (22). The receptor for preserving the volume of the SDN-POA is ERα, since WT and androgenized female rats treated with antisense oligonucleotides against ERα show a significantly smaller SDN-POA volume compared to their respective controls (23) though ERα expression levels are not significant between the sexes (**Table 1**). Non-genomic signaling is critical since male mice with a mutation that destroys the tethering of the ERα to the membrane and its ability to initiate non-genomic signaling showed decreased calbindin-immunoreactive (a marker for the SDN-POA) neurones (37). Knockdown of *Gper* in zebrafish resulted in a greater number of cells stained with acridine orange, a marker for apoptosis (38). However, specific brain regions were not identified, and it is not known if this role for GPER1 exists in rodent species. Indeed, the localization of GPER1 within the SDN-POA has not been directly investigated, though efferents from the mPOA to the VTA do express GPER1 (39). Yet, despite its role in non-genomic signaling, the establishment of sexual dimorphisms by GPER1 in the POA is unknown.

Anteroventral Periventricular Nucleus of the Hypothalamus

The anteroventral periventricular nucleus (AVPV) contrasts from the neighboring SDN-POA as females harbor greater cell volumes (40), greater numbers of glia (41), and greater numbers of dopaminergic neurones (40) compared to males. The surge of E_2 availability in the perinatal male brain upregulates caspase activity and cell death whilst new cells are added to the female AVPV during puberty (11) though the ER that mediates this is not clear.

Adult females express greater amounts of ERα than males (**Table 1**) and levels are not affected by gonadectomy (GDX) (32), which suggests that differences in expression occur prior to adulthood. ERKOα male mice have a greater AVPV volume (24) and a greater number of tyrosine hydroxylase (TH)-positive cells (42) than WT males. However, ERαKO males still have significantly less dopaminergic neurones than WT females (42), suggesting that another receptor contributes to the masculinization of the AVPV. ERβ may be a candidate since it is coexpressed with both ERα and TH in the female AVPV (43) and ERKOβ males have increased TH-ir compared to their WT counterparts (44). Together, this suggests that the sexual dimorphism in dopaminergic cell populations in the AVPV is driven by high levels of E_2 in the male brain acting through both ERα and ERβ to drive cell death. Interestingly, in cultured dopaminergic neurones shown to express both ERα and GPER1, E_2 is neuroprotective (5), suggesting that GPER1 may have a modulatory effect on ERα signaling. Though knockout of both α and β ERs (ERKO) has no effect on glial cell numbers in the male AVPV (24), the death of glial cells is an E_2-dependent process since aromatase KO (ArKO) mice have increased numbers of glial cells (24). This suggests that another ER, such as GPER1, that is abundant in glia, may contribute to the masculinization of the AVPV. Indeed, in an oxygen-glucose deprivation model, GPER1 increases apoptosis of cortical astrocytes (45). Neither expression of the GPER1 protein, nor its colocalization with other ERs in the AVPV, have been characterized in the male or female rodent.

The Medial and Extended Amygdala

The medial amygdala (MeA) is a major source of input to the medial (m)POA, responsible for relaying olfactory information that underlies social recognition. Similar to the AVPV, new cells are added to the MeA during puberty albeit solely in the male (11). Targeted knockdown of ERα in the MeA in pubertal male mice feminizes the volume of the MeA by reducing neuron numbers (46), suggesting ERα-mediated signaling is important in the establishment of volumetric sex differences.

Aromatase is strongly expressed in the MeA of male mice, particularly in nerve fibers (34) and may contribute to the modulation of synaptic properties of the female MeA across the estrous cycle (47), as E_2 inhibits neural transmission from the MeA (48). Indeed, administration of the GPER1 agonist G-1

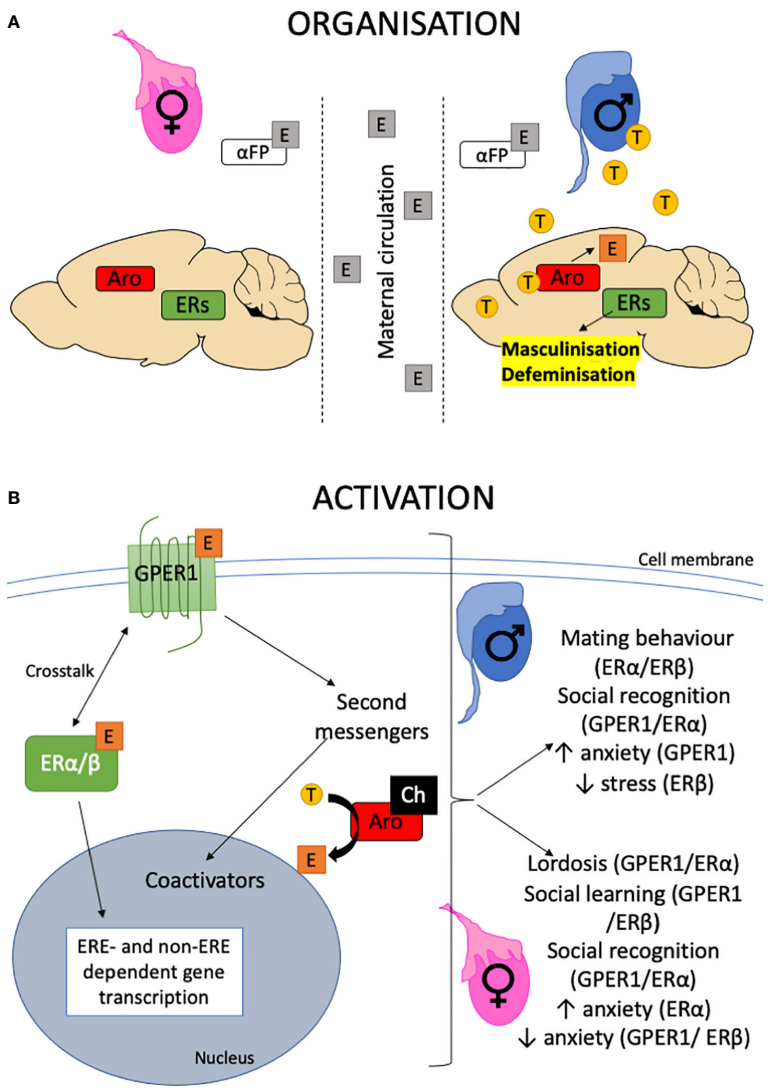

FIGURE 1 | Organizational-activational hypothesis. **(A)** The testes are active during perinatal development providing testosterone for central aromatase (Aro) to produce estrogen (E) within the brain. Estrogen organizes the brain by binding to ERs, leading to the masculinization and defeminization of the brain. By contrast, the perinatal ovary is quiescent. *In utero*, the brain is protected from estrogens that may enter *via* maternal circulation by the presence of α-fetoprotein that binds estrogen. The role of the GPER1 in this organizational period is largely unknown. For a detailed review, the reader is referred to (8) and references therein. **(B)** The organized neural substrate is activated following puberty when the gonads become active. Estrogen is released from the ovaries and testosterone (T) from the testes, which is then aromatized to estrogen in the brain. The availability of cholesterol (Ch) and presence of steroidogenic enzymes within the brain also allows for the *de novo* production of neuroestrogens. Estrogens activate neural circuits to express behaviors through activating second messenger pathways such as MAPK acutely and recruiting transcriptional coactivators such as fos and jun to regulate non-ERE containing promoters. This could be *via* multiple ERs, including GPER1 (9). Alternatively, the classical nuclear hormone receptors, ERα/β can translocate to the nucleus to directly bind estrogen-response-elements in DNA to regulate gene transcription. Both these pathways result in modulation of behaviors in both males and females.

attenuates the upregulation of NMDA receptors in the female basolateral amygdala and blocks the downregulation of $GABA_A$ receptors to increase inhibitory synaptic transmission (49).

The bed nucleus of the stria terminalis (BNST) is part of the extended amygdala and plays a key role in stress and anxiety-denoting behaviors (50), expressing both ERα and ERβ during developmental periods (**Table 1**). A subregion of the BNST, the principal nucleus of the BNST (BNSTp) is larger in males than females (51). Administration of testosterone propionate (TP) to females at P1 increases volume, although not to a level comparable with males (52–54). This may be a reflection of greater aromatase expression within the male BNST (34), allowing the brain to generate more estrogen to produce a greater magnitude of masculinization. In addition, it could be a reflection of less androgen receptor (AR) expression in the female brain (55), since masculinization of the BNSTp requires both E_2 signaling and

TABLE 1 | Sexual dimorphisms in central ER and aromatase expression across development.

Area	ERα			ERβ			GPER1			Aromatase		
	Pn	Pb	A	Pn	Pb	A	Pn	Pb	A	Pn	Pb	A
Hypothalamus												
ARH	= [7,8]	= [7]		X [3]	= [3]				= [13]			= [11]
VMH	= [7,8]	F [7]		F [6]	= [6]	=* [12]			= [13]	=* [4]		
PVH		= [7]				=* [12]						= [11]
LS	= [7]	= [7]							= [13]			
AVPV	F [1,9]		F [9]	X [1]	X [10]				= [13]			
mPOA	= [7,8]	F [7,8]		= [3]	F [3]	M* [12]			= [13]	X* [4]		M [2,11]
Extended amygdala												
BNST	F [1,9]	= [7,9]	F [9]	= [1]		M* [12]			= [13]	= [1] M* [4]		M [2]
MeA		= [7]				F* [12]			= [13]	=* [4]		M [2]
Bird song areas								M [5]	M [5]			

Relative expression of receptors and aromatase during perinatal (Pn), pubertal (Pb), and adult (A) periods. "F" denotes a greater expression in females, "M" a greater expression in males, "=" an equal expression between males and females, and "X" indicates undetectable expression. All referenced research uses mouse or rat (*) models, apart from one study which used zebra finches to study GPER1 expression in song areas. References 1–5 measured mRNA expression; references 7–12 measured protein expression; reference 6 measured both mRNA and protein. 1. (24). 2. (25). 3. (26). 4. (27). 5. (28). 6. (29). 7. (30). 8. (31). 9. (32). 10. (33). 11. (34). 12. (35). 13. (36).

testosterone signaling *via* the AR (24). Similar to the AVPV, both ERα and ERβ are required for complete masculinization of the BNSTp, since PPT and DPN (ERα and ERβ agonists respectively) given in the perinatal period increase cell number of the female BNSTp, but neither completely mimicked the effects of E_2 alone (56), suggesting that synergy between ERs, including GPER1 may maintain sexual dimorphism.

The pattern of expression of the ERs (**Table 1**) and the use of pharmacological and genetic studies to target them suggest that the development of the SBN frequently depends on a combination of ERα and GPER1 though it often appears that the role of ERα is predominant. This suggests that both membrane-initiated signaling and classical transcriptional signaling might be important for sex-typical behaviors that are responsive to external stimuli over longer time frames. The idea that GPER1 may facilitate or antagonize ERα signaling has been reviewed in (20) with examples given within and outside the brain. Given that the male brain expresses more aromatase and has more neuroestrogens (Section 3), we speculate that in most instances, neuromorphological organizational changes are driven by these ERs in the male, rather than the female brain.

A number of caveats exist to the localization data. First, most studies have compared the longitudinal dynamics of ER expression in the SBN of wildtype (WT) animals, focusing largely on sexual dimorphisms within one particular age window and/or nucleus. Unusually, a recent study showed that ERα and GPER1 were higher in the striatum of both male and female rats during development and perinatal life but then declined in a sexually dimorphic manner as development proceeded (57); however, they did not explore such developmental dynamics in the SBN. Secondly, due to antibody issues, colocalization studies of GPER1 with the other ERs have not been performed.

LOCAL ESTROGEN SYNTHESIS WITHIN THE SBN

Apart from the contribution of the ERs, another mechanism that affects SBN nuclei is the provision of local estrogens. The brain expresses the enzymes required to synthesize estrogens *de novo* (neuroestrogens) (58, 59). Developmentally, central aromatase may be important for allowing specific regions to access higher concentrations of E_2 to maintain cell numbers or drive apoptosis, although more evidence is required to support this idea. In an activational context, aromatase may be important for maintaining stable concentrations of neuroestrogens when systemic concentrations fluctuate across the estrous cycle, as seen in female baboons (60).

Regulation of Aromatase: Substrate Availability and Development

Is the regulation of aromatase sexually dimorphic? In limbic areas, aromatase activity appears to be constitutive (61). Therefore, the regulation of aromatase activity is proposed to rely on two different systems: a gonad-sensitive hypothalamic system and a non-gonad-sensitive limbic system (62, 63). Though there are no sex differences in aromatase mRNA expression in the BNST or AVPV during perinatal development (24), male rodents have greater levels of aromatase gene expression than females by adulthood (25). In line with this, prepubertal GDX in males reduces aromatase activity in adulthood (64), suggesting that aromatase expression is pubertally organized by pubertal gonadal hormones.

The regulation of central aromatase may also be determined by estrogens themselves. In MCF-7 cells, aromatase activity is upregulated by estrogens in a positive autocrine feedback loop *via* either ERα or GPER1 (65, 66). In transgenic mice that express EGFP in aromatase-positive neurons, EGFP is more highly co-expressed with ERα, ERβ and AR in the male BNST and MeA than in the adult female though co-expression of the ERs and AR with EGFP was prevalent in other nuclei of the SBN of both sexes (34). In contrast, aromatase is mostly co-expressed with ERα during the perinatal period (67), highlighting the potential to investigate developmental change in co-expression, which may be partly explained by the sexually dimorphic addition of new cells during puberty (11). How GPER1 regulates aromatase in the SBN is a question that is currently being investigated by us.

DISCUSSION: THERAPEUTIC POTENTIAL FOR GPER1

Why is the contribution of GPER1 to a sexually dimorphic SBN important? A sexually dimorphic brain results in sexually dimorphic disorders that are important to consider clinically. For example, neurodegenerative diseases disproportionately affect women (68), whereas learning difficulties such as those associated with autism spectrum disorder and dyslexia are more commonly observed in males (69). Females have a greater risk of developing depression, anxiety, or panic disorders (70) which are correlated with hormonal changes in puberty and menopause (71). E_2 can elicit anxiogenic or anxiolytic effects in the amygdala (72). ERα knockdown in the medial posterodorsal amygdala (MePDA) resulted in female rats spending more time in the light chamber in the light-dark test (LDT), implicating ERα as anxiogenic (73). On the other hand, there is a general consensus that ERβ is anxiolytic (72) while the role of GPER1 is less clear. Chronic administration of G-1 was anxiolytic in the open field test (OFT), but not the elevated plus maze (EPM) (74) in females while acute administration of G1 was anxiolytic in the EPM within 30 min of administration in males but not females (75). On the contrary, another study found that agonism of GPER1 produced anxiogenic effects in both the OFT and EPM (76) in male and female mice. In this study, G-1 was injected 2h before behavioral testing. Thus, the timeframe of administration may be an important factor in determining the roles of ERs in anxiety. The actions of GPER1 may also depend on the context of anxiety, i.e. whether the animal is previously stressed. Acute stress (imposed by restraint or forced swim tests) significantly decreased the time spent in the open arms and central area of the EPM, but this is ameliorated with G-1 treatment (49) in ovariectomized females. Moreover, acute stress significantly increased the levels of GluR1-containing AMPA receptors and NR2A-containing NMDA receptors, thus increasing small excitatory postsynaptic currents (sEPSCs). However, G-1 treatment reversed these effects, enhancing small inhibitory postsynaptic currents (sIPSCs) instead (49). Thus, GPER1 may be important in mitigating stress-induced anxiety, with little-to-no role in inhibiting behaviors that denote anxiety in the absence of stress. This specificity might allow for the development of personalized medications for anxiety. Furthermore, targeting GPER1 over ERα or ERβ may be preferable given the possible sexual dimorphism in anxiety modulation (75), involvement of the classical ERs in reproductive development and function, and the role of ERβ the in estrogenic modulation of GnRH (77)

FUTURE PERSPECTIVES

Clearly, understanding how GPER1 functions both independently and as a putative modulator of classical ERs in both sexes is imperative for uncovering its therapeutic potential in hormone-associated mood disorders. GPCRs such as the serotonin 1A receptor have been associated with the development of depression. SSRIs function by desensitizing serotonin 1A receptor signaling to decrease plasma levels of oxytocin and adrenocorticotropic hormone (ACTH) (78). The efficacy of SSRIs in attenuating serotonin 1A receptor mediated signaling and consequent oxytocin and ACTH release can be accelerated with G-1 treatment. Dual treatment targeting GPER1 means that symptoms of depression can be alleviated earlier, as it takes up to 12 weeks to reach clinical efficacy with SSRIs alone (79). Furthermore, a recent study has implicated GPER1 as a diagnostic tool for GAD and MDD. Drug-naïve patients with anxiety or depressive disorders exhibit increased serum levels of GPER1, which correlate with anxiety scores (80). This result was found to be independent of sex although mouse models suggest that the role of GPER1 in regulating anxiety is slightly more pronounced in males (76, 81). Though there is a general lack of sexual dimorphism in GPER1 expression with moderate to high distribution of GPER1 in the adult SBN (36), a recent study has shown that GPER1 concentrations decrease with approaching adulthood and the distribution shifts from multicompartment to predominantly cytoplasmic or membrane distribution in the striatum (57). This suggests that GPER1 expression is capable of being developmentally regulated though the significance of such regulation remains unknown. Moreover, the effects of GPER1 activation in adulthood on molecular mechanisms linked to sexual dimorphism raise the possibility that GPER1 may have similar effects in the perinatal and pubertal critical periods. This could be investigated by determining a) the expression of GPER1 in development *versus* adulthood in the SBN and its colocalization with ERα, ERβ; and aromatase; b) the effect of GPER1 agonism with G-1 and antagonism with specific antagonist G-15 and G-36 during the critical periods on sex differences in morphology, neuroestrogen production, and molecular signaling prevalent in the SBN; c) the nature of modulation of ERα action in the SBN. Some of this may be explored with the use of a conditional, regional GPER1KO model, though this is yet to be generated. Therefore, the distinct roles of GPER1 within specific limbic *vs* SBN nuclei in adulthood *versus* developmental periods need to be better understood to produce a targeted medication to alter mood without changing the expression of sex-typical organized behaviors involving GPER1, such as reproduction.

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Both JD and NV wrote and modified the text of the article after discussions. JD was responsible for the figure and the **Table 1** (which was further modified by NV). All authors contributed to the article and approved the submitted version.

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Soy Isoflavones Accelerate Glial Cell Migration *via* GPER-Mediated Signal Transduction Pathway

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Soybean isoflavones, such as genistein, daidzein, and its metabolite, S-equol, are widely known as phytoestrogens. Their biological actions are thought to be exerted *via* the estrogen signal transduction pathway. Estrogens, such as 17β-estradiol (E2), play a crucial role in the development and functional maintenance of the central nervous system. E2 bind to the nuclear estrogen receptor (ER) and regulates morphogenesis, migration, functional maturation, and intracellular metabolism of neurons and glial cells. In addition to binding to nuclear ER, E2 also binds to the G-protein-coupled estrogen receptor (GPER) and activates the nongenomic estrogen signaling pathway. Soybean isoflavones also bind to the ER and GPER. However, the effect of soybean isoflavone on brain development, particularly glial cell function, remains unclear. We examined the effects of soybean isoflavones using an astrocyte-enriched culture and astrocyte-derived C6 clonal cells. Isoflavones increased glial cell migration. This augmentation was suppressed by co-exposure with G15, a selective GPER antagonist, or knockdown of GPER expression using RNA interference. Isoflavones also activated actin cytoskeleton arrangement *via* increased actin polymerization and cortical actin, resulting in an increased number and length of filopodia. Isoflavones exposure increased the phosphorylation levels of FAK (Tyr397 and Tyr576/577), ERK1/2 (Thr202/Tyr204), Akt (Ser473), and Rac1/cdc42 (Ser71), and the expression levels of cortactin, paxillin and ERα. These effects were suppressed by knockdown of the GPER. Co-exposure of isoflavones to the selective RhoA inhibitor, rhosin, selective Cdc42 inhibitor, casin, or Rac1/Cdc42 inhibitor, ML-141, decreased the effects of isoflavones on cell migration. These findings indicate that soybean isoflavones exert their action *via* the GPER to activate the PI3K/FAK/Akt/RhoA/Rac1/Cdc42 signaling pathway, resulting in increased glial cell migration. Furthermore, *in silico* molecular docking studies to examine the binding mode of isoflavones to the GPER revealed the possibility that isoflavones bind directly to the GPER at the same position as E2, further confirming that the effects of the isoflavones are at least in part exerted *via* the GPER signal transduction pathway. The findings of the present study indicate that isoflavones may be an effective supplement to promote astrocyte migration in developing and/or injured adult brains.

Keywords: genistein, daidzein, S-equol, 17β-estradiol, astrocyte, F-actin, development, migration

INTRODUCTION

Soybean isoflavones are a natural class of isoflavones, exclusively produced by the legume family (1). They are well-known phytoestrogens that can bind and modulate the action of nuclear receptors including estrogen receptor (ER), thyroid hormone receptor, androgen receptor, pregnane X receptor, and aryl hydrocarbon receptor (2–6). Binding of isoflavones to receptors exerts various effects at the molecular, cellular, and organ levels (7). In addition, isoflavones also can affect other pathways by modulating membrane receptors, protein kinases, transcription factors, chromatin remodeling, antioxidants, and altering some enzyme activities (8, 9). Genistein, daidzein, and S-equol, a metabolite of isoflavones, are the main isoflavones that have been intensively studied. This wide variety of actions indicates that isoflavones act *via* several different signaling pathways.

Recent studies have shown that 17β-estradiol (E2) activates the G protein-coupled estrogen receptor (GPER; also known as GPR30), which then initiates several intracellular signal transduction pathways, such as the epidermal growth factor receptor-mediated pathway to activate extracellular signal-regulated kinase 1/2 (ERK1/2) and/or Akt-mediated pathways (10–13). In addition to E2, isoflavones may also interact with the GPER. *In vitro*, activation of the GPER by isoflavones has been demonstrated to trigger cell signaling pathways and growth factor receptor cross-talk (14, 15). Our previous study showed that S-equol could activate GPER to increase p-ERK1/2 leads to induced proliferation, growth, and differentiation in both neurons and astrocytes during cerebellar development (14). The K_d (dissociation constant) of E2 to the GPER is 3–6 nM. Meanwhile the effective concentration 50 (EC_{50}) values of isoflavones to the GPER based on functional dose-response 133 nM for genistein, < 1 nM for daidzein, and 100 nM for S-equol (16). Based on these findings, we hypothesized that isoflavones would affect the GPER signaling pathway and alter cellular function.

Estrogen plays a key role in the development and functional maintenance of the central nervous system (CNS) through genomic (*via* the ER) and rapid nongenomic responses *via* the GPER (17, 18). GPER is highly expressed in the CNS, including glial cells (19). GPER knockout mice showed altered anxiety levels and stress response (18), and this phenotype could not be fully rescued by estrogen treatment (18). These results indicate the involvement of the GPER in the normal development of the CNS. However, the role of the GPER on the function of each subset of cells remains unclear.

Glial cells are essential for brain functioning during development and in the adult brain and have been shown to play a significant role in neuronal migration, proliferation, differentiation, and synaptogenesis (20). Glial cells comprise astrocytes, oligodendrocytes, and microglia, among which, astrocytes are the most abundant cell type in the CNS (21). Astrocytes are most likely migrate to their final destination shortly after their birth in the ventricular zone or subventricular zone, cortical gray matter astrocytes were found to migrate along with radial glia processes, whereas white matter astrocytes migrated along developing axons of neurons (21, 22). Astrocytes are activated in injured or diseased CNS and begin to proliferate and migrate. This process is known as astrogliosis (21). High levels of GPER expression in astrocytes may affect the physiological response of astrocyte during development or in the adult brain.

Cell migration is a critical process in both physiological and pathological processes. The Rho family of GTPase is the core regulator of cell migration (23). In the CNS, Rho GTPase family members, such as RhoA, Rac1, and Cdc42, play fundamental roles in a wide variety of cellular processes, including rearrangement of the actin cytoskeleton, cell polarity, and controlling dynamic astrocyte morphology (24–26). Deletion of Rac1 and Rac3 in cerebellar granule neurons (CGNs) led to severe impairment of radial migration of CGNs, defects in the internal granule layer, and decreased cerebellum size (27). Cdc42 knockout mice also showed impaired radial migration of CGNs, disturbed alignment of Bergmann glia in the Purkinje cell layer, and aberrantly aligned Purkinje cells (24). In addition, astrocytes lacking Cdc42 were still able to form protrusions, although were unable to migrate in a directed manner toward the scratch/wound (26). Since isoflavones may bind to the GPER in astrocytes, these results raise the possibility that isoflavones affect astrocyte migration *via* the RhoGTPase signaling pathway.

Our previous study showed that S-equol, a daidzein metabolite, activates GPER to induced F-actin rearrangement lead to increase astrocyte migration during cerebellar development with unknown mechanisms (14). The present study examined the effects of isoflavones on cell migration of glial cells using astrocyte-enriched cultures of cerebral cortex and astrocyte-derived C6 clonal cells by wound healing and cell migration/invasion assays. We also examined changes in the actin cytoskeleton by labeling F-actin using phalloidin. Our findings revealed that isoflavones induced F-actin rearrangement and accelerated cell migration. These effects were reduced by the GPER inhibitor, G15, or short interfering RNA (siRNA) knockdown of GPER. Furthermore, activation of GPER by isoflavones activated the PI3K/Akt signaling pathway that induce RhoGTPase to accelerate cell migration. The results of our *in silico* molecular docking study revealed a common possible binding site of the isoflavones on the GPER.

MATERIAL AND METHODS

Chemicals

Genistein, daidzein, and E2 were purchased from Sigma (St. Louis, MO, USA). S-equol, G-15, casin, ML-141, LY294002, and U0126 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Rhosin HCl was purchased from Tocris Bioscience (Avonmouth, Bristol, UK). The purity of all chemicals was >98%.

Clonal Cell Culture

C6 rat glioma clonal cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C with 5% CO_2. The serum was stripped of hormones by constantly mixing with 5% (w/v) AGX1-8 resin (Bio-Rad, Hercules, CA, USA) prior to ultrafiltration (28).

Primary Culture of Mouse Cerebral Cortex Astrocytes

The animal experimentation protocol in the present study was approved by the Animal Care and Experimentation Committee, Gunma University (19-024, 17 December 2018), and all efforts were made to minimize animal suffering and the number of animals used.

A primary culture of mouse cerebral cortex astrocytes was prepared as previously described (29, 30) with slight modifications. A pregnant C57BL/6 strain mice were purchased from Japan SLC (Hamamatsu, Japan). Briefly, postnatal day 1 mouse cerebral cortices were dissected and digested with 2.5% trypsin (Wako, Japan) in Hank's balanced salt solution (Wako) for 30 min with continued shaking at 37°C. Cells were resuspended in an astrocyte culture medium (high-glucose DMEM, 10% heat-inactivated FBS, and 1% penicillin/streptomycin), and 10–15 million cells were plated on 10-cm dishes coated with Collagen I (Iwaki, Japan). Cells were incubated at 37°C in a CO_2 incubator. On day 3 *in vitro* (DIV3), astrocyte culture medium was replaced with phosphate-buffered saline (PBS). Dishes were then shaken by hand for 30–60 s until only the adherent monolayer of astrocytes was left. The PBS was then replaced with a fresh astrocyte culture medium. Astrocytes were harvested on DIV7 using 0.25% trypsin 1 mM disodium EDTA (Wako), and then plated on 12 or 24 well dishes. Cells were used for cell invasion assay or F-actin staining.

In Vitro Wound Healing (Scratch) Assay

C6 cells were plated in 24-well plate and cultured until confluent. Prior to making a scratch, cells were serum-starved in FBS-free DMEM for 6 h. A wound was created by scratching the monolayer with a 200-µl pipette tip. Floating cells were washed away using PBS. Serum-free DMEM and/or isoflavones, E2, G15, U1026, LY294002, rhosin, Casin, and/or ML-141 were added to the wells and incubated for a further 24 h. At 0 and 24 h, live-cell staining was performed using Cellstain-Hoechst 33258 solution (Dojindo Molecular Technologies, Inc., Japan) according to the manufacturer's protocol. Images of the scratched area were taken at 0 and 24 h. The cells were then visualized using a fluorescence microscope (Keyence BZ9000, Keyence Corporation of America, Itasca, IL, USA). Cell migration was determined at the edges of the wound, and the percentage migration was determined as the ratio between migrated distance and initial distance of the wound.

Matrigel Invasion Assay

In vitro invasion assays were performed using a 24-well Millicel hanging cell culture insert and a Corning Matrigel matrix according to the manufacturer's instructions. In brief, astrocytes were seeded at a density of 1×10^5/ml in serum- free DMEM in the upper chamber. The lower chamber was filled with serum-free DMEM and/or isoflavones, E2, G15, U1026, LY294002, rhosin, casin, and/or ML-141. After 16–18 h of incubation, noninvading cells in the upper chamber were removed with a sterile cotton swab. The filters from the inserts were fixed with 4% paraformaldehyde (PFA) and stained with DAPI. The cells were then inspected using a laser confocal scanning microscope (Zeiss LSM 880, Carl Zeiss Microscopy GmbH, Jena, Germany). The number of invaded cells on the lower surface of the filter was counted.

Filopodia Formation and Cortical F-Actin Score Index

Astrocytes were cultured on poly-L-lysine-coated coverslips and serum-starved DMEM for 24 h. The cells were then treated with either isoflavones or E2 for 30 min then washed with PBS and fixed with 4% PFA followed by blocking with 2% FBS. The cells were incubated with CytoPainter Phalloidin-iFluor 594 reagent (Abcam, Cambridge, UK) and nuclei were stained with DAPI and then visualized under a laser confocal scanning microscope (Zeiss LSM 880, Carl Zeiss Microscopy GmbH). The degree of cytoskeletal rearrangement was examined using the FiloQuant by ImageJ Fiji (NIH) or cortical F-actin score CFS index (31). The CFS index was determined based on at least three independent experiments. Briefly, F-actin cytoskeletal reorganization for each cell was scored on a scale ranging from 0 to 3, based on the degree of cortical F- actin ring formations 0, no cortical F-actin, normal stress fibers; 1, cortical F-actin deposits below half the cell border; 2, cortical F-actin deposits exceeding half the cell border; and 3, complete cortical ring formatting and/or total absence of central stress fiber. A minimum of 50 cells were examined from each group in each independent experiment, and the CFS index for treated astrocytes was the average score of the counted cells ± standard error of the mean (SEM).

Immunocytochemistry Analysis of Protein Phosphorylation and F-Actin Formation

Cultured cells were exposed to isoflavones or E2 for 30 min then rinsed three times with PBS, fixed with 4% PFA, and blocked with 2% FBS. Cells were then incubated with rabbit monoclonal anti-phospho-Akt (Ser473) (D9E) XP (1:200; Cell Signaling, MA, USA), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:200; Cell Signaling), or anti-phospho-Rac1/Cdc42 (Ser71) (1:200; Cell Signaling) antibodies, followed by CytoPainter Phalloidin-iFluor 594 reagent (Abcam) and donkey anti-rabbit IgG (H+L) secondary antibodies, Alexa Fluor® 488 conjugate (1:200; Thermo Fisher Scientific, Inc, Waltham, MA, USA). Cell nuclei were also stained with DAPI. The cells were then inspected using a laser confocal scanning microscope (Zeiss LSM 880, Carl Zeiss Microscopy GmbH).

RNA Interference Assay

Astrocyte-enriched cultures were transfected with siRNAs for ERα (Thermo Fisher Scientific.), ERβ (Thermo Fisher Scientific), GPER (Integrated DNA Technologies, Inc., Coralville, IA, USA), or negative control RNAs (nontargeting control [catalog no. SIC001; Sigma-Aldrich] or negative control DsiRNA [catalog no. 51-01-14-03; Integrated DNA Technologies, Inc.]), using lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The list of siRNA sequences used in this study is listed in **Table 1**. Briefly, siRNA lipid complexes [1 nM of control siRNA (scrambled RNA), ERα, ERβ or GPER siRNA] were incubated for 20 min, and then added to astrocytes at approximately 80% confluency in 35-mm

TABLE 1 | List of short interfering RNA (siRNA) sequences.

		Sequences
ERα	Sense (5'-3')	CGUCAAGUCGGUUCCGCAUGAUGAA
	Antisense (5'-3')	UUCAUCAUGCGGAACCGACUUGACG
ERβ	Sense (5'-3')	GCGUGGAAGGGAUUCUGGAAAUCUU
	Antisense (5'-3')	AAGAUUUCCAGAAUCCCUUCCACGC
GPER	Sense (5'-3')	GUGUUCAACCUGGACGA
	Antisense (5'-3')	AGUACUGCUCGUCCAGGU

dishes. After 16–24 h, the cells were subjected to matrigel invasion assay. The efficacy of the siRNA knockdown was verified by quantitative real-time PCR (qRT-PCR). Total RNA was extracted using *SuperPrep* cell lysis and RT kit for qPCR reagent (TOYOBO Bio-Technology, Japan) according to the manufacturer's instructions. qRT-PCR was performed using THUNDERBIRD SYBR qPCR mix (TOYOBO) as per the manufacturer's instructions and using a StepOne RT-PCR System (Thermo Fisher Scientific). The list of primers used in this study is listed in the supplementary information 1 (SI. 1). qRT-PCR was performed as follow: denaturation at 95°C for 20 s, followed by amplification at 95°C for 3 s and at 60°C for 30 s (40 cycles). All experiments were repeated three times, using independent RNA preparations to confirm the consistency of the results. All mRNA levels were normalized to that of Gapdh.

Western Blot Analysis

Cultured cells were homogenized in RIPA buffer (Cell Signaling) and protease inhibitors (Complete; Roche, IN, USA). Protein concentration was measured using the Bradford protein assay (Bio-Rad) according to manufacturer's instruction. After boiling for 5 min, protein samples (5 μg) were subjected to 5%–20% SDS-polyacrylamide Supersep Ace (Wako) gel electrophoresis, and the separated products were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, followed by an overnight incubation with the appropriate diluted primary antibodies for pFAK (1:1,000; Cell Signaling), FAK(1:1,000; Cell Signaling), pERK1/2 (1:1,000; Cell Signaling), ERK1/2 (1:1,000; Cell Signaling), pAkt (1:1000; Cell Signaling), Akt (1:1,000; Cell Signaling), pRac1/Cdc42 (1:1,000; Cell Signaling), Rac1/Cdc42 (1:1,000; Cell Signaling), Talin-1 (1:1,000; Cell Signaling), Vinculin (1:1,000; Cell Signaling), α-Actinin (1:1,000; Cell Signaling), Paxillin (1:1,000; Cell Signaling), ER-α (1:1,000; Abcam), Cortactin (1:1,000; Merck Milipore), GAPDH (1:1,000; Proteintech, IL, USA), and β-actin (1:5,000; Cell Signaling). After washing with Tris-buffered saline containing 0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (1:3,000; Cell Signaling) for 1 h at room temperature and detected using an ECL detection system (Wako). GAPDH or β-actin were used as loading controls.

In Silico Analysis of Ligand-Receptor Binding

In silico molecular docking analysis was performed as described previously (3), with slight modifications. All *in silico* calculations were performed using Dell XPS 8930 with Intel Core i7–8700 CPU @ 3.2 GHz, 16 GB DDR4 2666 MHz, NVIDIA GeForce GTX 1060 6 GB, running on a windows 10 professional operating system. Molecular structures for genistein (PubChem CID 5280961), daidzein (PubChem CID 5281708), S-equol (PubChem CID 91469), and E2 (PubChem CID 5757) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) in sdf format. The encoding sequence for GPER was retrieved from UniProt database (accession no Q6FHU6) and FASTA format was submitted to I-TASSER website (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), a specialized server for building three-dimensional (3D) models of seven-transmembrane domain receptors (32–34). The I-TASSER server yielded five models from 10 different templates (3oduA, 4mbsA, 4n6hA2, 4yayA, 5nddA, 5t1aA, 5vblB, 5zbhA, 6d26A, and 6me6A). The protein conformation was refined through molecular dynamics (MD) simulations performed with GROMACS package (35). The three-dimensional structure of GPER and ligands files were opened and modified with Discovery Studio structure-based design software, version 4.0 (BIOVIA/Accelrys Inc., San Diego, CA, USA). Water molecules and other substructures (bound molecules/ligand molecules) were removed from the coordinate file before docking. GPER models 1–5 were used for the docking of genistein, daidzein, S-equol or E2. Polar hydrogen atoms were added to the 3D structure of the GPER and generated input file in pdbqt format of GPER using AutoDockTools of MGLTools (http://autodock.scripps.edu/resources/adt). Docking coordinates were determined through a grid box in PyRx–Python Prescription 0.8 Virtual Screening software for Computer-Aided Drug Design (http://pyrx.sourceforge.net/) with AutoDock 4 and AutoDock Vina are used as a docking software (36). A blind docking strategy was utilized to include all possible ligand binding sites. MD simulations of the molecular complexes were carried out for each starting pose by using the AMBER ff99SB-ILDN force field (37) for the protein and GAFF (38) for the ligand. After an initial period of equilibration, conformational sampling was performed in the isobaric-isothermal ensemble in explicit water for 10 ns, with Cl-counterions added to obtain an overall neutral system. The system was first equilibrated for 2.5 ns and structures were afterwards sampled every 0.5 ns to evaluate the binding energy and the ligand location. At the end of the MD simulations, the binding modes and the affinity of the ligands were estimated from the structures of the protein–ligand complexes obtained every nanosecond. The binding energy was evaluated by using the AutoDock Vina energy evaluation function in score-only mode. LigPlot^{+} v.1.4 (http://www.ebi.ac.uk/thornton-srv/software/LigPlus/) was used to determine the interactions existing for the GPER and ligands complexes with best affinity score values. Binding affinity was expressed as a binding free energy (kcal/mol).

Statistical Analysis

Data are expressed as mean ± SEM of three individual experiments performed in triplicate. One-way or two-way ANOVA followed by Bonferoni's multiple comparison tests were performed using GraphPad Prism version 8.3.1 for windows (GraphPad Software, San Diego, USA, www.graphpad.com). All p-values < 0.05 were considered statistically significant.

RESULTS

Isoflavone Increased Cell Migration

Extensive research using flat, two-dimensional (2D) glass and plastic cell migration analyses has elucidated the detailed molecular and biophysical mechanisms of the migrating process in cultured cells. However, most cells migrating through tissues undergo 3D migration under the physical constraints of the surrounding cells and extracellular matrix (39, 40). Therefore, we examined the effects of isoflavones or E2 (**Figure 1A**) in 2D and 3D migration using wound healing and invasion assays, respectively.

Representative photomicrograph images of cells stained using live-cell Hoechst staining in wound healing assays with 10 nM of isoflavones or E2 for 24 h are shown (**Figure 1B**). In the wound healing assay, genistein, daidzein, S-equol and E2 increased cell migration of C6 astrocyte clonal cells without an evident concentration dependency. Genistein accelerated cell migration at concentrations of 1 and 10 nM, whereas this effect decreased at 100 nM. Daidzein accelerated cell migration in a concentration-dependent manner and reached a peak at 100 nM. S-equol (1 nM) showed the greatest acceleration (**Figure 1C**). These results indicate that isoflavones accelerated 2D cell migration, but, was independent concentration.

We also examined 3D astrocyte migration using an invasion assay. Representative photomicrographs of invaded astrocytes stained with DAPI after isoflavones or E2 exposure are shown (**Figure 1D**). Isoflavones and E2 exposure accelerated the astrocyte migration in a dose-dependent manner, except genistein, which showed greatest acceleration at 10 nM (**Figure 1E**). These results indicate that isoflavones and E2 can induce cell migration in C6 clonal cells and astrocytes.

Isoflavones Increased Cell Migration *via* the GPER Pathway

Isoflavones are known phytoestrogens that activate the estrogen-mediated signaling pathway *via* nuclear ER and GPER. To examine whether further isoflavones affect cell migration, *via* ER and GPER, we used siRNA against ERα, ERβ, or GPER to knockdown their RNA expression. Knock down of GPER in astrocytes significantly reduced isoflavone and E2-accelerated cell migration (**Figures 2A, B** and **Figure SI.1**). On the other hand, knock down of ERα also significantly but weakly reduced genistein or E2 accelerated cell migration (**Figure 2C** and **Figure SI.1**). Knock down of ERβ also weakly reduced S-equol or E2-accelerated cell migration (**Figure 2D** and **Figure SI. 1**). Furthermore, co-exposure with the GPER inhibitor, G15 (10 nM), significantly reduced isoflavone or E2-accelerated cell migration in cell invasion (**Figure 2E**) and wound healing (**Figure 2F**) assays. These results indicate that isoflavones and E2 accelerate cell migration mainly *via* activation of GPER.

Acceleration of Cell Migration by Isoflavones Was Associated With F-Actin Induction

The ability of cells to migrate requires complex molecular events that are initiated by the assembly of F-actin to alter the cellular morphology to move through interstitial submicron size pores in tissues (40, 41). In order to further examine the mechanisms involved in isoflavone-induced acceleration of cell migration, we visualized F-actin with Phalloidin-iFlour 594 reagent. At first, we examined filopodia formation using FiloQuant by ImageJ Fiji. Isoflavones or E2 exposure for 30 min increased filopodia formation (**Figure 3A**, upper panel). Quantitative analysis showed the increase in the number and length of the filopodia (**Figure 3A**, lower panel). Then we continued to examine the formation of stress fibers in the cortical actin filaments using cortical F-actin score (CFS) index. The CFS index was determined based on F-actin cytoskeletal reorganization for each cell. It was scored on a scale ranging from 0 to 3, based on the degree of cortical F- actin ring formations 0, no cortical F-actin, normal stress fibers; 1, cortical F-actin deposits below half the cell border; 2, cortical F-actin deposits exceeding half the cell border; and 3, complete cortical ring formatting and/or total absence of central stress fiber. Isoflavones or E2 attenuated stress fibers to increase cortical actin filaments in astrocytes after 30-min exposure (**Figure 3B**, upper panel). The CFS index significantly increased after 10 nM exposure of isoflavone or E2 and these effects were reduced by knockdown of GPER (**Figure 3B**, lower panel). In addition, we also examined the effects of isoflavones and E2 on focal adhesion proteins related to actin reorganization. We found that 10 nM isoflavones or E2 increased the protein expression levels of vinculin, cortactin and paxillin, and these effects were reduced by knockdown of GPER (**Figure 3C**). We also found that both isoflavones and E2 increased the protein expression levels of ERα and it reduced after silencing the GPER. However, there is no significant changes in the talin-1 and α-actinin protein expression levels after the exposure of isoflavones or E2 (**Figure 3C**). These results indicate the exposure of isoflavones induced F-actin formation, which may have accelerated the migration of astrocytes.

Isoflavones Accelerated Cell Migration *via* the GPER/PI3K/FAK/Akt Pathway

Activation of GPER can induce FAK, Akt, and ERK phosphorylation signaling. To examine the downstream targets of GPER activation by isoflavones, we performed Western blot analysis to measure phosphorylation of FAK, Akt, and ERK1/2 after knockdown of GPER. Isoflavones or E2 increased pFAK, pAkt, and pERK1/2 protein levels, and these effects were reduced after knockdown of GPER (**Figure 4A**). Moreover, immunofluorescence study showed increased F-actin expression concurrent with pAkt, but not with pERK1/2 (**Figure 4B** and **Figure SI.2**). To examine further, we cultured cells with isoflavones and either PI3K inhibitor (LY294002) or ERK1/2 inhibitor (U0126) prior to performing wound healing and invasion assays. LY294002 suppressed isoflavones or E2-accelerated cell migration in C6 cells. No significant effects were observed after co-exposure of isoflavones with U0126 (**Figures 4C, D**). However, we found significant difference in cell invasion after co-exposure of E2 and U0126 (**Figure 4D**). These results indicate isoflavones increased cell migration *via* GPER/PI3K/FAK/Akt pathway.

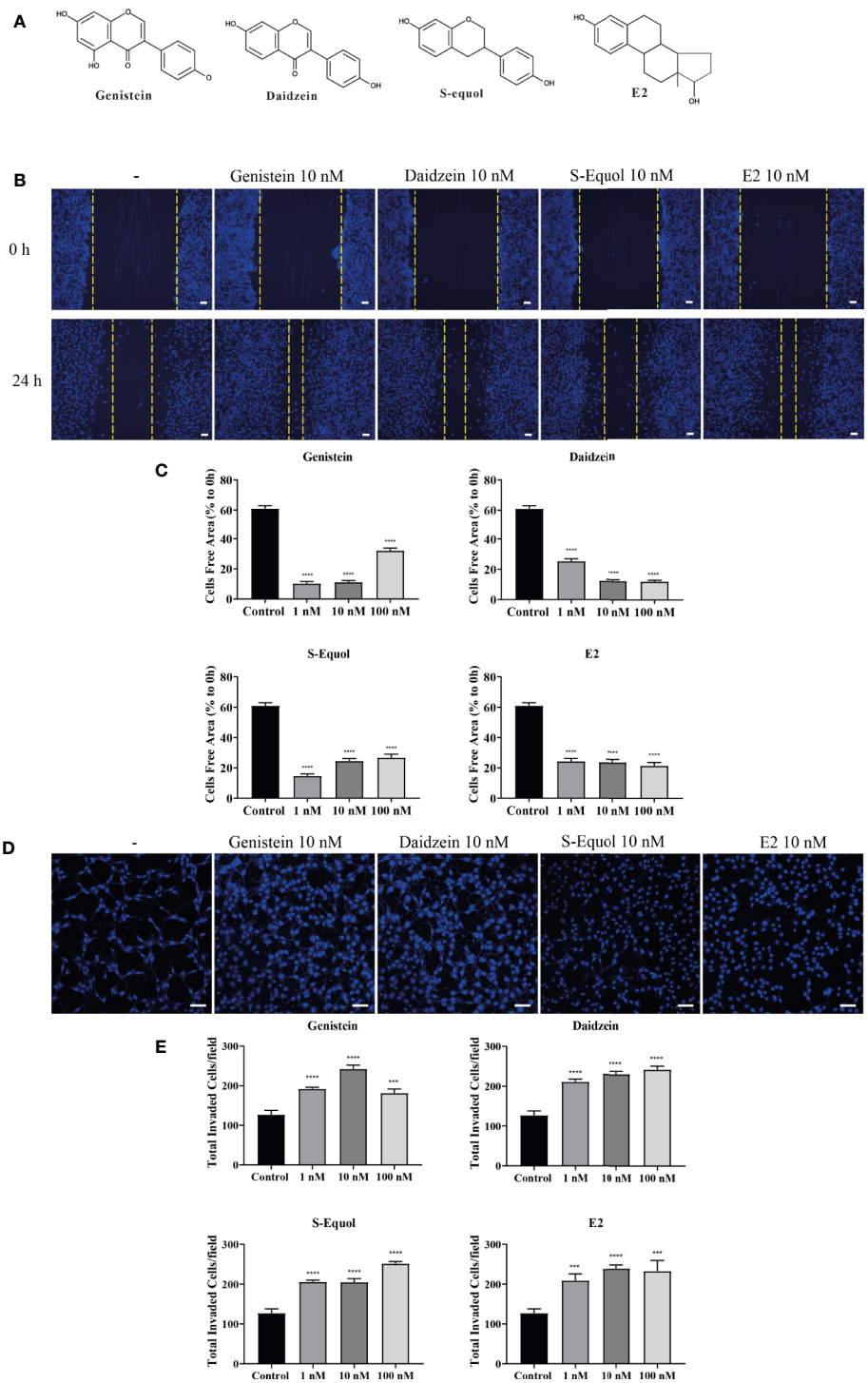

FIGURE 1 | Effects of isoflavones on two-dimensional (2D) or three-dimensional (3D) cell migration. **(A)** Chemicals structures of isoflavones and E2. **(B)** Representative photomicrographs showing the effects of isoflavones on 2D wound healing assays using C6 cells. Live-cell staining was performed using Cellstain-Hoechst 33258 (Dojindo Molecular Technologies, Inc., Japan). **(C)** Quantitative analysis of the effect of isoflavones or E2 (1 – 100 nM) on cell migration measured by wound healing assay. **(D)** Representative photomicrographs showing the effects of isoflavone on 3D matrigel invasion assays using astrocytes. Cell nuclei were stained with DAPI. **(E)** Quantitative analysis of the effect of isoflavones or E2 (1 – 100 nM) on cell invasion measured by matrigel invasion assay. The total number of cells was quantified using ImageJ software (NIH). Bars represent 50 μm. Data are expressed as the mean ± SEM (n = 30 determinations) of at least three independent experiments. $^{****}p < 0.0001$, $^{***}p < 0.001$, indicates statistical significance measured using Bonferroni's test compared with the control (–).

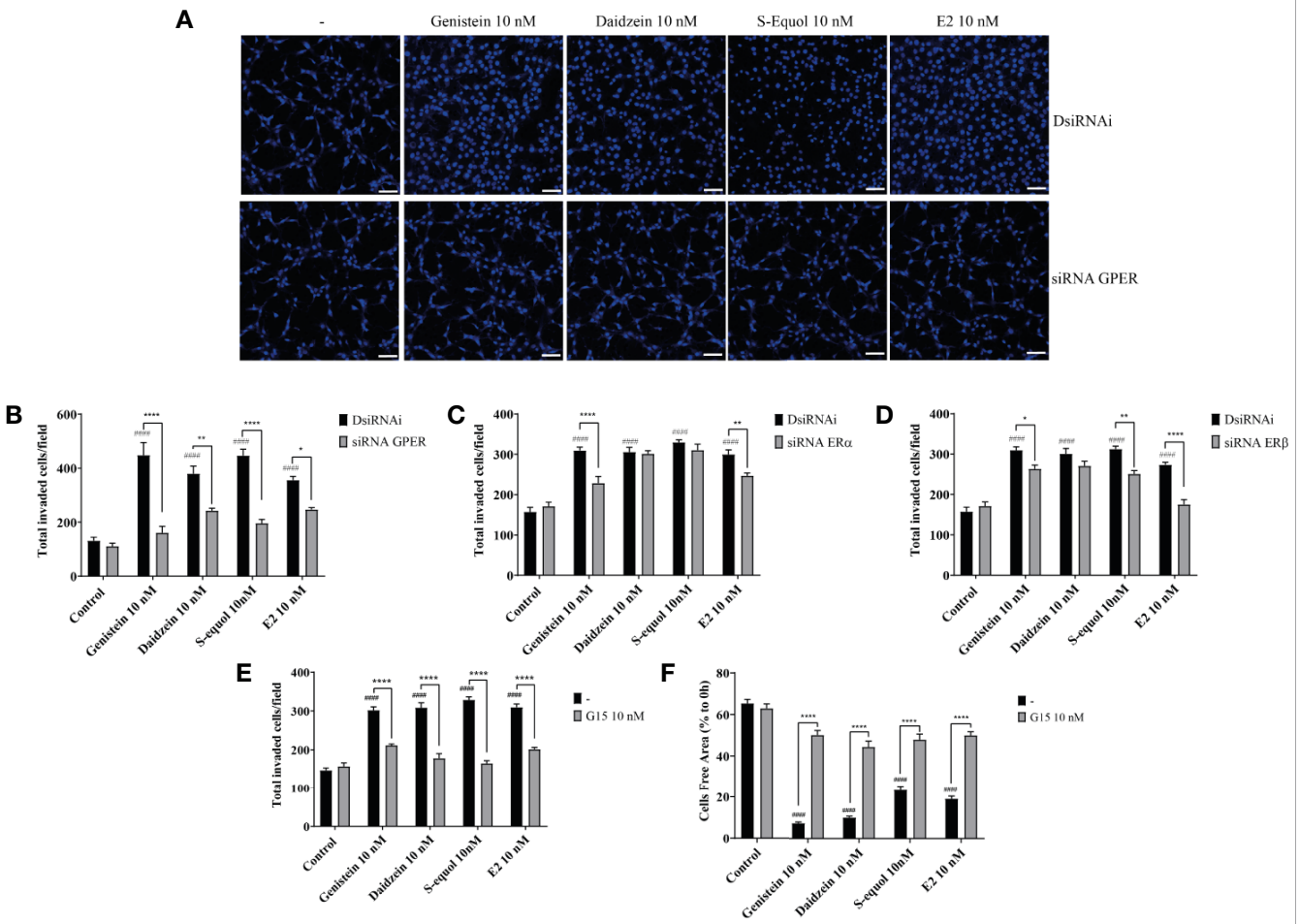

FIGURE 2 | Isoflavones increased astrocyte migration *via* G-protein-coupled estrogen receptor (GPER) activation. Mouse primary cerebellar astrocytes were cultured for seven days prior to siRNA transfection, and matrigel invasion assay followed by DAPI staining was performed **(A–D)**. **(A)** Representative photomicrographs showing the effects of isoflavones on cell migration after deletion of GPER. (B–D) Quantitative analysis of the effect of isoflavones on cell invasion after deletion of GPER **(B)**, ERα **(C)**, or ERβ **(D)**. **(E)** Quantitative analysis of the effect of G15, a GPER inhibitor, on isoflavone-accelerated cell invasion using astrocytes. **(F)** Quantitative analysis of the effect of G15 on isoflavone-accelerated C6 cell migration. Bars represent 50 μm. Data are expressed as mean ± SEM (n = 15 determinations) and are representative of at least three independent experiments. $^{\#\#\#\#}p < 0.0001$, indicates statistical significance measured using Bonferroni's test compared with the control. $^{****}p < 0.0001$, $^{**}p < 0.01$, $^{*}p < 0.05$, indicates statistical significance measured using Bonferroni's test.

Isoflavone Activated p-Akt Led to Increase RhoGTPase Levels

Cell movement is depended on the involvement of Rho GTPase activation on actin. RhoA, Rac1, and Cdc42 play major roles in actin polymerization that leads to cell movement. We examined the effects of isoflavones on Rho GTPase signaling using Western blot, wound healing assay, and immunocytochemistry analyses. Western blot analysis showed that 10 nM isoflavones or E2 increased protein levels of pRac1/Cdc42 (**Figures 5A, B** and **Figure SI. 3**). The phosphorylation of Rac1/Cdc42 significantly decreased after knockdown of GPER or co-exposure with LY294002 (**Figure 5A**). Immunocytochemistry analysis also showed an overlap between F-actin and pRac1/Cdc42 (**Figure 5B**). Co-exposure with RhoA inhibitor (rhosin), Rac1/Cdc42 inhibitor (ML-141), or Cdc42 inhibitor (chasin) significantly suppressed isoflavone or E2-accelerated cell migration in the wound healing and cell invasion assays (**Figures 5C, D**). These results indicate that exposure to isoflavones increased the expression levels of Rho GTPase to induce F-actin formation and subsequent activation of cell motility.

Potential Binding of Isoflavones to the GPER

To investigate the plausible binding modes of isoflavones to GPER, we generated *in silico* binding models using molecular docking study with AutoDocks Vina. Since the crystal structure of GPER remains unknown, the 3D protein structure was predicted using the I-TASSER website. The encoding sequence for GPER was retrieved from the UniProt database (accession number Q6FHU6)

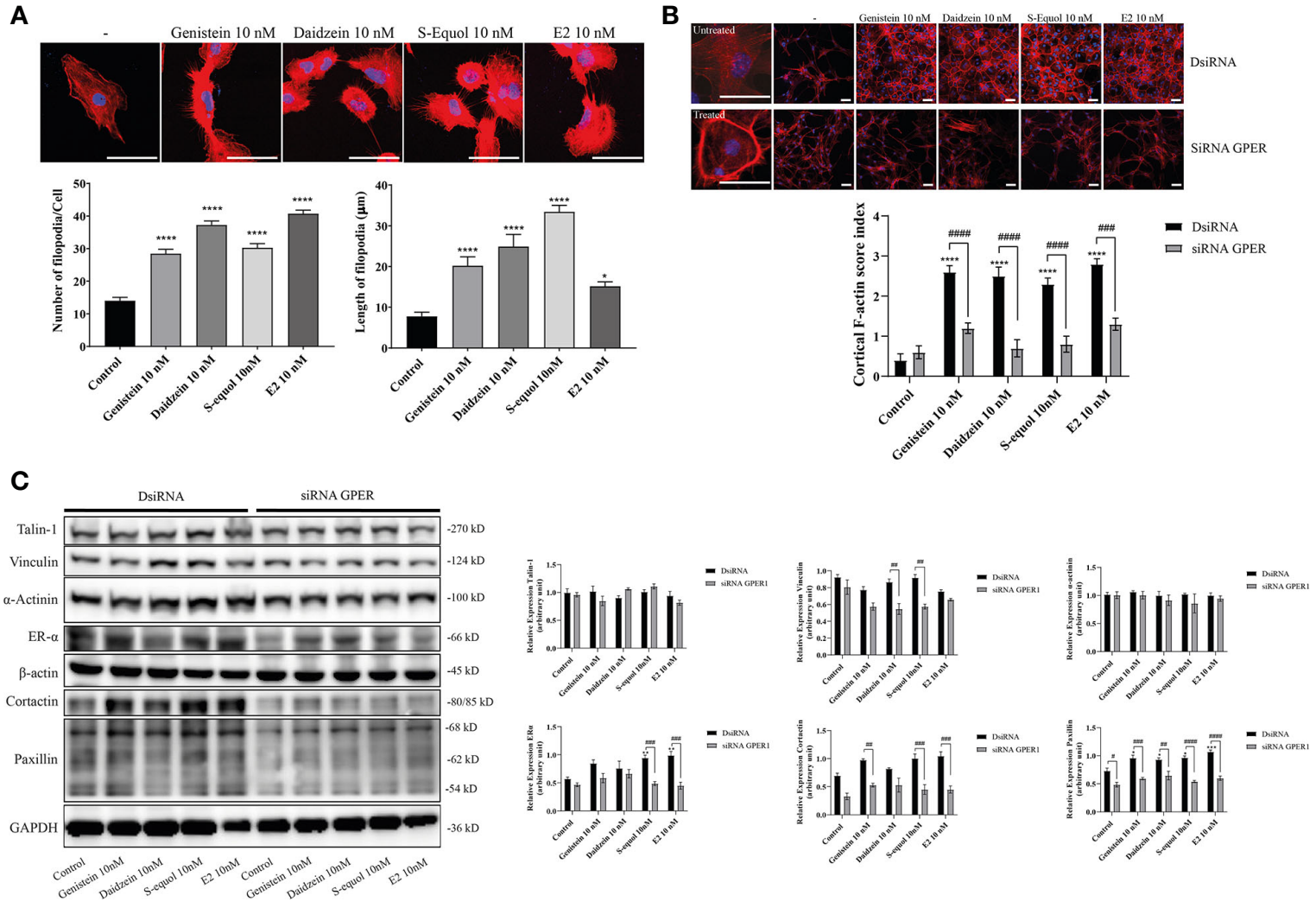

FIGURE 3 | Effects of isoflavones on F-actin formation in astrocytes. Mouse primary cerebral cortex astrocytes were cultured for seven days, followed by fluorescence labeling analysis using phalloidin F-actin and DAPI. **(A)** Upper panel: Representative photomicrographs showing the F-actin and DAPI staining to examine the effects of isoflavones and E2 on filopodia formation. Lower panel: Changes in the number and length of filopodia. The number and length of filopodia were quantified using FiloQuant Fiji ImageJ software (NIH). **(B)** Upper panel: Representative photomicrographs showing F-actin and DAPI staining to examine the effects of isoflavones and E2. The first column shown the overview images with higher magnification to differentiate between the treated and untreated cells. Astrocytes were transfected with DsiRNA or siRNA of GPER, then exposed to isoflavones or E2 for 60 min after serum-starvation for 6 h. Lower panel: Changes in the cortical F-actin score index. Bars represent 50 µm. **(C)** Western blot analysis of talin-1, vinculin, α-actinin, ERα, β-actin, cortactin, paxillin, and GAPDH levels after 60 min exposure of isoflavones or E2 in C6 cells. Representative blots are shown in the left panel, whereas quantitative analysis results are shown in the right panel. Data are expressed as mean ± SEM and are representative of at least three independent experiments. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, indicates statistical significance measured using Bonferroni's test compared with control (-). $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, indicates statistical significance measured using Bonferroni's.

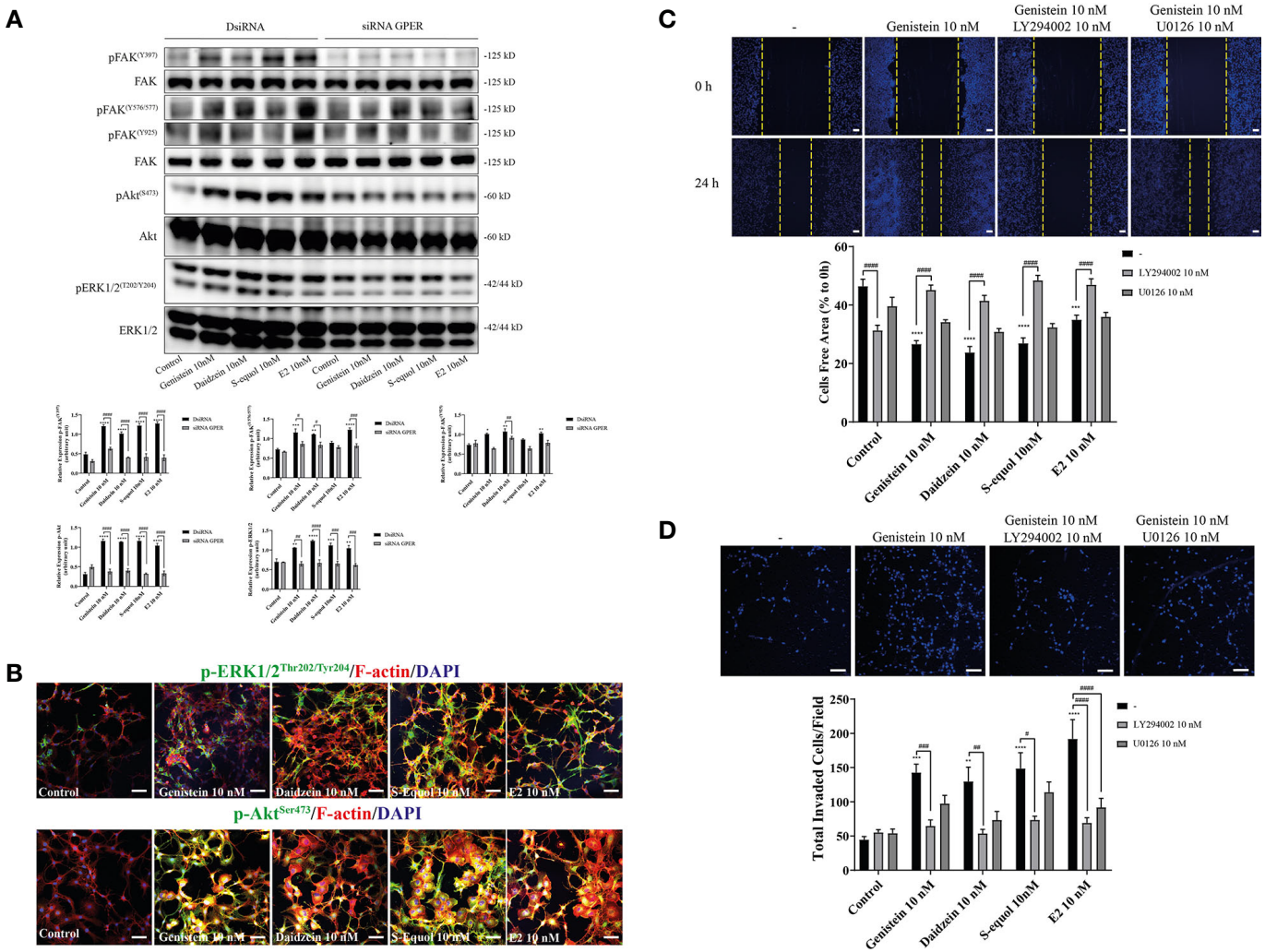

FIGURE 4 | Effects of isoflavones on activation of the PI3K/FAK/Akt axis. **(A)** Western blot analysis of p-FAK, FAK, pERK1/2, ERK1/2, pAkt, and Akt levels after 30 min exposure of isoflavones or E2 in the astrocytes that have been transfected with DsiRNA or short interfering RNA (siRNA) G-protein-coupled estrogen receptor (GPER). Representative blots are shown in the upper panel, whereas quantitative analysis results are shown in the lower panel. **(B)** Representative photomicrographs showing immunocytochemistry results for pERK1/2 and pAkt, with F-actin and DAPI staining to examine the effects of isoflavones or E2 in C6 cells. C6 clonal cells were exposed to isoflavones for 30 min after serum starvation for 6 h. **(C)** Upper panel: Representative photomicrographs showing the effect of LY294002, a PI3K inhibitor, and/or U0126, an ERK1/2 inhibitor, on genistein-accelerated C6 cell migration measured using wound healing assay. Lower panel: Quantitative analysis of the effect of LY294002 or U0126 on isoflavones-accelerated C6 cell migration. **(D)** Upper panel: Representative photomicrographs showing the effect of LY294002, a PI3K inhibitor, and/or U0126, an ERK1/2 inhibitor, on genistein-accelerated C6 cell migration measured using cell invasion assay. Lower panel: Quantitative analysis of the effect of LY294002 or U0126 on isoflavones-accelerated C6 cell invasion. Bars represent 50 μm. Data are expressed as mean ± SEM and are representative of at least three independent experiments. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, indicates statistical significance measured using Bonferroni's test compared with control (-). $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, indicates statistical significance measured using Bonferroni's.

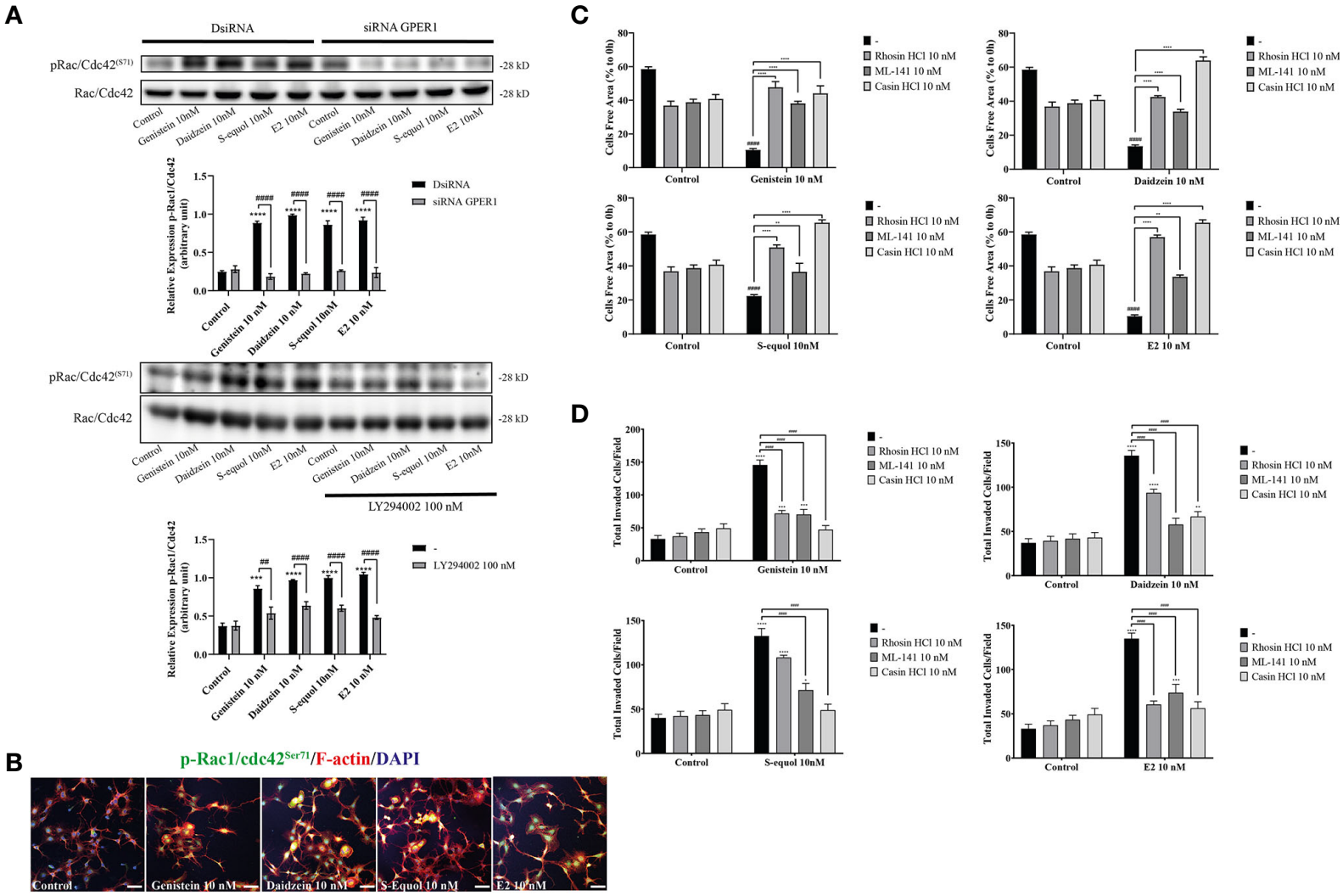

FIGURE 5 | Effects of isoflavones on phosphorylation of RhoGTPase and their involvement on cell migration and F-actin formation. **(A)** Western blot analysis for pRac1/Cdc42 and Rac1/Cdc42 levels after 30 min exposure of isoflavones or E2 in the astrocytes that have been transfected with DsiRNA or short interfering RNA (siRNA) G-protein-coupled estrogen receptor (GPER), or co-exposure with LY194002. Representative blots are shown in the upper panel, whereas quantitative analysis results are shown in the lower panel. **(B)** Representative photomicrographs showing immunocytochemistry results for pRac1/Cdc42 with F-actin and DAPI staining to examine the effects of isoflavones or E2 in astrocytes. Astrocytes were exposed to isoflavones for 60 min after serum starvation for 6 h. **(C)** Quantitative analysis of the effect of rhosin HCl (RhoA inhibitor), ML-141 (Rac1/Cdc42 inhibitor), and casin HCl (Cdc42 inhibitor) on isoflavone-accelerated cell migration measured by wound healing assay. **(D)** Quantitative analysis of the effect of rhosin HCl (RhoA inhibitor), ML-141 (Rac1/Cdc42 inhibitor), and casin HCl (Cdc42 inhibitor) on isoflavone-accelerated cell migration measured by cell invasion assay. Bars represent 50 μm. Data are expressed as mean ± SEM and are representative of at least three independent experiments. $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, indicates statistical significance measured using Bonferroni's test compared with control (-). $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, indicates statistical significance measured using Bonferroni's.

and submitted to the I-TASSER website, a specialized server for building 3D models of seven transmembrane receptors. The I-TASSER server yielded five predicted models from 10 different templates. Before generating the MD simulations, several geometrical observables such as area per lipid, the root mean square deviation (RMSD) of heavy atom with respect to the starting conformation, and the atomic fluctuation were evaluated to observe if the systems reached equilibrium. RMSD is known standards for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modeling. MD simulations shows that after reached the equilibrium, the RMSD values of 8.4 ± 4.5, 5.6 ± 0.19, 5.5 ± 1.2, 5.0 ± 0.13, and 5.9 ± 1.1 Å for GPER, GPER-genistein, GPER-daidzein, GPER-S-equol, and GPER-E2, respectively. The 3D docking results of GPER showed isoflavones and E2 possess a similar binding pose under blind docking procedures. Isoflavones and E2 could form a hydrogen bond with Glu 329 and have the same amino acid residues that have equivalent 3D positions concerning the residues in the first plot, as shown in red circles and ellipses (**Figures 6A–D**). An additional possibility to form hydrogen bond was also found between genistein and Arg 169 and Arg 253 (**Figure 6A**), daidzein and Arg 169 (**Figure 6B**), S-equol and Thr 330 (**Figure 6C**), and E2 and Thr 330 (**Figure 6D**). The binding affinities for genistein, daidzein, S-equol and E2 were -8.8, -8.6, -8.9, and -8.3 kcal/mol, respectively, with GPER. The docking poses in 3D model of isoflavones and E2 bound to GPER also shown in SI. 4. These results indicate that isoflavones may bind directly to GPER to accelerate cell migration.

DISCUSSION

The present study examined the effects of isoflavones and E2 on glial cell migration. Previously we reported that S-equol, a daidzein metabolite, activates GPER to induced F-actin rearrangement lead to increase astrocyte migration during cerebellar development with mechanisms remains unclear (14). This study reveals a novel mechanism of isoflavones (genistein, daidzein, and S-equol) in cell migration *via* GPER that may play an important role not only during brain development but also brain injury. We found that isoflavones increased 2D and 3D glial cell migration of primary astrocytes and C6 clonal cells. Isoflavone-accelerated cell migration was suppressed by knock down of GPER expression or coexposure with a GPER inhibitor. Isoflavone exposure also increased phosphorylation of FAK and Akt, which is a downstream target of GPER, leading to increased phosphorylation levels of RhoGTPase signaling, including Rac1 and Cdc42 that play a major role in F-actin formation. *In silico* analysis revealed that these isoflavones may directly interact with GPER. Our results showed the novel action of isoflavones in promoting glial cell migration *via* GPER signaling pathway.

The estrogenic activity of isoflavones has been well demonstrated (16, 42). Genistein exhibits >20-fold higher affinity for ERβ than ERα. Binding of isoflavones to ERs leads to shuttling of the ligand–ER complex to the nucleus and induces the transcription of target genes *via* the classical genomic pathway (16). In addition, recent studies have shown that isoflavones can interact with GPER and mediate rapid cellular signaling in neurons and endothelial cells (43–45). The present study also showed that the effects of isoflavones may be exerted by binding to the GPER to accelerate cell migration, since GPER knock down or co-exposure with GPER antagonist, at least in part, inhibited its action on astrocytes migration. On the other hand, knock down of nuclear ERs led to a weaker effect than that seen after GPER knockdown. These results indicate that the accelerated migration of astrocyte by isoflavones is mainly exerted *via* the GPER. The action may be slightly different to that of E2, since E2 action was inhibited by knock down of both nuclear ERs and GPER. We were unable to clarify the mechanisms involved in these differences. One possibility may be due to the difference in the affinities of the ER and GPER. The finding of our *in silico* study revealed a higher affinity of isoflavones compared with E2. However, further studies that include crystallization of GPER are required to confirm this difference.

The GPER belongs to the family of seven transmembrane-spanning GPCR and specifically binds estrogens, thereby activating intracellular signaling cascades (15, 16). In addition to cell migration, the GPER regulates various cellular functions, such as apoptosis, autophagy, proliferation, and differentiation, *via* a wide variety of signal transduction pathway including Ras/ERK (46, 47), PI3K/Akt (47–49), receptor tyrosine kinase (16), PLC-mediated pathway (50), and cAMP-mediated pathway (16). GPER induces rapid cellular effects including the production of cAMP, the mobilization of intracellular calcium, and the activation of kinase, such as ERK and PI3K, as well as ion channels and endothelial nitric oxide synthase (eNOS) (16). In addition, ERs (especially ERα) also activate such pathways. However, in cells that express both ERα and GPER there is a possibility of crosstalk or squelching between receptors (16, 51). The GPER also mediates estrogenic regulation of actin polymerization involves SRC-1 and PI3K/mTORC2 pathways in the hippocampus of female mice (49). In addition, the GPER acts *via* the PLCβ-PKC and Rho/ROCK/LIMK/cofilin pathways to regulate F-actin cytoskeleton assembly, thereby enhancing TAZ nuclear localization and activation, leading to increased cell migration and invasion (50). These varied GPER-activated pathways to regulate diverse cellular functions indicate the profound implications of GPER under physiological and pathophysiological conditions. In the present study, although isoflavones increased phosphorylation of ERK1/2 and Akt, inhibition of the PI3K/Akt pathway significantly suppressed the cell migration of astrocytes. These results indicate that, although various signal transduction pathways may be activated by isoflavones *via* the GPER, the Akt signaling pathway plays a major role in accelerating cell migration. Each signal transduction pathway of GPER may play a distinct role in cellular function.

The small GTPases of the Rho family (RhoA, Rac1 and Cdc42) appear to be at the heart of the initial signals leading to cellular polarization, and stress fiber, filopodia, and lamellipodia formation in migrating cells (40, 41, 52). Classic RhoGTPases are regulated by the opposing actions of Rho-

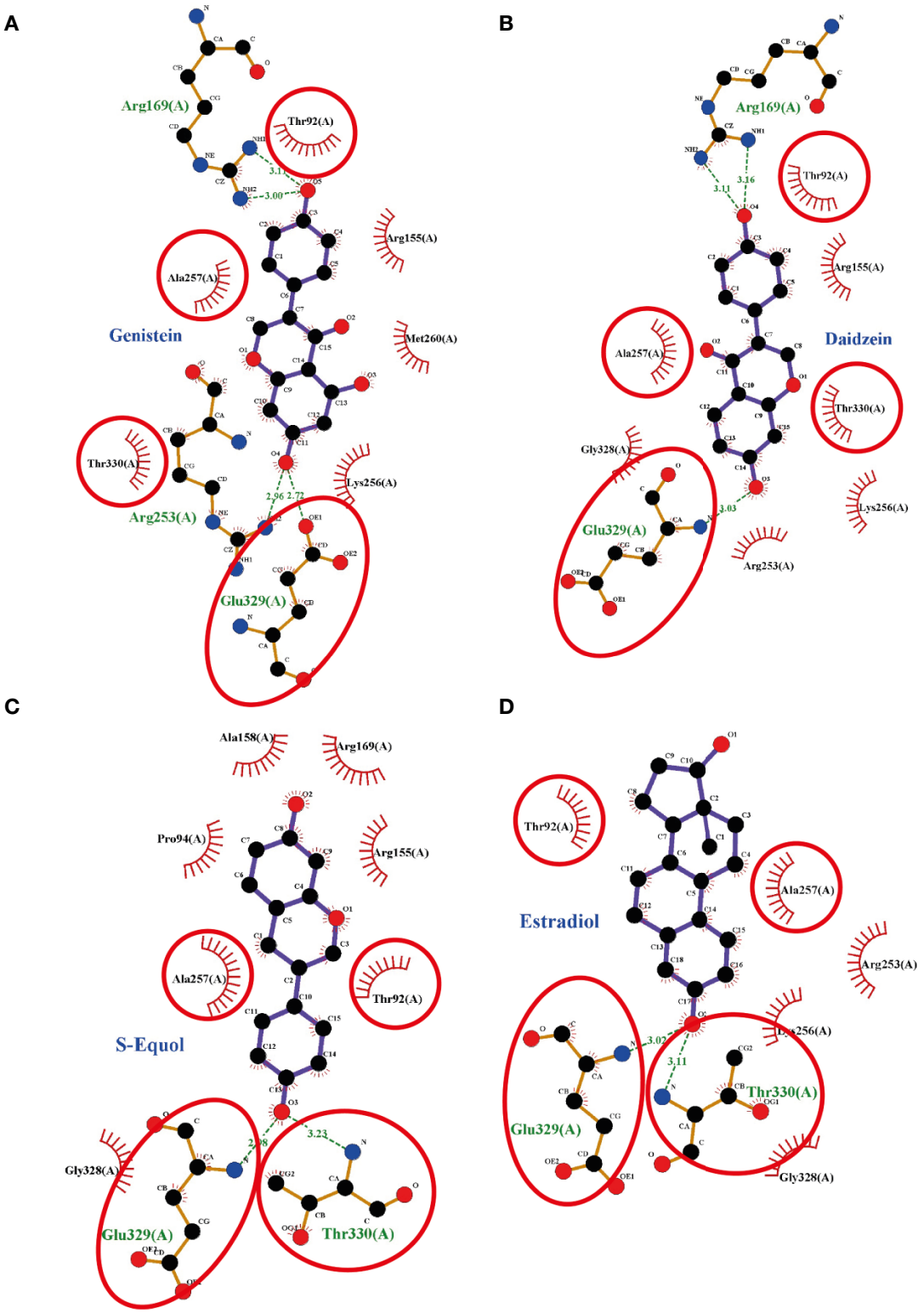

FIGURE 6 | Interaction plots between **(A)** genistein, **(B)** daidzein, **(C)** S-equols, or **(D)** E2 and the G-protein-coupled estrogen receptor (GPER) generated by LigPlot⁺ v.2.0, with each subsequent plot being automatically fitted. Red circles and ellipses in each plot indicate amino acid residues that have equivalent 3D positions with respect to the residues in the first plot. Hydrogen bonds are shown as green dotted lines, while spooked arcs represent residues making nonbonded contacts with the ligand.

specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (53). PI3K activates Rac and Cdc42 *via* activation of PIP3-regulated GEFs, and inhibition of Cdc42 in Ras-transformed cells decreased Akt signaling, leading to reduced migration/invasion (54). In addition to PI3K activates Rac and Cdc42, FAK also can influence the activity of RhoGTPases through a direct interaction or phosphorylation of the protein activators or inhibitors of RhoGTPases (55). It has been reported that estrogen activated FAK tyrosine phosphorylation ($Tyr^{397/576/577}$) *via* Src, then regulated Cdc42 and Cdc42 effector Wiskott-Aldrich syndrome protein N-WASP (Neuronal-WASP) (56). N-WASP is a scaffold protein that links upstream signals to activate of the Arp2/3 complex, leading to actin nucleation for the rapid formation of actin network at the leading edge of the cell (55, 56). It has been known that paxillin and cortactin are direct target of FAK in the regulation of focal adhesion dynamics to promotes cell motility or invasion (55). In the present study, it is highly possible that activation of the PI3K/Akt axis and FAK induced the activation of Rac1, Cdc42, and focal adhesion protein, leading to accelerated cell migration. Cdc42 is essential for the formation of protrusions leading to elongated morphology. Deletion of Cdc42 in astrocytes revealed that the cells were still able to form protrusions, but in a nonoriented manner (26). Consequently, astrocytes failed to migrate in a directed manner toward a scratch. On the other hand, Rac is essential for both the development and maintenance of protrusions during migration. Rac1 also plays a role in local restructuring of the cytoskeleton coordinate with surface expansion, leading to astrocyte stellation (57). Isoflavones activated of Rac1/Cdc42 and increased the filopodia and cortical actin (**Figure 5**). Moreover, coexposure of isoflavones with ML-141, a Rac1/Cdc42 inhibitor, or casin, a Cdc42 inhibitor, significantly suppressed isoflavones-accelerated cell migration, indicating the involvement of Rac1/Cdc42 in this process. In addition, rhosin, a RhoA inhibitor also suppressed migration, indicating its involvement. These results are consistent with our hypothesis that isoflavones bind to the GPER (**Figure 6**) and activate PI3K/Akt axis signaling pathways to induce activation of RhoGTPase, resulting in F-actin formation and activation of astrocytes cell migration.

Astrocytes contribute to physiological brain function on many levels, including monitoring normal function of neurotransmitter uptake, synapse formation, regulation of the blood–brain barrier, and development of the CNS (21, 25). Astrocytes become dynamic migratory cells under certain physiological action and/or pathological conditions (21, 26, 40). Astrocyte migration requires coordination of complex signaling pathways, such as actin polymerization, delivery of membrane to the leading edge, and formation of attachments at the leading edge to provide traction, contraction, and disassembly of attachment at the rear (21, 26). Cell migration also depends on the mechanical and chemical interaction between the cells and their extracellular environment. Mechanical interaction depends on the polarization, adhesion, deformability, contractility, and proteolytic ability of cells (58). Our study showed that exposure to isoflavones increased the 2D or 3D migration of astrocytes by activating F-actin formation. Filopodia and stress fiber formation significantly increased after the exposure to isoflavones. F-actin in migrating cells are polarized with their plus (barbed)-ends toward the cell periphery against the plasma membrane, resulting in the formation of filopodia or lamellipodia, anchored focal adhesion, and extension at the front of the cells (40, 59). Filopodia are thin, finger-like, highly dynamic actin–rich membrane protrusions that extend out from the cell edge, thus extension of filopodia is driven by linear polymerization of actin filaments (41). During 3D migration, the mode of migration depends on the extracellular environment in which cells adopt round, amoeboid shapes, and extend lamellipodia. Extensive studies have revealed that amoeboid migration does not require focal adhesion-dependent force transmission, but instead relies on the global retrograde flow of cortical actomyosin (40). Cortical actin networks are involved in aligning along cell–cell junctions, supporting both stable and dynamics contacts in stationary epithelial and during collective cell migration (58). In summary, isoflavone-induced cell migration and F-actin rearrangement are processes that cannot be separated. However, further studies are required to understand how isoflavones induce cell migration as a result of the F-actin rearrangement.

The effect of isoflavones on human health remains controversial. While studies into the use of phytoestrogens as dietary supplements have reported various health benefits, such as antioxidant, anti-inflammatory, anti-cancer, and neuroprotective effects, there is a concern about potential adverse effects as results of modulating or disrupting endocrine function (8, 16, 60). However, most studies showing adverse effects used a higher dose of soy isoflavones than those found in the plasma of the population who regularly consume soy. Isoflavone dose is crucial to examine the effects of isoflavones in human health. For example, isoflavone dose affects risk of cancer. It was demonstrated that genistein enhanced cell proliferation in MCF-7 cells at concentrations of 10–100 nM. However, at higher concentrations (> 20 μM), genistein inhibited MCF-7 cell growth (8, 61). The total isoflavones plasma concentration in the Asian population consuming a traditional diet, including soy-based food, is in the range of 525–775 nM. In contrast, the total isoflavones plasma concentration in European countries was found to be <10 nM in individuals with a non-vegetarian diet and 79–148 nM in those with vegetarian and vegan diet (62). For the practical application of isoflavones in human health, studies using doses of isoflavones that represent plasma concentrations should be undertaken. The dose of isoflavones used in the present study ranged from 1–100 nM. Since this was an *in vitro* study, the results cannot be compared with those from *in vivo* conditions. However, our highlight the novel possibility that isoflavones activate astrocyte, indicating that they can be a useful supplementary compound during brain development or in the injured brain.

In summary, our results showed that exposure of physiological concentrations of isoflavones increased cell migration *via* direct binding to GPER and subsequent activation of the PI3K/FAK/Akt/RhoGTPase signaling pathway, which induces F-actin formation (**Figure 7**). The present study highlights the potential use of

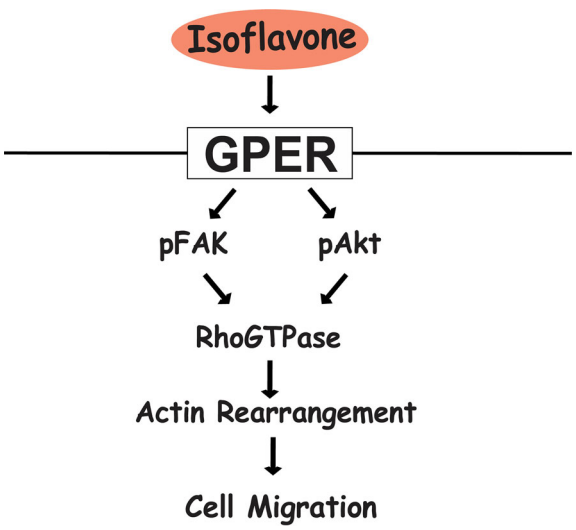

FIGURE 7 | Proposed mechanism of isoflavone action on astrocyte migration *via* G-protein-coupled estrogen receptor (GPER). Isoflavones bind to the GPER and activate the PI3K/Akt and FAK signaling pathway, leading to phosphorylation of Rac1/Cdc42 and resulting in actin polymerization and formation of filopodia and stress fibers to promote cell migration in astrocytes.

isoflavones as an effective supplement to promote astrocyte migration during brain development or brain injury.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Experimentation Committee, Gunma University (19-024, 17 December 2018).

AUTHOR CONTRIBUTIONS

WA designed and performed the experiments, analyzed the results, and wrote the manuscript. IA and KH performed some of the experiments and analyzed the results. WM, TS, and NK designed the experiments, evaluated the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.554941/full#supplementary-material

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