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Extra-nuclear estrogen receptor GPR30 regulates serotonin function in rat hypothalamus

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Abstract

Selective serotonin reuptake inhibitors (SSRIs), such as Prozac®, are used to treat mood disorders. SSRIs attenuate (i.e., desensitize) serotonin 1A (5-HT_{1A}) receptor signaling, as demonstrated in rats through decreased release of oxytocin and adrenocorticotropin hormone (ACTH) following 5-HT_{1A} receptor stimulation. Maximal therapeutic effects of SSRIs for treatment of mood disorders, as well as effects on hypothalamic 5-HT_{1A} receptor signaling in animals, take one to two weeks to develop. Estradiol also attenuates 5-HT_{1A} receptor signaling, but, in rats, these effects occur within 2 days; thus, estrogens or selective estrogen receptor modulators may serve as useful short-term tools to accelerate desensitization of 5-HT_{1A} receptors in response to SSRIs if candidate estrogen receptor targets in the hypothalamus are identified. We found high levels of GPR30, which has been identified recently as a pertussis-toxin (PTX) sensitive G-protein coupled estrogen receptor, in the hypothalamic paraventricular nucleus (PVN) of rats. Double-label immunohistochemistry revealed that GPR30 co-localizes with 5-HT_{1A} receptors, corticotrophin releasing factor (CRF) and oxytocin in neurons in the PVN. Pretreatment with PTX to the PVN before peripheral injections of 17-β-estradiol 3-benzoate completely prevented the reduction of the oxytocin response to the 5-HT_{1A} receptor agonist, (+)-8-hydroxy-2-dipropylaminotetralin (DPAT). Treatment with the selective GPR30 agonist, G-1, attenuated 5-HT_{1A} receptor signaling in the PVN as measured by an attenuated oxytocin (by 29%) and ACTH (by 31%) response to DPAT. This study indicates that a putative extra-nuclear estrogen receptor, GPR30, may play a role in estradiol-mediated attenuation of 5-HT_{1A} receptor signaling, and potentially in accelerating the effects of SSRIs in treatment of mood disorders.

Keywords

serotonin 1A receptor; hypothalamic paraventricular nucleus; estrogen receptor; membrane estrogen receptor; oxytocin; adrenocorticotropin hormone

Estrogens have been shown to enhance the clinical efficacy of selective serotonin reuptake inhibitors (SSRIs) for treatment of mood disorders and hot flushes in women (Schneider et al., 1997; Lomax and Schonbaum, 1993), but mechanisms underlying this effect are unknown. Attenuation of serotonin 1A (5-HT_{1A}) receptor signaling, including 5-HT_{1A} receptor signaling in the hypothalamus, is considered important for the therapeutic effectiveness of SSRIs (Lerer et al., 1999). Using well established neuroendocrine challenge tests, we and others previously demonstrated that, like SSRIs, estradiol can produce a desensitization of 5-HT_{1A} receptors, i.e., an attenuation of signaling, but the time course and magnitude of this response is different; estradiol produces a partial desensitization of 5-HT_{1A} receptors in the hypothalamic paraventricular nucleus (PVN) that develops within 2 days (Raap et al., 2000), whereas SSRIs produce a full desensitization that develops within 7-14 days (Li et al., 1996; Raap et al., 1999). Thus, combining estrogens with SSRIs might lead to a more rapid onset and potentiation of the therapeutic effects of SSRIs.

Desensitization of 5-HT_{1A} receptors in the PVN can be measured by neuroendocrine challenge tests that detect changes in hormone responses to specific 5-HT_{1A} receptor agonists (Osei-Owusu et al., 2005; Li et al., 1997). In humans, chronic treatment with SSRIs reduces the release of oxytocin and ACTH in response to stimulation of 5-HT_{1A} receptors (Lerer et al., 1999), providing diagnostically useful peripheral indicators of central (particularly of PVN) 5-HT_{1A} receptor function. Plasma oxytocin levels are a direct blood-borne marker of events occurring in the hypothalamus, whereas secretion of ACTH by the pituitary serves as a useful indicator of activation of hypothalamic-pituitary communication.

The receptors mediating the effects of estradiol on 5-HT_{1A} receptor signaling in the PVN are not known but may involve GPR30, a newly identified extranuclear estrogen receptor GPR30 is expressed in several hypothalamic nuclei including the PVN, supraoptic, arcuate and suprachiasmatic nucleus (O'Dowd et al., 1998; Brailoiu et al., 2007). GPR30 is a seven-transmembrane G protein-coupled receptor that binds estrogens with high affinity (Revankar et al., 2005; Thomas et al., 2005). GPR30 acts independently of the classical alpha and beta estrogen receptors (ER) to stimulate adenylyl cyclase via Gas protein (Filardo et al., 2002) and mitogen-activated protein kinases ERK1/2 via a bordatella pertussis toxin (PTX)-sensitive, Gβγ pathway (Filardo et al., 2000). GPR30 mediates increased c-fos expression stimulated by estrogens and phytoestrogens (Maggiolini et al., 2004).

The purpose of this study is to test the hypothesis that stimulation of GPR30 in the PVN contributes to the desensitization of 5-HT_{1A} receptors in the PVN. We first used double-label immunohistochemistry to determine whether GPR30 is localized in the relevant cell groups in the PVN. Second, we used well-established neuroendocrine challenge tests to assay potential effects on 5-HT_{1A} receptor signaling following either non-specific stimulation of estrogen receptors combined with inhibition of GPR30 with PTX or selective stimulation of GPR30; no selective GPR30 antagonist is currently available.

Experimental Procedures

Rats

Female Sprague-Dawley rats (225-250 g) were purchased from Harlan Laboratories (Indianapolis, IN., USA). The rats were housed two per cage in a temperature-, humidity-, and light-controlled room (12-hour light/dark cycle; lights on from 7:00 a.m. to 7:00 p.m.). Food and water were available ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by Institutional Animal Care and Use Committee of Loyola University Chicago or the University of Kansas. Every effort was made to minimize suffering and discomfort to the

animals. Twelve rats per group were used for all experiments in which hormone levels were measured based on the minimum needed for statistically meaningful evaluation of the data.

Immunofluorescence Labeling

Tissue Preparation—Gonadally intact female Sprague-Dawley rats (225-250 g) were anesthetized with a mixture of ketamine and xylazine (100 mg/kg ketamine plus 7 mg/kg xylazine, 1.4 ml/kg, i.p.) and were perfused intracardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 0.1M phosphate-buffered 4% formaldehyde (pH 7.4). The brains were removed and post-fixed for 2h at 4°C, then stored overnight at 4°C in 0.01 M PBS containing 30% sucrose. Serial coronal sections of the whole brain including the hypothalamus (30 µm) were cut with a freezing microtome and transferred into 0.01 M PBS containing 0.2% sodium azide and stored at 4°C. For labeling CRF-containing cells, stereotaxic injections of 5 µl of colchicine solution (10mg/ml in 0.9% saline; Sigma, St. Louis, MO) were made into the lateral ventricle of anesthetized rats to block axonal transport and increase CRF immunoreactivity in somata (Norstrom, 1975; Lantos et al., 1995). Food pellets were placed directly on the bedding to allow easier access for the colchicine treated rats. Animals were allowed to survive 48h before they were anesthetized and perfused as described above.

Double labeling of GPR30 receptor with 5-HT_{1A} receptor, oxytocin or CRF—

Double immunofluorescence was performed to colocalize GPR30 with 5-HT_{1A} receptor. After washes in PBS (3×10 min) sections were (a) permeated with 0.2% Triton X-100 (Fisher Scientific Co., Pittsburgh, PA) for 40 min and placed in 3% hydrogen peroxide for 10 min.; (b) incubated in 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1h at room temperature (RT) to block nonspecific staining; (c) incubated with polyclonal rabbit anti-GPR30 IgG (1:250 dilution; Lifespan Bioscience, Seattle, WA) for 1h at RT and overnight at 4°C; (e) incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes, Inc. Eugene, OR) diluted 1:200 in PBS for 1h at RT. After the incubation with GPR30 antiserum, sections were (a) washed in PBS (3 × 10 min); (b) incubated with polyclonal guinea pig anti-5-HT_{1A} IgG (1:200 dilution; Chemicon International, Inc. Temecula, CA) for 1h at RT and overnight at 4°C; (c) washed in PBS (3× 10 min); (d) incubated with the Texas Red anti-guinea pig IgG (TI-7000, Vector Laboratories) diluted 1:200 in PBS for 1h at RT; and (e) washed in PBS (3×10 min). Finally, the sections were mounted and coverslipped with immunofluorescence mounting medium (H-1000; Vector Laboratories) and examined under an Olympus Model IX81 Inverted Research Microscope (Tokyo, Japan) configured with Image-Pro Plus 4.5 software (VayTek, Fairfield, IA). Monochrome images were captured using Retiga EX 1350 camera and VolumeScan 3.1 software (VayTek) and then were pseudocolored and merged using Image-Pro Plus 4.5 software. Three sections (-1.33, -1.53 and -1.78 mm posterior from bregma) were sampled per rat (with three rats in each group). Each tissue section was examined bilaterally for neurons immunopositive for GPR30 and double-labeled neurons. The number of neurons immunopositive for GPR30 and the number of double-labeled neurons for both GPR30 and 5-HT_{1A} receptors were counted on each side of the PVN in each of the nine tissue sections (3 sections per rat in 3 rats). The percentages of double-labeled neurons with respect to GPR30 and 5-HT_{1A} receptor immunopositive neurons were then calculated in each rat and then averaged for the three rats. As a control for non-specific labeling, some tissue sections were incubated with PBS buffer in place of the primary antibodies (GPR30, 5-HT_{1A} receptor, oxytocin or CRF); these sections had either no labeling or very light diffuse background. Previous studies by us and others had demonstrated the specificity of these antibodies including the reduction or elimination of labeling with these antibodies following pre-adsorption of the antibodies with specific antigen (Zhang et al., 2004; Revankar et al., 2005; Brailoiu et al., 2007).

Similar procedures and controls as described above were used to double label GPR30 receptor and oxytocin-expressing cells. For labeling oxytocin-expressing cells, the oxytocin antibody was a mouse monoclonal antibody (1:10,000 dilution; Chemicon International, Inc. Temecula, CA) and it was used in conjunction with Alexa Fluor 594 goat anti-mouse IgG (H+L) (Molecular Probes, Inc. Eugene, OR). The same procedure as described above was used to determine the percentage of double-labeled GPR30 and oxytocin-expressing cells.

The processing for double-labeling of GPR30 and CRF was essentially the same as that described for GPR30 and 5-HT_{1A} receptor or oxytocin, with some minor modifications. Sections were not pretreated in hydrogen peroxide, and the blocking serum was 5% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA). Sections were incubated with guinea pig anti-CRF serum (1:200 dilution; Peninsula Laboratories, San Carlos, CA) for 1h at RT and overnight at 4°C, followed by rhodamine-conjugated Donkey anti-guinea pig IgG (1:200 dilution; Jackson Immunoresearch Laboratories, West Grove, PA) for 2h at RT, then GPR30 IgG (1:250 dilution) for 1h at RT and overnight at 4°C, and finally Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (Molecular Probes, Inc. Eugene, OR) diluted 1:400 in PBS for 2h at RT. Sections were washed extensively with PBS between incubations. Sections were mounted on slides, air dried, coverslipped with Vectorshield (Vector laboratories, Burlingame, CA) and examined on a Zeiss Upright Epifluorescence Microscope using appropriate filters and analyzed as described above.

Neuroendocrine challenge tests

Drugs: The drugs were purchased or donated as follow: (+)-8-hydroxy-2-dipropylaminotetralin (DPAT) (Tocris Bioscience, Ellisville, MO), GPR30 agonist G-1 (ChemDiv, Inc., San Diego, CA), pertussis toxin (PTX) (EMD Bioscience, Inc. San Diego, CA), EB (17- β -estradiol 3-benzoate) and sesame oil were purchased from Sigma (St Louis, MO). DPAT was dissolved in 0.9% saline and PTX was dissolved in ddH₂O. EB (10 μ g/ml) was prepared by sonicating it in sesame oil. EB or sesame oil was injected subcutaneously in a volume of 1 ml/kg/day. All solutions were freshly made before administration.

Surgery: All rats were anesthetized with a mixture of ketamine and xylazine (100 mg/kg ketamine plus 7 mg/kg xylazine, 1.4 ml/kg, i.p.) and secured in the stereotaxic instrument with the incisor bar set so that bregma and lambda were level. A double-barreled guide cannula (26 gauge, 1.2 mm center-to-center distance) with its corresponding dummy cannula inserted inside (Plastic One, Roanoke, VA) was implanted into the brain above both sides of the PVN according to the following stereotaxic coordinates: 1.8 mm caudal, $\pm$ 0.6 mm lateral with respect to bregma, and 6.4 mm ventral from the skull surface. The cannulae were kept in place by dental cement attached to microscrews embedded in the skull. Post-surgery dehydration and infection were prevented by injecting saline (1ml, s.c.) and ampicillin (50 mg/kg, s.c.) immediately after surgery and followed by 4 days of administration of sulfamethoxazole and trimethoprim suspended in the drinking water. These procedures for dual cannulae placement have been used extensively in this laboratory and reliably target the PVN. One week after implantation of cannulae, all animals were reanesthetized and ovariectomy was performed via single ventral midline incision. Post-surgery care was the same as for the previous surgery. Rats were allowed to recover from the ovariectomy for 3 days, and then they were handled for 2 consecutive days immediately before treatment with estradiol or G-1 as described below.

On the day of the intra-PVN injection, after removal of the dummy cannula, an injection cannula (33 gauge, 1.2 mm center-to-center distance, 0.5 mm projection from the tip of the double guide cannula) was inserted through the implanted guide cannula to the top of the PVN. After subsequent injections of vehicle or specific drugs as described below, rats were rapidly decapitated and the trunk blood was collected in centrifuge tubes containing a 0.5ml solution

of 0.3 M EDTA, pH 7.4. After centrifugation, the plasma was aliquoted and stored at -80°C for radioimmunoassays of plasma hormones. The brains were sectioned on a cryostat to carefully verify the position of the cannula tracks in each animal.

Experiment 1. Injection of PTX

Rats were ovariectomized and then double barrel cannulae were placed directly above the PVN to allow direct injections of PTX to block GPR30 mediated effects of estradiol on 5-HT_{1A} receptor signaling in the PVN. Rats received an intra-PVN injection (0.5 µl/side) of vehicle or PTX (1 µg/µl) over a 1 min period. The injection cannula was left *in situ* for an additional 1 min before removal. The next two days, rats received daily injections of EB (10 µg/kg, s.c.) and the rats were handled for these 2 days. 72 hours after the injection of PTX or vehicle all rats received a challenge injection of saline (1 ml/kg, s.c.) or the 5-HT_{1A} receptor agonist, DPAT (200 µg/kg, s.c.), 15 min prior to decapitation and subsequent collection of trunk blood.

Experiment 2. Injection of the GPR30 agonist, G-1

Ovariectomized rats received intra-PVN injections of vehicle or G-1 for 2 consecutive days to mimic the EB-induced effects on GPR30. Rats received an intra-PVN injection (0.5 µl/side) of vehicle or G-1 (10 nmol/µl or 100 nmol/µl) over a 1 min period. The injection cannula was left *in situ* for an additional 1 min before removal. 48 hours after the first injection of vehicle or G-1, all rats received a challenge injection of saline (1 ml/kg, s.c.) or the 5-HT_{1A} receptor agonist, DPAT (200 µg/kg, s.c.), 15 min prior to the decapitation and collection of trunk blood.

Radioimmunoassay for plasma hormone concentrations

Plasma oxytocin and ACTH concentrations were determined in all animals by radioimmunoassays as previously described in detail (Li et al., 1992). Estradiol concentrations (pg/ml) were determined in plasma samples obtained from vehicle- and EB- treated rats using a double-antibody estradiol RIA kit from Diagnostic Products Corp (Los Angeles, CA.).

Statistical analyses

All data are expressed as the means ± SEM, where *n* indicates the number of rats in each group. Oxytocin and ACTH data were analyzed by a three-way analysis of variance (ANOVA). Plasma estradiol levels were analyzed by a two-way analysis of variance (ANOVA). Group means were compared by Newman-Keuls' multiple-range test. GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for all statistical analyses. A probability level of $p \leq 0.05$ was considered to be statistically significant for all statistical tests.

Results

GPR30 colocalizes with 5-HT_{1A} receptors in the PVN

Based on the knowledge that the putative membrane estrogen receptor, GPR30, is expressed in the PVN and that estradiol modulates 5-HT_{1A} receptor signaling in the hypothalamus, we hypothesized that GPR30 plays a role in estradiol-induced modulation of hypothalamic serotonergic function. We first examined coexpression of GPR30 and 5-HT_{1A} receptors in the PVN. GPR30 immunoreactivity was localized in discrete cell groups in the hypothalamus, including the PVN and supraoptic nucleus (SON). Double-label immunofluorescence revealed that GPR30 and 5-HT_{1A} receptors were coexpressed by neurons in the PVN (Fig. 1A). Virtually all magnocellular neurons were immunoreactive for GPR30 in the PVN. A moderate number of GPR30 immunoreactive neurons were located in the parvocellular part of the PVN. In the magnocellular regions of the PVN, 84.1 ± 2.4 percent (mean ± standard error of the mean) of the neurons that are GPR30-positive, express 5-HT_{1A} receptors. An average of 147 cells are GPR30 positive per tissue section for a total of 1325 cells examined in the magnocellular region

in 9 tissue sections. In the parvocellular region, 77.4 ± 2.6 percent of the neurons that are GPR30-positive are also 5-HT_{1A} receptor positive. A total of 357 cells and an average of 40 cells per section were examined in the parvocellular region of the PVN in the 9 tissue sections. We also observed coexpression of GPR30 and 5-HT_{1A} receptors in the SON as well as in accessory magnocellular neurons scattered between the PVN and SON of the hypothalamus (data not shown).

GPR30 colocalizes with oxytocin in the PVN

All oxytocin immunoreactive neurons in the PVN were also GPR30 immunopositive (Fig. 1B). In the magnocellular region, 58.2 ± 3.3 percent of the GPR30 immunopositive neurons and 93.5 ± 1.4 percent in the parvocellular region were immunopositive for oxytocin. In the magnocellular region 768 cells were counted in the 9 tissue sections and in the parvocellular region 332 cells were counted. Coexpression of GPR30 and oxytocin also occurred in the SON.

GPR30 colocalizes with CRF in the PVN

In animals treated with colchicine to allow visualization of CRF in neuronal cell bodies, GPR30 immunoreactivity was present in PVN neurons, but the distribution in the soma was uneven compared with that in animals that did not receive colchicine, probably due to the disruption of normal cellular physiology. All the CRF expressing neurons in the PVN were GPR30 immunoreactive (Fig. 1C). Many GPR30 immunopositive neurons in the magnocellular region and in the parvocellular region of the PVN were immunopositive for CRF.

PTX inhibits modulation of 5-HT_{1A} receptor signaling by estradiol

Next, we used two complementary approaches and neuroendocrine challenge tests to assess the potential role of GPR30 in 5-HT_{1A} receptor signaling. First, we injected PTX directly to the PVN to inhibit estradiol-induced GPR30 signaling (Vivacqua et al., 2006). Pretreatment with PTX had no effect on the plasma estradiol levels of rats nor were significant differences observed in plasma estradiol levels among any of the estradiol-treated groups (Fig. 2). Plasma estradiol levels in ovariectomized rats were 15.09 ± 2.05 and 17.37 ± 2.18 pg/ml in the vehicle and PTX treated groups respectively. Treatment with EB significantly increased plasma estradiol levels to 159.72 ± 18.85 (vehicle group) and 150.6 ± 25.79 pg/ml (PTX group) ($p < 0.01$).

Although basal levels of oxytocin and ACTH were not affected by EB treatment (Fig. 3), EB altered the response of oxytocin and ACTH to the 5-HT_{1A} receptor agonist, DPAT. DPAT treatment increased circulating oxytocin and ACTH levels in both vehicle- and EB treated-ovariectomized females, but the magnitude of the increase was different depending on whether animals had received EB treatment or not. Without EB, DPAT produced a significant increase (over 2,000%) in the plasma levels of oxytocin (Fig. 3A). With EB, DPAT also significantly elevated (by 1,200%) plasma oxytocin levels compared to basal levels in rats given two daily injections of EB. However, this oxytocin response to DPAT was significantly lower (by 40%) than what was observed in rats that received no estrogen after ovariectomy. The two-way ANOVA for plasma oxytocin indicated a significant effect for EB ($F_{(1,69)} = 6.76$, $p < 0.05$), a significant effect for DPAT ($F_{(1,69)} = 218.47$, $p < 0.05$) and a significant interaction between EB and DPAT ($F_{(1,69)} = 6.49$, $p < 0.05$). Pretreatment with PTX, before the 2 consecutive days of injections of EB, blocked the EB-induced attenuation of the oxytocin response to DPAT (Fig. 3A).

DPAT produced a significant increase in the plasma levels of ACTH (Fig. 3B) in animals that received no EB or received two daily injections of EB; plasma ACTH levels in response to DPAT were elevated by 400% compared to basal levels. In contrast with oxytocin, the ACTH response to DPAT was not significantly different in animals that received no EB after

ovariectomy compared with those that did. The two-way ANOVA for plasma ACTH indicated a significant effect of DPAT ($F_{(1,69)}=92372$, $p<0.01$), but no attenuation of the response by EB ($F_{(1,69)}=1.53$, $p=0.22$), nor was there a significant interaction between DPAT and EB ($F_{(1,69)}=2.17$, $p=0.15$). Moreover, pretreatment with PTX or vehicle had no effect on the plasma ACTH levels in response to DPAT in any group (Fig. 3B).

GPR30 Agonist Decreases 5-HT_{1A} Receptor Signaling

In a subsequent experiment, the selective GPR30 agonist, G-1, was injected directly above the PVN. Basal levels of oxytocin in ovariectomized female rats were not altered after injection of G-1 (Fig. 4). DPAT produced a significant increase in the plasma levels of oxytocin (over 1,500% of saline-treated control) in control animals that did not receive G-1, as well as in animals that received the lower dose of 10 nmol of G-1, directly into the PVN. However, at the higher dose of 100 nmol of G-1 above the PVN, the GPR30 agonist significantly attenuated (by 29%) the plasma oxytocin response to DPAT. The two-way ANOVA for plasma oxytocin indicated a significant effect for G-1 ($F_{(2,52)}=8.79$, $p<0.01$), a significant effect for DPAT ($F_{(1,52)}=632.33$, $P<0.01$), and a significant interaction between G-1 and DPAT ($F_{(2,52)}=8.43$, $p<0.01$) indicating that G-1 administration influenced 5-HT_{1A} receptor signaling affecting oxytocin neurons.

As shown in Figure 4B, treatment with G-1 did not significantly alter the basal levels of plasma ACTH, but did attenuate the response to DPAT. Injections of either 10 or 100 nmol G-1 into the PVN significantly reduced the DPAT-stimulated levels of plasma ACTH by 26% and 31%, respectively, compared with vehicle-treated rats. The two-way ANOVA for plasma ACTH indicated a significant effect for G-1 ($F_{(2,54)}=3.69$, $p<0.05$), a significant effect for DPAT ($F_{(1,52)}=217.87$, $P<0.0001$) and a significant interaction between G-1 and DPAT ($F_{(2,54)}=4.28$, $p<0.05$) indicating that G-1 influenced the 5-HT_{1A} receptor mediated release of ACTH.

Discussion

The present results suggest that the putative extranuclear estrogen receptor, GPR30, at least partially mediates the desensitization of post-synaptic 5-HT_{1A} receptor signaling by estrogens. First, GPR30 colocalizes with 5-HT_{1A} receptors, CRF and oxytocin in PVN neurons. Second, PTX, an inhibitor of GPR30-mediated signaling, blocked the effects of EB on 5-HT_{1A} receptor signaling and finally, a selective GPR30 agonist, injected directly into the PVN, mimicked the effects of EB on 5-HT_{1A} receptor signaling with respect to oxytocin, but not ACTH.

The double-label immunofluorescent data demonstrated a high degree of co-localization of GPR30 and 5-HT_{1A} receptors in the PVN and within oxytocin and CRF containing neurons in the magnocellular and parvocellular neurons of the PVN. The presence of GPR30 in oxytocin-containing neurons is consistent with our findings that repeated stimulation of GPR30 desensitizes 5-HT_{1A} receptor-stimulated oxytocin release. During the preparation of this manuscript, a paper was published also demonstrating the presence of GPR30 in oxytocin magnocellular cells in the PVN (Sakamoto et al., 2007). Colchicine pretreatment was required to cause accumulation of detectable levels of CRF in PVN neurons so that we could address the question of whether estrogen has the potential to directly affect 5-HT_{1A} receptor signaling in CRF neurons via GPR30. Although the colchicine was predictably toxic to the neurons, nevertheless CRF was colocalized in both parvocellular and magnocellular PVN neurons that were also labeled for GPR30 receptors. The rats used for studying colocalization were cycling females, since there is no evidence in the literature suggesting that the expression of either 5-HT_{1A} receptors or GPR30 is altered in the PVN by estrous cycle stage. In any case, although colocalization of GPR30 with oxytocin, CRF or 5-HT_{1A} receptors does not constitute proof that the functional responses observed, and discussed below, are direct or indirect, nevertheless,

such colocalization does demonstrate that the potential exists for estradiol to directly affect 5-HT_{1A} signaling in PVN neurons by acting via GPR30.

Central PTX injections were used to block the effects of EB on GPR30, presumably through blocking the GPR30 mediated activation of ERK1/2 via a G $\beta\gamma$ pathway as previously demonstrated in cells in culture (Filardo et al., 2000). PTX injections could have had many non-specific effects especially via other PTX-sensitive ER. However, PTX injections into the PVN did not disrupt basal hormone release, nor did they directly interfere with the activation of 5-HT_{1A} receptors, as PTX did not disrupt 5-HT_{1A} receptor-stimulated hormone release; only the desensitization response to EB was attenuated by PTX. Moreover, it is known that DPAT stimulates hormone release via activation of 5-HT_{1A} receptors coupled to G α_z (Serres et al., 2000); G α_z proteins are PTX insensitive. Furthermore, our previous experiments had demonstrated that the effects of PTX in the PVN persist at least 72 hours (unpublished observation). Although these effects could conceivably occur through alterations in classical estrogen receptors coupled to G_{i/o} (we speculate since it is not known if classical estrogen receptors signal via G_{i/o} proteins), it is equally plausible that the effects of estradiol following PTX treatment on 5-HT_{1A} receptor-stimulated hormone release were mediated via GPR30.

The idea that GPR30 mediates the effects of estradiol on 5-HT_{1A} receptor signaling was more strongly supported by examining the effects of a GPR30 selective agonist on 5-HT_{1A} receptor mediated hormone release. The GPR30 agonist, G-1, was injected directly into the PVN for two days prior to the peripheral challenge injection of the 5-HT_{1A} receptor agonist, DPAT. G-1 exhibits high affinity and selectivity for GPR30; in competition with fluorescent estrogen, the K_i for G-1 is 11 nM for GPR30 whereas 1 μ M G-1 inhibits less than 20% of binding to ER α or ER β (Bologa et al., 2006). G-1 did not alter basal hormone levels, suggesting that neurons exposed to G-1 were functioning normally and that G-1 was not generally toxic to cells in the PVN. Similar to peripheral EB, central G-1 reduced the oxytocin release stimulated by the 5-HT_{1A} receptor agonist DPAT, suggesting a desensitization of 5-HT_{1A} receptor signaling in oxytocin cells in the PVN. Although EB does not consistently desensitize the DPAT-stimulated ACTH response (Raap et al., 2000; Carrasco et al., 2004), we found that the GPR30 agonist produced a desensitization of the DPAT-stimulated ACTH response. Given that EB did not affect DPAT-stimulated ACTH release in these studies, whereas the GPR30 agonist, G-1, significantly attenuated the release of both oxytocin and ACTH in the response to DPAT, it will be important to determine whether the effects of EB on GPR30 activation in CRF-containing cells are modulated by other ER receptors. Activation of other estrogen receptors by estradiol could counter the effect of GPR30 signaling either in the hypothalamus or pituitary. In particular, ER β , which functions at least in part as a nuclear transcription factor, is localized to the PVN (Hrabovszky et al., 1998). A large percentage of oxytocin producing cells in the PVN are ER β positive, whereas a small percentage (about 10%) of CRF cells in the PVN are also ER β immunopositive (Simonian and Herbison, 1997; Suzuki and Handa, 2005; Alves et al., 1998; Hrabovszky et al., 1998; Shughrue et al., 1997). Therefore, these two estrogen receptor subtypes with quite different structures and modes of action are in an ideal position to interact and produce integrated responses to EB within the same 5-HT_{1A} receptor producing neurons especially in oxytocin containing cells. Similar integrated responses have been proposed for other cell types and tissues (Levin, 2005; Shughrue et al., 1997; Miller et al., 2004) and for other recently identified estrogen receptors (Toran-Allerand et al., 2002). For example, ER-X is a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury.

Our previous studies suggest that estrogen and SSRIs desensitize 5-HT_{1A} receptor function by different mechanisms. Estrogens produce a more rapid (2 days) dose-dependent desensitization of 5-HT_{1A} receptor signaling that coincides with a dose-dependent increase in regulators of G protein signaling protein Z-1 (RGSZ-1) levels in the PVN (Carrasco et al., 2004), whereas

fluoxetine decreases $G_{\alpha z}$ protein levels (Raap et al., 1999). RGS proteins limit activated G_{α} protein signaling at least in part by increasing the rate of hydrolysis of GTP, such that increasing RGS protein activity could cause a desensitization response. Similarly, a decrease in $G_{\alpha z}$ protein levels directly could cause desensitization of 5-HT_{1A} receptor signaling. Although the desensitization response is presumably occurring via rapidly responding G-protein coupled receptors, i.e., GPR30 and serotonin receptors respectively, the desensitization response takes a number of days to occur, likely due to the necessity of these suggested changes in protein expression.

GPR30 mediates the stimulation of c-fos expression by estrogens and phytoestrogens (Maggiolini et al., 2004) as well as the increased transcriptional activity of c-fos promoter constructs (Vivacqua et al., 2006). Induction of c-fos expression via GPR30 is prevented by PTX and involves $G\beta\gamma$, Src, and epidermal growth factor receptor (Maggiolini et al., 2004). The increase in RGSZ-1 protein induced by estradiol could result from c-fos-mediated changes in gene expression regulated by GPR30 since PTX blocked the desensitization response induced by estradiol. The 2 day time period needed to cause the desensitization response by estradiol may be due to the time needed to increase expression of RGSZ-1 protein.

Desensitization of post-synaptic 5-HT_{1A} receptor signaling in the hypothalamus, other forebrain regions, and the midbrain, may be an underlying mechanism for the therapeutic effects of estrogens and of SSRIs, for mood disorders and hot flushes in peri- and post-menopausal women (Raap et al., 2000; Shen et al., 2002; Czachura and Rasmussen, 2000; Jackson and Etgen, 2001; Albert and Lemonde, 2004; Lifschytz et al., 2006). Mood disorders have been associated not only with dysregulation of serotonergic function, but also with increased expression of CRF, oxytocin and vasopressin in the PVN of depressed patients (Scott and Dinan, 2002; Purba et al., 1996; Raadsheer et al., 1995). Hypothalamic neurons, along with the neurotransmitters, neuropeptides and hormones they produce, comprise a central component of the limbic system circuits that control mood (Swanson and Sawchenko, 1983). Moreover, the hypothalamus is the central brain region responsible for the development of hot flushes in women (Gambone et al., 1984; Woodward and Freedman, 1994). Animal studies have shown that DPAT enhances the anti-depressant effects of estrogens (Estrada-Camarena et al., 2006) and, conversely, clinical studies have demonstrated a benefit of estrogens, superimposed on SSRIs, in treating depression in post-menopausal women (Schneider et al., 1997). Thus, behavioral experiments with GPR30 agonists and antagonists in the PVN, using models for depression such as the forced swim test, should be of benefit in determining whether the proposed mechanism for estradiol effects on 5-HT_{1A} receptor signaling have direct behavioral relevance, as well as relevance to hormonal responses.

Since estrogens as therapeutic agents may increase the probability of breast and endometrial cancer (Rossouw et al., 2002), and GPR30 itself may be associated with cell growth and proliferation and increased expression of growth related genes in endometrial and breast cancer cells lines (Vivacqua et al., 2006; Maggiolini et al., 2004), identifying the estrogen receptors that underlie the positive effects of estrogens in the relatively rapid estrogen-dependent desensitization of 5-HT_{1A} receptor signaling is of paramount importance. Further dissection of the integrative responses of different estrogen receptor subtypes may allow for the use of exquisitely selective estrogen receptor agonists as alternative medications for the treatment of symptoms such as hot flushes and mood disorders in peri- and post-menopausal women.

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Abbreviations

ACTH, adrenocorticotropin hormone; CRF, corticotrophin releasing factor; DPAT, (+)-8-hydroxy-2-dipropylaminotetralin; EB, 17- β -estradiol 3-benzoate; ER, estrogen receptors; PVN, paraventricular nucleus; PTX, pertussis-toxin; RT, room temperature; SSRIs, selective serotonin reuptake inhibitors; 5-HT_{1A}, serotonin 1A.

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Figure 1.

(A) Immunohistochemical double-labeling of GPR30 and 5-HT_{1A} receptors in the PVN. Arrowheads point to GPR30 positive neurons that are immunopositive for 5-HT_{1A} receptors. Arrows point to neurons that are immunopositive for GPR30 but immunonegative for 5-HT_{1A} receptors. (B) Immunohistochemical double-labeling of GPR30 and oxytocin (oxy) in the PVN. Arrowheads point to oxytocin neurons that are immunopositive for GPR30; Arrows point to neurons that are immunopositive for GPR30 but immunonegative for oxytocin. (C) Immunohistochemical double-labeling of GPR30 and CRF in the parvocellular region of the PVN. Arrowheads point to CRF neurons that are immunopositive for GPR30. (D) Low power image of immunohistochemical double-labeling of GPR30 and CRF in the PVN. 3V indicates the third ventricle and box shows the location of the higher power image in (C).

Figure 2.

Plasma estradiol levels in ovariectomized rats. Plasma estradiol levels were significantly different in the groups that received 2 days injection of EB versus vehicle. Pretreatment with vehicle or PTX produced no difference in plasma estradiol levels in ovariectomized rats. * indicates significantly different from rats treated with sesame oil at $p < 0.05$.

Figure 3.

(A) Pre-treatment with PTX blocked the reduction of the oxytocin response to DPAT in ovariectomized rats with 2 day injections of EB. EB significantly reduces the oxytocin responses to DPAT. * indicates significantly different from vehicle-treated DPAT challenged group at $p < 0.05$ using Newman-Keuls post hoc test. (B) Pretreatment with PTX produced no change in basal plasma ACTH levels or the response to DPAT in ovariectomized rats with 2 day injections of EB in sesame oil. V = vehicle

Figure 4.

Injection of GPR30 agonist G-1 into the PVN daily for two days significantly decreased the oxytocin and ACTH responses to DPAT in ovariectomized rats. * indicates statistically different at $p < 0.05$ and ** indicates statistically different at $p < 0.01$ compared vehicle treated DPAT stimulated rats using a Newman-Keuls post hoc test.