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The Serotonin Type 3_A Receptor Facilitates Luteinizing Hormone Release and LHβ Promoter Activity in Immortalized Pituitary Gonadotropes

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The 5-hydroxytryptamine type 3_A receptor (5-HT3_AR) is a ligand-gated cation channel activated by serotonin. This receptor is expressed throughout the nervous system as well as in the pituitary gland. Although it has been documented that the 5-HT3_AR modulates exocytosis in neurons, its role in the pituitary gland has not been determined. Previous research has shown that the 5-HT3_AR modulates circulating gonadotropin levels in vivo. It is unclear, however, if its activation in the pituitary gland mediates these effects or if receptors elsewhere in the hypothalamus–pituitary–gonadal axis are responsible. To investigate the potential for the 5-HT3_AR to modulate gonadotropin release from pituitary gonadotropes, the gonadotrope-derived LβT2 cell line was used as a model system and radioimmunoassays were employed to investigate how 5-HT3_AR activation influences luteinizing hormone (LH) release. Our studies demonstrate that gonadotropin releasing hormone (GnRH)–stimulated LH release was decreased by the 5-HT3_AR–specific antagonist MDL 72222 in a concentration-dependent manner. In addition, it was found that overexpressing the 5-HT3_AR in LβT2 cells enhanced both basal and GnRH-stimulated LH release and also increased LHβ gene promoter activity. These results suggest that the 5-HT3_AR may participate in the hypothalamus–pituitary–gonadal axis at the level of the pituitary gonadotrope to mediate pituitary hormone release.

Key Words: Hypothalamic–pituitary–gonadal axis; reproduction; ligand-gated ion channel; adenohypophysis; secretion.

Introduction

The 5-hydroxytryptamine type 3_A receptor (5-HT3_AR) is a ligand-gated cation channel expressed in the central and peripheral nervous systems as well as in the pituitary gland *(1–3)*. The endogenous ligand for the 5-HT3_AR, serotonin (5-HT), plays a central role in a myriad of cognitive and physiological processes *(4)* that include regulation of the hypothalamus–pituitary–adrenal (HPA) axis *(5–7)* and the hypothalamus–pituitary–gonad (HPG) axis *(8–11)*. Of the 14 5-HT receptors that have been cloned to date, the 5-HT3_AR is the only ligand-gated ion channel (LGIC). As such, this receptor represents a distinct mechanism by which 5-HT can induce cellular responses not directly related to second messengers typically associated with stimulation of G protein–coupled 5-HT receptor activation *(12,13)*.

Previously it has been shown that the 5-HT3_AR facilitates exocytosis of neurotransmitters. 5-HT3_ARs in the central nervous system (CNS) are involved with release of dopamine in the nucleus accumbens *(14–16)* and striatum *(17,18)* as well as with release of γ-aminobutyric acid in the dorsal horn *(19)* and acetylcholine in the hippocampus *(20)*. Outside the CNS, blocking the 5-HT3_AR reduces exocytosis from intact neuroepithelial body cells *(21)* and regulates pancreatic secretion *(22,23)*. In addition, several studies have identified the 5-HT3_AR as a potential mediator of pituitary hormone secretion in vivo *(11,24,25)*; however, it is unclear where in the HPG axis the 5-HT3_AR activity exerts its effects.

Although much is known about the mechanisms underlying LH release from gonadotropes, the possibility that the 5-HT3_AR may be directly involved with this process has not yet been explored. To this end, we employed the gonadotrope-derived LβT2 cell line *(26–28)* as a model system to determine if 5-HT3_AR activity can directly regulate LH release. In these studies we provide novel data showing that the 5-HT3_AR is expressed in LβT2 cells where its activation is necessary for maximal LH release. Furthermore, we provide data showing that overexpression of the 5-HT3_AR in LβT2 cells enhances basal and GnRH-induced LH release as well as LHβ gene promoter activity. Together, these data suggest that the 5-HT3_AR participates in regulation of the HPG axis.

Results

The 5-HT3_AR Is Expressed in Pituitary Gonadotrope-Derived LβT2 Cells

The role of the 5-HT3_AR in LH release was investigated using the pituitary gonadotrope-derived LβT2 cell line. LβT2

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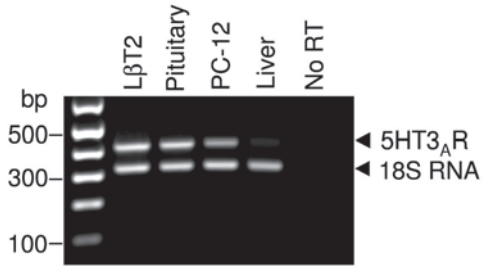

Fig. 1. The 5-HT3$_A$R is expressed in the pituitary gland and in gonadotrope-derived LβT2 cells. Using RT-PCR a 425 bp fragment of the 5-HT3$_A$R was amplified from LβT2 cells using primers designed to amplify the rat or mouse sequence. RT-PCR was performed using 400 ng template cDNA transcribed from total RNA isolated from LβT2 cells, adult rat pituitary, PC-12 cells, or adult rat liver. Primers to amplify a 318 bp band of 18S rRNA were included as a loading control (n = 3).

cells represent a well-characterized model of mature pituitary gonadotropes and have been used previously to study GnRH-stimulated LH release *(27,28)*. To verify that these cells express the 5-HT3$_A$R, RT-PCR was performed using RNA isolated from cultured LβT2 cells and from control tissues (Fig. 1). In these experiments a 425 bp fragment of the 5-HT3$_A$R was amplified from LβT2 cells, rat pituitary gland, and from the rat pheochromocytoma cell line, PC-12, which was included as a positive control *(29,30)*. Although significant levels of 5-HT3$_A$R mRNA were detected in LβT2 cells, no band was observed when rat liver RNA was used as template or when RT was omitted from a reaction containing PC-12 RNA. These results suggest that functional 5-HT3$_A$Rs are expressed in LβT2 cells.

Blocking the 5-HT3$_A$R Attenuates GnRH-stimulated LH Release

In response to GnRH stimulation, LβT2 cells release LH in a time- and concentration-dependent manner *(27,28)*. This response is attenuated by pretreating cells with the 5-HT3$_A$R-specific antagonist, MDL-72222 (5 μ*M*; Fig. 2A) or with Y25130, a selective 5-HT3$_A$R antagonist that is structurally unrelated to MDL 72222 (not shown). Moreover, the attenuating effect of MDL-72222 cannot be overcome by increasing the concentration of GnRH. In cultures of LβT2 cells treated with increasing concentrations of GnRH, a concentration-dependent increase in LH release was observed, but 30 min pretreatment with MDL-72222 (5 μ*M*) reduced LH release by a similar amount at every GnRH concentration tested (Fig. 2B).

5-HT3$_A$R Activation Alone Does Not Stimulate LH Release

When cells are pretreated with increasing concentrations of MDL-72222, GnRH-stimulated LH release is decreased in a concentration-dependent manner (Fig. 3A), indicating that receptor activation is necessary for maximal LH release. However, stimulating the receptor with the 5-HT3$_A$R–specific agonist, mCPBG (up to 1 m*M*) or 5-HT (not shown) does not elicit LH release from LβT2 cells (Fig. 3B).

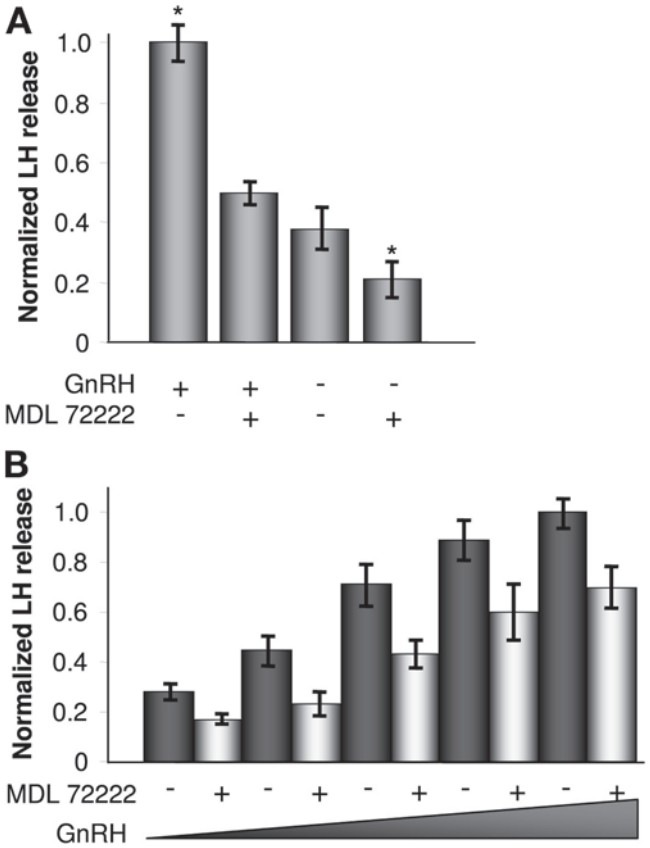

Fig. 2. (**A**) Blocking the 5-HT3$_A$R decreases GnRH-stimulated LH release. Confluent plates of LβT2 cells were serum-starved overnight and then stimulated with the indicated combinations of compounds for 15 min. When MDL-72222 was used, it was applied 30 min prior to stimulating the cells. Radioimmunoassays were then performed to determine the concentration of LH in the media. All data were normalized to the maximal GnRH-stimulated LH release. The data presented are pooled from three independent experiments (mean ± SEM; asterisks indicate values that are significantly different from those obtained in the vehicle control condition, $p < 0.05$; $n = 3$). (**B**) MDL-72222 attenuates dose-dependent GnRH-stimulated release of LH from LβT2 cells. LβT2 cells were treated for 15 min with increasing concentrations of GnRH (0, 1, 10, 100, 1000 nM) in the presence (+) or absence of (–) MDL-72222 (5 μ*M*) and the LH concentration in the media was then determined by radioimmuoassay. At every concentration of GnRH tested, LH secretion was significantly ($p < 0.05$) attenuated by MDL-72222 (mean ± SEM; n = 3).

The inability of *m*CPBG or 5-HT to elicit LH release may be due to relatively low levels of 5-HT3$_A$R expression in the plasma membrane. If few receptors are present on the cell surface, 5-HT in the serum added to the culture media could fully occupy those receptors. If this is the case, constitutive activity of the 5-HT3$_A$R may mask any effect that would result from adding agonist. To address this possibility stable LβT2 cell lines were generated that overexpress either the full-length 5-HT3$_A$R (LβT2-5-HT3$_A$) or the empty

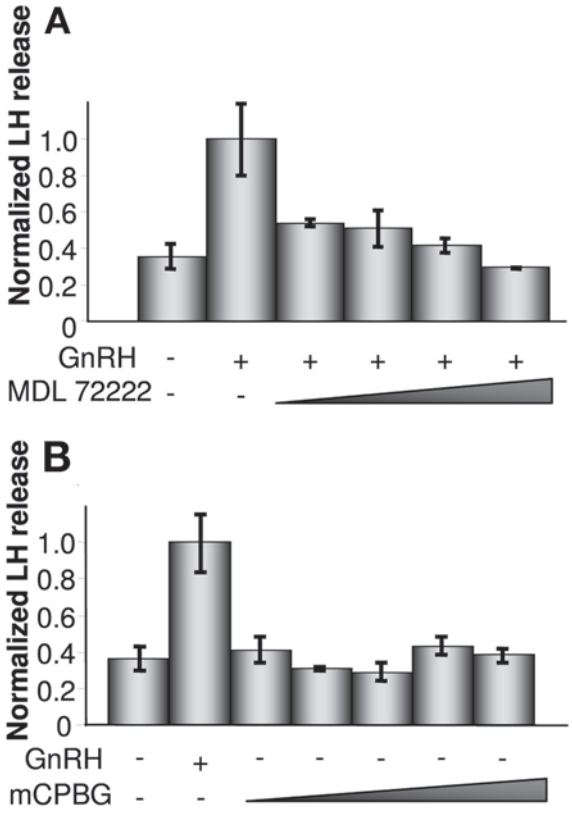

Fig. 3. Activation of the 5-HT3$_A$R receptor is necessary, but not sufficient, for inducing maximal GnRH-stimulated LH release from LβT2 cells. LβT2 cells were serum starved for 16 h and then stimulated with GnRH (100 n*M*) for 15 min in the presence of increasing concentrations of the 5-HT3$_A$R antagonist, MDL-72222 (**A**), or the 5-HT3$_A$R agonist mCPBG (**B**). In the presence of MDL-72222 (0, 0.5, 5, 10, 50 μ*M*), a dose-dependent decrease in GnRH-stimulated LH release was observed, while the 5-HT3$_A$R agonist mCPBG (0, 0.4, 4, 40, 400, 4000 n*M*) did not elicit LH release even at the highest concentration ($n = 3$; mean ± SEM).

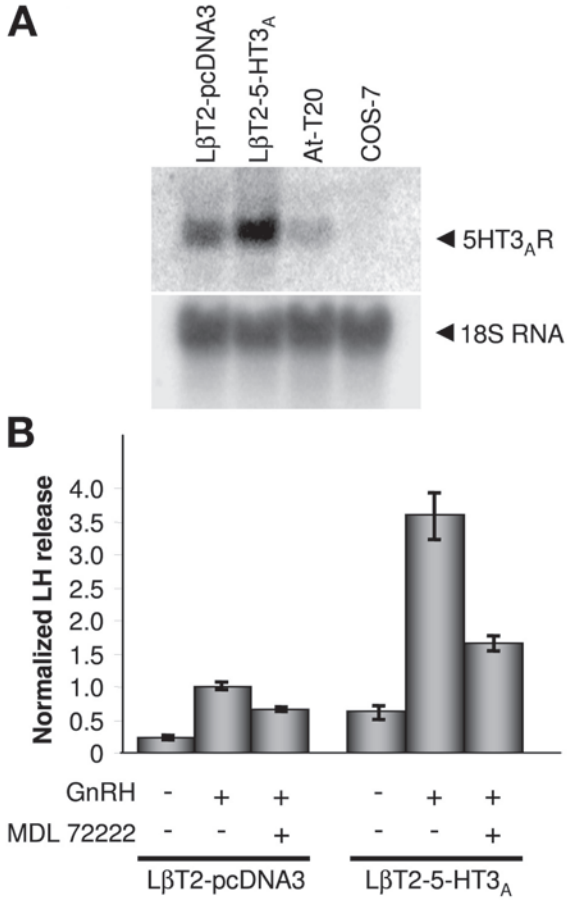

Fig. 4. LβT2 cells that overexpress the 5-HT3$_A$R exhibit enhanced basal and GnRH-stimulated LH release. (**A**) A representative Northern blot demonstrates that LβT2-5-HT3$_A$R cells express higher levels of 5-HT3$_A$R mRNA than LβT2-pcDNA3 cells. Total RNA (20 μg) from LβT2-5-HT3$_A$, LβT2-pcDNA3, At-T20 (positive control), and COS-7 (negative control) cells were probed with the full-length 5-HT3$_A$R cDNA and then the blot was stripped and reprobed with an 18S rRNA cDNA to normalize for RNA content ($n = 3$). (**B**) LβT2-5-HT3$_A$ and LβT2-pcDNA3 cells were serum-starved for 16 h and then stimulated with GnRH (100 n*M*) for 15 min in the presence or absence of the MDL-72222 (5 μ*M*). LβT2-5-HT3$_A$R cells demonstrated enhanced basal LH release and a greater than threefold increase in GnRH-stimulated LH release relative to LβT2-pcDNA3 cells ($n = 3$; mean ± SEM).

expression vector (LβT2-pcDNA3). Northern blot analysis (Fig. 4A) shows that LβT2-5-HT3$_A$ cells express considerably higher levels of 5-HT3$_A$R mRNA than the LβT2-pcDNA3 cells. The specificity of the 5-HT3$_A$R radiolabeled probe was confirmed by including RNA from COS-7 cells, which do not express 5-HT3$_A$Rs *(31)* and RNA from At-T20 cells was included as a positive control.

Overexpressing the 5-HT3$_A$R in LβT2 cells had a significant effect on LH release. Compared with control LβT2-pcDNA3 cells, the LβT2-5-HT3$_A$ cells exhibited higher basal LH release. In addition, GnRH-stimulated LH release was increased approximately fourfold by overexpression of the 5-HT3$_A$R (Fig. 4B). This difference was not the result of differing growth rates in the two cell lines. After collecting the media for radioimmunoassay, the cells were counted and no difference in cell number was detected (not shown). These data verify that the 5-HT3$_A$R plays a direct role in modulating basal and GnRH-stimulated LH release from LβT2 cells. However, in these experiments neither mCPBG nor the structurally unrelated 5-HT3$_A$R agonist N-methylquipazine, elicited LH release from LβT2-pcDNA3 or LβT2-5-HT3$_A$ cells (not shown).

Overexpressing the 5-HT3$_A$R Enhances LHβ Promoter Activity in LβT2 Cells

To determine if increased LH secretion, secondary to 5-HT3$_A$R overexpression, results in a genetic response in LHβ transcriptional activity, luciferase reporter experiments were performed. For these studies LβT2-5-HT3$_A$ and LβT2-pcDNA cells were transfected with a reporter vector containing –779 bp of the proximal LHβ gene promoter driving luciferase expression *(32,33)*. Differences in promoter activity between the two cell lines was then determined by mea-

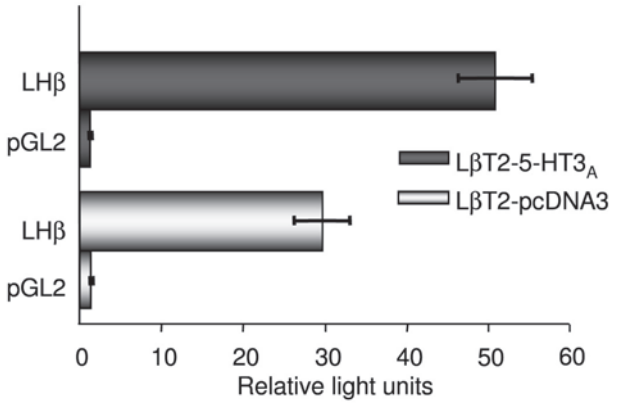

Fig. 5. Overexpressing the 5-HT3$_A$R enhances LHβ promoter activity. LβT2-5-HT3$_A$ and LβT2-pcDNA3 cells were transiently transfected with a luciferase reporter construct containing –779 bp of the LHβ promoter and luciferase activity was assayed after 24 h. The LHβ promoter activity in LβT2-5-HT3$_A$ cells was significantly enhanced relative that observed in LβT2-pcDNA3 cells ($p < 0.0001$; $n = 3$).

suring luciferase activity (Fig. 5). In both cell lines robust LHβ promoter activity was detected, but luciferase activity was significantly enhanced in the LβT2-5-HT3$_A$R cell line relative to that measured in LβT2-pcDNA3 cells. This result was anticipated, as enhanced LH release from LβT2-5-HT3$_A$ cells should result in a compensatory increase in LHβ gene transcription. To ensure that differences in luciferase expression between the cell lines were not due to systematic changes in cell viability, cells in sister cultures were counted to ensure similar numbers of live cells from each line were used in the assays (not shown).

Discussion

Our studies offer novel data that identify the 5-HT3$_A$R as an effector of basal and GnRH-stimulated LH release from immortalized gonadotropes. In addition, we show that overexpressing the 5-HT3$_A$R in these cells increases LH release and enhances LHβ gene promoter activity. Together, these findings suggest that the 5-HT3$_A$R may play a role in regulating serotonergic control of the HPG axis through its effects on pituitary gonadotropes.

Previous studies have implicated the 5-HT3$_A$R as a participant in HPG axis regulation, but the conclusions drawn have been inconsistent *(11,24,25)*. The discrepancies among these studies are likely due to the variety of experimental systems employed. In immature female rats LH release is enhanced by 5-HT3$_A$R agonists, and 5-hydroxytryptophan–induced LH release is decreased by 5-HT3$_A$R antagonists *(24)*. In prepubertal male rats 5-HT3$_A$R agonists decrease LH release. However, they have no effect in adults *(11)*. Thus, in rats the contribution of the 5-HT3$_A$R to gonadotropin regulation appears to be developmentally regulated and sex-dependent. It is therefore likely that the ability of the 5-HT3$_A$R to modulate LH levels in gonadotropes is dependent on the hormonal background against which it is tested. This notion is supported by the fact that the 5-HT3$_A$R is functionally *(34,35)* and transcriptionally *(36,37)* regulated by steroid hormones.

In whole-animal models it is difficult to separate the effects of receptors in the pituitary gland from those of receptors expressed in other tissues comprising the HPG axis. The LβT2 gonadotrope cell line allows precise control over the extracellular milieu and provides a valid and reliable model with which to characterize the function of the 5-HT3$_A$R in HPG axis regulation.

The fact that the 5-HT3$_A$R is modulated by components of the extracellular milieu may also help explain the inability of 5-HT3$_A$R agonists to affect LH release in our system. Fetal bovine serum (FBS) in culture media contains relatively high levels of 5-HT. As plasma membrane targeting of the 5-HT3$_A$R is tightly regulated, even in LβT2-5-HT3$_A$ cells functional receptors may not be abundant in the plasma membrane. It is possible that the receptors present on the cell surface are constitutively activated by 5-HT in the media and no further activation can be attained by adding agonist. This possibility is supported by our data showing that overexpression of the 5-HT3$_A$R enhances basal LH release in the absence of GnRH. To fully test this, it would be necessary to selectively remove 5-HT from the culture media and then determine if 5-HT3$_A$R activation elicits LH release. Unfortunately, removal of serum from the culture media results in an overall loss of LH release and LHβ promoter activity even in the presence of GnRH (data not shown). When serum was dialyzed to remove 5-HT, GnRH responsiveness was restored, but 5-HT3$_A$R agonists still failed to elicit a response (data not shown). This suggests that additional unidentified factors in the serum are required for agonist-induced LH release. The ability of 5-HT3$_A$R specific agonists to elicit LH release may be dependent on concomitant inputs from separate systems. Such is the case for the potent LH secretegogue Kisspeptin (KiSS-1). In the presence of GnRH receptor activation, KiSS-1, acting through its receptor GPR54, elicits maximal LH release, but when the GnRH receptor is blocked, KiSS-1 is ineffective *(38)*. Thus, there may be a particular combination of factors not present in our culture conditions that is required for 5-HT3$_A$R agonists to be effective.

Although it is well established that GnRH is the primary secretagogue driving LH release in pituitary gonadotropes, LH is released constitutively at low levels. Our studies show that overexpression of the 5-HT3$_A$R enhances this basal LH release in the absence of GnRH. The molecular mechanisms that control LH exocytosis are plastic and complex *(39)*. Studies on LH release have shown it is a Ca^{2+}-dependent *(40)* event and that multiple, pharmacologically distinct, Ca^{2+}-mobilizing mechanisms *(27,40–42)* are tightly

coordinated to modulate intracellular calcium concentrations, and therefore LH release, in gonadotropes *(39,43)*. Since the 5-HT3$_A$R is a Ca^{2+}-permeable ion channel *(31)*, it is likely that Ca^{2+} entry through the 5-HT3$_A$R is the mechanism by which it affects LH release in gonadotropes.

Using LβT2-5-HT3$_A$ cells we showed that increasing 5-HT3$_A$R expression caused an increase in both basal and GnRH-stimulated LH release. We expected this increased LH secretion to result in a compensatory increase in LHβ gene expression. Because LβT2-5-HT3$_A$ cells in culture are not influenced by negative feedback from gonadal steroids, as is the case in vivo, they continue to release LH constitutively at high levels, which depletes intracellular LH stores. To maintain consistent intracellular releasable LH pools, cells must increase LH production. It has been shown that LHβ gene transcription *(44,45)* and translation *(46)* is increased by GnRH in a calcium-dependent manner that requires Ca^{2+} influx. Thus, it is possible that Ca^{2+} influx through the 5-HT3$_A$R contributes to transcriptional and/or translational regulation of the LHβ gene. However, 5-HT3$_A$-LβT2 cells exhibit enhanced LHβ gene transcription and LH release in the absence of GnRH. Thus, an effector other than activation of the GnRH receptor must drive LHβ gene transcription and LH release in these cells. It is likely that Ca^{2+} entry via the 5-HT3$_A$R plays a role in this enhanced LHβ activity. Certainly, the 5-HT3$_A$R is not the primary mechanism for Ca^{2+} influx in LβT2 cells, but it may represent a serotonergic rheostat for fine-tuning the cellular response to depolarizing events that activate voltage-gated calcium channels.

Having gained insight into the effects of 5-HT3$_A$R activation in gonadotropes, it will now be possible to utilize an in vivo approach to verify and expand our findings. Employing gonadotrope-specific overexpression of the 5-HT3$_A$R in a transgenic model would be useful to unambiguously characterize the role of this receptor against the complex hormonal influences of the HPG axis. By introducing a transgene under the control of the α-glycoprotein subunit promoter, gonadotrope-specific overexpression of transgenes has previously been demonstrated *(47)*, and such a strategy could be used to study the effects of 5-HT3$_A$R overexpression.

Our studies have determined that the 5-HT3$_A$R, a LGIC, enhances release of LH and increases LHβ gene transcription in immortalized pituitary gonadotropes. Although it is known that this receptor plays a role in neuronal neurotransmitter release, its importance for hormone exocytosis in pituitary secretory cells has been controversial. This is largely due to the conflicting data derived from experiments in whole animals and the difficulty of isolating the inputs from individual components of the HPG axis. Using LβT2 cells as a model of pituitary gonadotropes, we have shown that the 5-HT3$_A$R is a regulator of gonadotrope function. Because this receptor may also be expressed in other pituitary cell types *(48)*, it may, in fact, serve as a general mechanism of serotonergic control over pituitary hormone regulation.

Materials and Methods

The 5-HT3$_A$R antagonist, MDL-72222, and agonist, *m*-chlorophenylbiguanide hydrochloride (mCPBG) were purchased from Tocris (Ballwin, MO). GnRH and 5-HT were purchased from Sigma (St. Louis, MO). Fugene6® transfection reagent was from Roche Molecular Biochemicals (Indianapolis, IN). All reagents for RNA isolation, RT-PCR, and cell culture were purchased from Invitrogen (Carlsbad, CA). Primers for RT-PCR were purchased from Sigma-Genosys (The Woolands, TX). The primer sequences used amplify a 425 bp 5-HT3$_A$R subunit fragment have been described elsewhere *(49)*. The primers used to amplify 18S rRNA were purchased from Ambion (Austin, TX) and the Dual Luciferease® Reporter Assay System used for LHβ promoter assays was purchased from Promega (Madison, WI). Primary tissue for RT-PCR experiments was collected from Sprague–Dawley rats obtained at the specified ages from Zivic Laboratories, Inc. (Pittsburgh, PA). All procedures involving animals were performed in accordance with applicable local and federal regulations under a protocol approved by the Case Western Reserve University Institutional Animal Care and Use Committee. The gonadotrope-derived LβT2 cell line was obtained from Dr. Pamela Mellon (UC-San Diego) and the murine 5-HT3$_A$R cDNA was obtained from Dr. David Julius (UC-San Francisco). The full-length 5-HT3$_A$R cDNA was subcloned into the mammalian expression vector pcDNA3 from Invitrogen. The –779 bp LHβ-pGL2 promoter construct was kindly provided by Dr. John Nilson (Washington State University).

Cell Culture and Treatments

LβT2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2. The cells were serum-starved for 16 h prior to treatments. In cultures where antagonist was applied, cells were pretreated with MDL-72222 at the appropriate concentration 30 min prior to treatment with agonists and/or hormone. Drug treatments were applied to cells for 15 min, after which the media were collected and immediately frozen. Media samples were stored at –80°C until used in radioimmunoassays.

Generation of Stable Cell Lines

To generate cell lines stably expressing the 5-HT3$_A$R (LβT2-5-HT3$_A$) or the empty pcDNA3 expression vector (LβT2-pcDNA3), LβT2 cells were transfected in six-well plates with 3.5 μg of cDNA using Fugene6® transfection reagent according to the manufacturer's protocol. The day following transfections G418 (500 μg/mL) was added to the media to select stably transfected cells. Cultures were maintained under antibiotic selection until individual colonies

could be picked. For each cell line, several colonies were picked and maintained under antibiotic selection. Colonies from each cell line were then pooled and each line was passed three times prior to storage in liquid N_2.

Semi-quantitative RT-PCR

Total RNA was isolated from freshly dissected rat pituitary glands or monolayer cultures of LβT2 cells using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 2 μg total RNA using 1 μL Superscript II® reverse transcriptase (Invitrogen) in 10 μL reactions. Duplex PCR was then carried out using 2 μL of the RT reaction as template in 50 μL reactions. In initial PCR studies to detect the 5-HT3$_A$R transcript, no attempt was made to maintain the amplicon in the linear range and 40 cycles of PCR were performed. For semiquantitative RT-PCR, the PCR was carried out for 28 cycles using a primer ratio that maintained both amplicons in the linear phase of amplification. PCR products were separated on 1.5% agarose gels and then stained in 0.005% (w/v) ethidium bromide for 20 min. After destaining in deionized distilled H_2O for 1 h, digital images of the gels were obtained under UV light with the Gene Genius Bioimaging system running GeneSnap 6.00.20 software (SynGene, Cambridge, UK). Digital images were then analyzed with GeneTools Analysis Software 3.02.00 (SynGene). Relative 5-HT3$_A$R transcript expression is reported as a ratio of the pixel density of the 5-HT3$_A$R band to that of the 18S rRNA band on digital images of agarose gels. RT-PCR experiments were a minimum of three times.

Radioimmunoassay

LH concentrations in culture media from treated LβT2 cells were determined using radioimmunoassay. Radioimmunoassays were performed by the Reproductive Endocrinology Laboratory at Colorado State University using previously described antibodies and procedures *(50)* and by the University of Virginia Ligand Core Facility using a mouse LH sandwich assay. The complete protocol is available on their website (http://www.healthsystem.virginia.edu/internet/crr/methodspage.cfm). To analyze RIA data, one-way analyses of variance (ANOVA) were performed on the mean relative LH release across conditions from three separate experiments. In each case data were normalized to the mean LH release from GnRH-treated cells. Each experiment was performed in triplicate, and the data from all three experiments were pooled and analyzed together. To identify significant differences among the conditions, Tukey's *post hoc* multiple comparison procedure was employed ($\alpha = 0.05$).

Northern Blot

To assess 5-HT3$_A$R mRNA levels in 5-HT3$_A$R-LβT2 cells, Northern blot analysis was performed as previously described *(33)*. Briefly, total RNA was isolated from confluent plate LβT2-pcDNA3 cells, LβT2-5-HT3$_A$R cells, or COS-7 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Fifteen micrograms of total RNA was separated on a 1.0% denaturing agarose/formaldehyde gel, transferred to a nylon membrane, and then cross-linked with UV radiation. Prior to hybridization with radiolabeled probe, the membrane was blocked in hybridization buffer [40% deionized formamide, 20 m*M* PIPES, 800 m*M* NaCl, 2 m*M* EDTA, 4% sodium dodecylsulfate (SDS), 80 μg/mL salmon sperm DNA] for 5 h. [^{32}P]deoxy-CTP-labeled (3000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) cDNA probe was then added and the membrane was incubated overnight at 45°C. After several washes at 65°C with saline-sodium citrate containing 0.5% SDS, the membrane was placed on a phosphor screen (Amersham Biosciences) for 1 (18S rRNA) or 4 (5-HT3$_A$R) d and then analyzed using a Typhoon blot imager and ImageQuant software (Amersham Biosciences). Following hybridization with the 5-HT3$_A$R probe, the membrane was stripped and reprobed using a radiolabeled cDNA amplicon generated with primers that amplify a 425 bp fragment of murine 18S rRNA (Ambion). This experiment was repeated three times with similar results.

Dual Luciferase® Reporter Assay

LβT2-pcDNA3 or LβT2-5-HT3$_A$R cells were plated on six-well culture plates at 3.5×10^6 cells/well the day prior to transfection. Cells were then co-transfected with 2.0 μg of reporter plasmid or control vector along with 100 ng of the pRL-CMV expression vector using Fugene6® transfection reagent according to the manufacturer's protocol. After 24 h the cells were harvested and reporter activity was measured in a luminometer. To control for differences in cell number and transfection efficiency, LHβ promoter reporter-luciferase activity was first normalized against the activity of pRL-CMV activity and then normalized to the promoterless pGL2 expression vector. In these experiments, cells from sister cultures (three wells per cell line) were counted after incubation with trypan blue to ensure that similar numbers of live cells from each cell line were included in the analyses. Data reported are from three independent experiments.

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