Neuronal Excitability

The Dopamine D4 Receptor Regulates Gonadotropin-Releasing Hormone Neuron Excitability in Male Mice

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Abstract

Gonadotropin-releasing hormone (GnRH)-secreting neurons control fertility. The release of GnRH peptide regulates the synthesis and release of both luteinizing hormone (LH) and Follicle stimulation hormone (FSH) from the anterior pituitary. While it is known that dopamine regulates GnRH neurons, the specific dopamine receptor subtype(s) involved remain unclear. Previous studies in adult rodents have reported juxtaposition of fibers containing tyrosine hydroxylase (TH), a marker of catecholaminergic cells, onto GnRH neurons and that exogenous dopamine inhibits GnRH neurons postsynaptically through dopamine D1-like and/or D2-like receptors. Our microarray data from GnRH neurons revealed a high level of Drd4 transcripts [i.e., dopamine D4 receptor (D4R)]. Single-cell RT-PCR and immunocytochemistry confirmed GnRH cells express the Drd4 transcript and protein, respectively. Calcium imaging identified changes in GnRH neuronal activity during application of subtype-specific dopamine receptor agonists and antagonists when GABAergic and glutamatergic transmission was blocked. Dopamine, dopamine with D1/5R-specific or D2/3R-specific antagonists or D4R-specific agonists decreased the frequency of calcium oscillations. In contrast, D1/5R-specific agonists increased the frequency of calcium oscillations. The D4R-mediated inhibition was dependent on Ga protein coupling, while the D1/5R-mediated excitation required Gs protein coupling. Together, these results indicate that D4R plays an important role in the dopaminergic inhibition of GnRH neurons.

Key words: calcium imaging; dopamine; fertility; GnRH; patch clamp

Significance Statement

The convergence of information on neurons secreting gonadotropin-releasing hormone (GnRH) shape their secretory profile and consequently, fertility. Dopamine inhibits GnRH neurons, yet the specific dopamine receptor subtype(s) involved remain unclear. Using single RT-PCR, immunofluorescence, and dopamine receptor-specific pharmacological tools, we show here that dopamine D4 receptor (D4R) plays a role in the dopaminergic inhibition of GnRH neurons, adding a new tool for the modulation of reproductive function.

Introduction

Dopamine is a regulator of reproductive function at many levels along the hypothalamic-pituitary-gonadal axis. A female-specific dopaminergic inhibitory tone, observed in developing rats (Lacau de Mengido et al., 1987; Becú-Villalobos and Libertun, 1995), influences the timing of puberty (Ruf and Holmes, 1974; Lamberts and Wuttke, 1981; Gerber et al., 1984; de Mengido et al., 1989). In seasonal breeders, dopamine is a component of anestrus (Lehman et al., 1996; Saxena et al., 2015). Ovarian dopamine receptors regulate ovulation (Venegas-Meneses et al., 2015) and dopaminergic neurons control anterior

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Dopamine receptors, based on their structural, pharmacological, and signaling properties, are divided into two families of G-protein-coupled receptors: D1-like (D1 and D5 subtypes) and D2-like (D2, D3, and D4 subtypes; Beaulieu et al., 2015). Both the tuberoinfundibular dopaminergic pathway (Leranth et al., 1988; Mitchell et al., 2003) and the anteroventral periventricular nucleus (AVPV; Horvath et al., 1993) contribute to the TH innervation of GnRH-secreting neurons. Notably, the kisspeptin neuron subpopulation in the arcuate nucleus (ARC) acts on the tuberoinfundibular dopaminergic pathway (Ribeiro et al., 2015) and the sexually dimorphic kisspeptin neuron subpopulation in the AVPV coexpresses TH (Simerly et al., 1985; Clarkson and Herbison, 2011), indicating that dopamine may influence GnRH neuronal activity both directly and indirectly. Throughout the brain, dopamine modulates neuronal excitability (Beaulieu et al., 2015). However, dopamine hyperpolarizes GnRH neurons, via the activation of potassium channels (Liu and Herbison, 2013).

Dopamine receptors couple to G-protein signaling pathways, and the G-protein subtypes that they interact with determine the nature of the receptor’s physiological effects (Bourque et al., 2002). While each GnRH neuron did not express all five dopamine receptors, all dopamine receptors were present within the GnRH neuron population. In primary mouse GnRH neurons maintained in explants, devoid of kisspeptin neurons and TH containing neurons, calcium imaging revealed that activation of D1-like receptors only increased GnRH neuronal activity, via G<sub>αs</sub> protein coupling. In contrast, activation of D2-like receptors, including D4R, decreased GnRH neuronal activity, via G<sub>αi/o</sub> protein coupling. Consistent with these results, patch clamp revealed D4R specific activation decreased GnRH neuron firing in brain slices. These data demonstrate that dopamine inhibits GnRH neuronal activity via activation of D2-like receptors and identifies D4R as a new contributor to this inhibition.

**Materials and Methods**

**Nasal explants**

All procedures were conducted in accordance with the Society for Neuroscience’s Policies on the Use of Animals and Humans in Research. Unsexed embryos from timed mated NIH Swiss mice were used to generate nasal explants, as previously described (Klenke and Taylor-Burds, 2012). One embryo gives one explant. Explants were maintained at 37°C in serum-free medium (SFM) in a 5% CO<sub>2</sub> humidified incubator. Fresh media containing fluoro-deoxyuridine (2.3 μM; Sigma-Aldrich) was applied on culture day 3 to inhibit proliferation of dividing olfactory neurons and nonneuronal explant tissue. On culture day 6, and every other day afterward, the medium was changed with fresh SFM. After days 2–3 in vitro, GnRH neurons migrate out of the explant and can be recorded in the periphery.

**Single GnRH cell isolation and microarray data**

This dataset was previously generated (Messina et al., 2011). Briefly, the cytoplasmic content of single GnRH neurons in 10-d in vitro explants was extracted and poly(A) amplified cDNA libraries were generated (Kramer, 2002).

cDNA libraries from nine individual GnRH neurons 10 div (see below) were randomly grouped into three samples. This material was amplified, processed for microarray data generation and analysis. All cDNA were labeled and hybridized to the DIRP NIH microarray core facility with GeneChip Mouse Genome 430 2.0 arrays (Affymetrix). Custom R scripts, including covariance-based PCA, correlation heat maps, LOWESS analysis, and clustering checked the dataset for noise and outliers. Normalization, i.e., the average expression of genes, was performed identically throughout the dataset and log-transformed. Gene expression values were calculated using robust multigroup average (RMA) procedure and compared with repeated-measurement one-way ANOVA, using data from one probe set (Drd1_629) as a reference.

**PCR on single cells and explants**

Poly(A) amplified cDNA libraries were previously created from whole explants and individual GnRH cells in explants (Kramer, 2002). The quality of cDNA from each cell
was verified by PCR for GnRH, L19, and β-tubulin (Giacobini et al., 2004). Primers were designed in the 3′-untranslated region of genes Drd1, Drd2, Drd3, Drd4, and Drd5 (Table 1). All primers were screened with BLAST to ensure specificity. For each reaction, 1× PCR buffer, 2 mM MgCl₂, 250 μM of each deoxynucleotide (Life Technologies), 250 nM forward primer, 250 nM reverse primer, and 2.5 U Ampli-Taq Gold (Life Technologies) were combined with 1-μl template cDNA. PCR for Drd1 and Drd2 was performed as follows: initial 10-min denaturation (94°C), 40 cycles of 30-s denaturation (94°C), 30-s annealing (55°C), and 2-min extension (72°C), followed by a 10 min after elongation (72°C). PCR for Drd3, Drd4, and Drd5 used the same steps, but included a “touch-up” annealing temperature with 0.1°C increment per cycle for the first 20 cycles (55–58°C, shown in Table 1), followed by 30 cycles at the highest annealing temperature (57–60°C). PCR for Drd3 and Drd5 used a “nested” technique in which PCR product from an initial reaction was used as template in a subsequent reaction with the same protocol and different primers. Table 1 shows the annealing temperature of the 40 cycles (Drd1, Drd2) and the annealing temperature range of the 20 cycles and the annealing temperature of the subsequent 30 cycles (Drd3, Drd4, Drd5) and the product size for each primer combinations. Amplified products were run on a 1.5% agarose gel. Specific bands of the predicted size were observed in control brain, whereas no bands were seen in water.

### Table 1: Primer sequences

| Gene (NCBI/GenBank reference sequence) | Primers sequences (5′→3′) | Annealing temperature | Product size |
|----------------------------------------|--------------------------|-----------------------|--------------|
| D1 dopamine receptor (Drd1) (NM_001291801.1, NM_010076.3) | F: GTACCATCAAGTCCCCCTCGG R: CAGGCCCTCTCTTGATCTATC | 55°C (40×) | 120 bp |
| D2 dopamine receptor (Drd2) (XM_006509998.3) | F: GCTGAATATGGAGTTGATAA R: CCAGACCCAAATGTTACAGCA | 55°C (40×) | 145 bp |
| D3 dopamine receptor (Drd3) (NM_007877.2, XM_006521777.2) *Nested: F2/R2 | F1: GGCTTCTCTGTGTTGGGC R1: AAGTGGGTAAAGGGAATGTCTC 57.5°C → 59.5°C (20×, +0.1°C/cycle) + 59.5°C (30×) | 228 bp |
| D4 dopamine receptor (Drd4) (XM_006536156.3, XM_017321972.1) | F: TCTCTGAGAGTTGGAAAAGT R: GCCAGAAAAAAGACAAAAA 55°C → 57°C (20×, +0.1°C/cycle) + 57°C (30×) | 284 bp |
| D5 dopamine receptor (Drd5) (NM_013503.3) *Nested: F1/R2 | F1: GAGTACGGTGAAATGTCTTTAT R1: GAGTACGGTGAAATGTCTTTAT R2: CGGCTGTTCAGAAGACTCATAA 55°C → 57°C (20×, +0.1°C/cycle) + 57°C (30×) | 245 bp |

### Calcium imaging

Calcium imaging recordings were performed on explants between 6 and 11 d in vitro (d) as previously described (Klenke and Taylor-Burds, 2012; Fig. 1A,B). Briefly, cells were loaded with 13.5 μM Calcium Green-1 for 20 min at 37°C in a 5% CO₂ humidified incubator, then washed for 20 min in fresh SFM. After loading, explants were mounted into a perfusion chamber (Warner Instruments) and continuously perfused with SFM at a rate of 300 μl/min using a peristaltic pump (Instech). All experiments had a control period (SFM; 5 min), amino acid blocking period (AAB; 5 min), one or two drug treatment periods (5 or 10 min each), and a final washout period (SFM; 5 min). Time-lapse images piloted by imaging software (BioVision) were taken of cells every 2 s for up to 30 min. Images were obtained through a 20× fluorescence objective using an inverted Nikon microscope and a charge-coupled device camera (QImaging) connected to a computer. Excitation wavelengths of 465-495 nm were provided through a medium-width excitation bandpass filter, and emission was monitored through a 40-nm bandpass centered on 535 nm. All recordings were completed with a 40 mV KCl stimulation to ensure viability of cells. Changes in levels of gray over time [optical density (OD), arbitrary unit] were quantified in single neurons a posteriori with iVision and calcium oscillations, reflecting neuronal activity (Constantin and Wray, 2008), detected with MATLAB (MathWorks). The frequency of oscillations was expressed in peaks per minute. The phenotype of cells included in the results was confirmed by immunocytochemistry using anti-GnRH primary antibody previously described (Wray et al., 1989).

### Electrophysiology

Loose patch clamp was performed on GnRH neurons from acute brain slices. Since sex hormone (estradiol, progesterone) fluctuations during the estrous cycle in females are known to influence GnRH neuron excitability (Liu and Herbison, 2011; Farkas et al., 2013; Silveira et al., 2017; Adams et al., 2018), male mice were used for slice recording (Lee et al., 2012; Herde et al., 2013; Constantin and Wray, 2018; Constantin et al., 2021a, b).

GnRH-GFP mice (MGI:6158458; Spergel et al., 1999) were killed at ~10:30 A.M. by cervical dislocation then decapitated. The brain was glued to a vibratome plate and submersed into ice-cold low [Ca²⁺]/high [Mg²⁺] (0.5/6
mM, respectively) artificial CSF (aCSF), bubbled with 95% O2/5% CO2. After vibratome sectioning (Leica VT1000S), coronal 200-μm slices were incubated at 30°C in normal aCSF containing the following: 118 mM NaCl, 3 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES, 25 mM NaHCO3, and 11 mM D-glucose (pH 7.3), bubbled with 95% O2/5% CO2.

Slices were transferred into a recording chamber mounted on an upright microscope (Nikon Eclipse FN1) and continuously perfused with oxygenated normal aCSF maintained at 28–30°C, at a rate of ~2 ml/min (Constantin et al., 2012). GnRH neurons were identified in slices under fluorescence (20-nm narrow bandpass EGFP filter centered at 480 nm) using a 40× water immersion objective.

**Figure 1.** GnRH neurons respond to dopamine. **A**, GnRH neurons obtained from an E11.5 mouse and maintained in culture for 7 d recorded for calcium imaging. GnRH cells (arrows) were identified under brightfield by their bipolar morphology (left panel), recorded after loading with Calcium Green 1-AM (middle panel), and then stained for GnRH after recording (right panel). Scale bar: 50 μm. **B**, Representative calcium imaging recording of two single GnRH neurons showing spontaneous intracellular calcium oscillations (peaks) in SFM. Recordings were terminated with 40 mM KCl to ensure viability of cells. y-axis, arbitrary OD units; x-axis, 2 min. **C**, Analysis of single GnRH neuron cDNA libraries show all five dopamine receptors, dopamine D1-like receptors (D1 and D5) and dopamine D2-like dopamine receptors (D2, D3, and D4), within the GnRH neuronal population. GnRHsc, GnRH single cells; Br, brain; H20, water. **D**, Representative calcium imaging recordings of two GnRH neurons in AAB (20 μM BIC, 10 μM CNQX, 10 μM AP5) plus dopamine (1 μM). The rate of spontaneous calcium oscillations in GnRH neurons was reduced after blocking the GABAergic and glutamatergic excitatory inputs, then further inhibited with dopamine, indicating a direct effect of dopamine on GnRH neuronal activity. Bar graph (mean ± SEM) indicates the quantification in peaks/min from all cells (n = 162, N = 6). Nonparametric Friedman tests, followed by Dunn’s multiple comparisons test with significance shown using GraphPad style: *0.01 < p < 0.05, **0.001 < p < 0.01, ***0.0001 < p < 0.001, ****p < 0.0001. Beeswarm plot indicates the spread of individual cell changes in the frequency of calcium oscillations in response to dopamine (DA) compared with the spread of individual cell spontaneous changes when maintained in AAB (mean ± SD). The Δ values are calculated at the drug switch highlighted on the bar graph. Nonparametric Kruskal–Wallis, followed by Dunn’s multiple comparisons test, was used with DA as the reference.
Table 2: Antibody table

| Peptide/protein target | Name of antibody | Manufacturer, catalog number, and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used |
|------------------------|------------------|---------------------------------------------------------------------------------|---------------------------------|--------------|
| rbGnRH                 | SW-1             | S. Wray                                                                          | Rabbit; polyclonal              | 1:15,000 (in vivo) |
|                        |                  |                                                                                  |                                 | 1:5000 (in vitro)       |
| mGnRH                  | F1D3C5 + SMI-41  | A. Karande + Abcam                                                                | Mouse; monoclonal               | 1:4000         |
|                        |                  |                                                                                  |                                 | +1:6000         |
| GFP                    | Anti-GFP         | Abcam, ab92456                                                                   | Chicken; polyclonal             | 1:2000         |
| Dopamine receptor D4   | Anti-D4          | Abcam, ab135978                                                                  | Rabbit; polyclonal              | 1:24,000       |
| Dopamine receptor D1   | clone 1–1–F11    | A. Buonanno                                                                      | Rabbit; polyclonal              | 2 µg/ml         |
|                        |                  |                                                                                  | Rat; monoclonal                 | 1:100          |

Analysis

For calcium imaging, nonparametric Friedman tests, followed by Dunn’s multiple comparisons test, were used to compare peaks/min of cells between treatment periods of an experiment (Fig. 1D, bar plot). The magnitude of the effect evoked by an agonist was calculated for each cell as the difference (δ) between the frequency of calcium oscillations during the period before the agonist and the frequency of calcium oscillations during the period with agonist. Nonparametric Mann–Whitney rank tests were used to compare δ between two paradigms only and non-parametric Kruskall–Wallis tests, followed by Dunn’s multiple comparisons test, were used to compare δ of the effects between more than two paradigms. A response, inhibition or excitation, to a pharmacological challenge was defined as δ < −1 or δ > 1 peak/min, respectively.

For electrophysiology, action potentials (APs) were detected with Clampfit 10 on continuous recordings. The average firing frequency was calculated over the last 3 min of two consecutive recording periods. The firing rate during the second period (dopamine application) was normalized to the firing rate during the first period and expressed as a percentage. A response to dopamine was defined as a change in firing rate >20%. Individual cell changes in firing between two consecutive treatments were analyzed with paired t test. Changes in firing rate between two paradigms were analyzed with unpaired t test. Significance was determined by p < 0.05, and data are presented as mean ± SEM (n and N, representing the number of cells and animals used, respectively).

Drugs

(-)-Bicuculline methochloride (BIC; GABAA receptor antagonist), D-(-)-2-amino-5-phosphonopentanoic acid (AP5; NMDA glutamatergic receptor antagonist), CNQX disodium salt (AMPA/kainate receptor antagonist), A 68930 hydrochloride (D1/5R agonist), SCH-23390 hydrochloride (D1/5R agonist), (S)-(-)-Sulpiride (D2/D3R antagonist), A 412997 dihydrochloride (D4R agonist), L-745870 trihydrochloride, and PD 168568 dihydrochloride (D4R antagonist) and (−)-quinpirole hydrochloride (D2-like receptor agonist) were obtained from Tocris.

Cholera toxin (CTX; Gαs protein uncoupling agent) and pertussis toxin (PTX; Gαi/o protein uncoupling agent) were obtained from Sigma-Aldrich. Dopamine hydrochloride was purchased from Sigma-Aldrich. (−)-Quinpirole hydrochloride (Sigma-Aldrich) and spiperone (D2-like receptor antagonist; Sigma-Aldrich) were generously provided by David Sibley (National Institutes of Health, National Institute of Neurological Disorders and Stroke). All stock solutions were aliquoted and stored at −20°C in either DMSO or distilled water, and solutions were prepared immediately before each experiment by diluting 1:1000 stock into SFM to minimize oxidation. DMSO (up to 1:500) is known to have no effect on the frequency of calcium oscillations (Constantin et al., 2009). All drugs were applied by perfusion to the explants during imaging except CTX and PTX which were applied for >4 h before recording.

Immunocytochemistry

After calcium imaging, explants (6–11 d) were fixed for 30 min with 0.1 M PBS pH 7.4 containing 4% formaldehyde at room temperature. After a few washes in PBS, explants were incubated in a blocking solution (10% normal horse serum + 0.3% Triton X-100 + 0.1% NaAzide) for 1 h and washed several times in PBS. The explants were incubated at 4°C overnight in the primary antibody (in PBS with 1% BSA + 0.1% NaAzide; SW-1, Wray et al., 1989; Table 2). The next day, explants were washed in PBS, incubated for 1 h with biotinylated secondary donkey anti-rabbit antibody (1:500 in PBS/0.3% Triton X-100; Vector Laboratories, Inc), washed in PBS, and processed for avidin-biotin horseradish peroxidase/3,3′-diaminobenzidine (Fig. 1A).

Immunofluorescence

Primary antibodies are listed in the antibody table (Table 2).

Explants were fixed for 1 h in 4% formaldehyde. After washing in PBS, explants were incubated in blocking
solution (1 h; 10% normal horse serum + 0.3% Triton X-100), washed in PBS, and then incubated in primary antibody (anti-D4 or anti-D1; 2 nights at 4°C). The next day, explants were washed in PBS, incubated in either Alexa Fluor 555-conjugated secondary anti-rabbit antibody for anti-D4 antibody (1 h; 1:1000; Life Technologies) or biotinylated secondary anti-mouse or anti-rabbit antibody, for anti-D4 clone 2B9 and H/L/420 AP antibody, respectively, or biotinylated secondary anti-rat antibody for anti-D1 antibody (1 h; 1:500 + 0.3% Triton X-100; Vector Laboratories, Inc.). Explants treated with a biotinylated secondary antibody were washed then incubated with Alexa Fluor 555-conjugated avidin (1 h; 1:1000; Life Technologies). After few washes in PBS, explants were briefly fixed, washed, and incubated in a second primary antibody raised in different species (anti-GnRH; one to two nights at 4°C). The next day explants were washed, treated with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit antibody (1 h; 1:1000; Invitrogen) for anti-GnRH F1D3C5 + SMI-41 or SW-1 antibody, respectively, washed in PBS, and coveredslipped with an antifade mounting solution (Electron Microscopy Sciences). No anti-D4R primary antibody controls were run for each antibody to determine non-specific background staining on GnRH-labeled neurons.

**Results**

All dopamine receptors are detected in prenatal GnRH neurons

The relevance of prenatal GnRH neurons is often questioned when assessing the physiology of adult GnRH neurons. Therefore, 48 genes previously detected in adult GnRH neurons (Todman et al., 2005; Burger et al., 2018) were compared with the microarray dataset obtained from GnRH cells in explants (Table 3). We also included transcripts for 6 genes previously detected by single-cell PCR of GnRH neurons in explants (Sharifi et al., 2002; Klenke et al., 2010; Constantin et al., 2009, 2016; Constantin and Wray, 2018).

The RMA values for transcripts for Drd1, Drd2, Drd3, and Drd4 dopamine receptors ranged between 2.71 and 6.34. Compared with the gene expression values from the probe set Drd1_629, gene expression values for both probe sets for Drd4 were higher, while gene expression values for Drd2 and Drd3 were lower. Analysis of cDNAs generated from single GnRH neurons (n = 5–7) demonstrated that transcripts for each dopamine receptor are present in a subset of GnRH neurons (Fig. 1C).

Exogenous dopamine directly reduces GnRH neuron activity

Exogenous dopamine (1 µM) was applied to explants and changes in frequency of intracellular calcium in GnRH neurons were determined using calcium imaging. Since GABAergic and glutamatergic inputs to GnRH neurons are robust in explants (Constantin et al., 2010), excitatory inputs were blocked by treatment with AABs (20 µM BIC, 10 µM CNQX, 10 µM AP5). After a control period (SFM 5 min, followed by AAB 5 min), dopamine was added (Fig. 1D, top). Dopamine decreased the mean frequency of calcium oscillations in GnRH neurons, indicating mainly inhibition (Fig. 1D, bottom left). Using ± 1 peaks/min as cutoff values in changes in the frequency of calcium oscillations, ~25% of the GnRH cells were inhibited by dopamine (δ < −1) while ~5% were excited (δ > 1; Fig. 1D, bottom right).

The dopamine D2-like receptor, D4R, is required for the dopamine inhibition

Canonically, the D1-like subfamily of dopamine receptors couple to the stimulatory G<sub>α</sub> protein subunit while the D2-like subfamily couple to the inhibitory G<sub>i/o</sub> protein subunit. Thus, the decrease in GnRH neuronal activity after exposure to dopamine suggested the activation of D2-like receptors. Consistent with this observation, dopamine-mediated inhibition persisted in presence of the D1-like receptor antagonist SCH-23390 (10 nM; Fig. 2A, traces) and the magnitude of the dopamine inhibition, with or without SCH-23390, were the same (Fig. 2A, beeswarm plot) suggesting a minimal role of D1-like receptors, if any. Conversely, dopamine-mediated inhibition was abolished in presence of D2-like receptor antagonist spiperone (50 nM; Fig. 2B, beeswarm plot). Notably, while the numbers of cells showing inhibition decreased (~25% down to ~8%) by spiperone + dopamine (Fig. 2B, top trace), the numbers of cells showing excitation increased (~5% up to ~16%; Fig. 2B, bottom trace). Consistent with the SCH-23390/dopamine experiment, the D2-like receptor agonist quinpirole (50 nM) decreased the frequency of calcium oscillations in GnRH neurons (Fig. 2C, traces) and the magnitudes of the quinpirole and dopamine inhibition were the same (Fig. 2C, beeswarm plot).

Since the three members of the D2-like receptor family were found by PCR, we used pharmacology to determine which receptor(s) transduced the dopamine-mediated inhibition. In presence of the D2/3R antagonist, sulpiride (20 nM) and SCH-23390 (D1/5R antagonist), dopamine still inhibited the activity of GnRH neurons (Fig. 3A, traces) with the same magnitude as dopamine alone (Fig. 3A, beeswarm plot), indicating a major role for D4R in the inhibition. Accordingly, the D4R agonist A 412997 (50 nM) alone decreased the frequency of calcium oscillations in GnRH neurons (Fig. 3B, traces) while approximately about 14% of the GnRH cells were inhibited by A 412997 while ~1% were excited. The magnitude of the inhibition with A 412997 was similar to the one with dopamine (Fig. 3B, beeswarm plot). To ensure the subtype specificity of A 412997, the D4R antagonists L-745870 (100 nM) was co-applied with A 412997 and the inhibition was prevented (Fig. 3C, traces and beeswarm plot). Finally, to test the role of D2/3R in the dopamine-mediated inhibition, quinpirole (D2-like agonist) was applied in presence of another D4R antagonist PD 168568 (50 nM), also effective on the A 412997-mediated inhibition, to assess the contribution of D4R in the D2-like-mediated inhibition. The inhibition was partially prevented by the D4R antagonist (Fig. 3D, traces and beeswarm plot). Together, the data indicate that dopaminergic inhibition of GnRH neurons required the activation of D4R.
The dopamine D1-like receptor activates GnRH neuronal activity

The D1-like receptor agonist A 68930 (10 nM) was applied to test the function of D1/5R. The frequency of calcium oscillations in GnRH neurons increased (Fig. 4A, traces). No GnRH cells were inhibited by A 68930 while ~21% were excited (Fig. 4A, beeswarm plot). The co-application of A 68930 and SCH-23390 (D1/5R antagonist) prevented the increase of GnRH neuronal activity (Fig. 4B, traces and beeswarm plot) but co-application of A 68930 and L-745870 (D4R antagonist) did not (Fig. 4C, traces and beeswarm plot), validating a D1/5R-mediated excitation. These data indicate that dopaminergic inhibition of GnRH neurons did not require D1/5R activation and was entirely mediated through D2-like receptors, specifically D4R.

D4R triggered G\(\alpha_{i/o}\) protein signaling while D1-like receptors triggered G\(\alpha_s\) protein signaling

Canonically, the D2-like subfamily of dopamine receptors couples to the inhibitory G\(\alpha_{i/o}\) protein subunit (Beaulieu et al., 2015). Explants were treated with PTX (250 ng/ml) for >4 h to uncouple G\(\alpha_{i/o}\) proteins from their receptors. The D4R was selectively activated using a cocktail of subtype specific antagonists (SCH-23390 for D1/5R and sulpiride for D2/3R) with dopamine. Compared with previous observation, the PTX...
pretreatment prevented the dopamine inhibition (Fig. 5A, beeswarm plot), confirming the Goi/o-mediated inhibition. Since the D1-like subfamily of dopamine receptors couples to the excitatory Go protein subunit (Beaulieu et al., 2015), another group of explants were treated with CTX (10 ng/ml) for >4 h to uncouple the Go protein from their receptors. Compared with previous observation, CTX pretreatment prevented the excitatory effect of the D1-like receptor agonist A68930 (Fig. 5B, beeswarm plot), supporting Go-mediated excitation.
The dopamine D4R is present in GnRH neurons

To support pharmacological data of D4Rs inhibiting GnRH neuronal activity, three different antibodies raised against the D4R were used and confirmed in single plan confocal images, that GnRH neurons in explants express the protein (Fig. 6A). The presence of D1R was also confirmed with immunostaining (Fig. 6B). No staining was present when the primary antibodies were omitted (Fig. 6C, nonconfocal image).

The dopamine D4R is functional in adult GnRH neurons from acute brain slices

D4R activation was next analyzed in GnRH cells in adult brain slices. Similar to that shown in Figure 3A for GnRH cells maintained in explants, blocking D1/5R and D2/3R with SCH-23390 (S; 5 μM) and sulpiride (Su; 5 μM), respectively, then applying dopamine (5 μM; Fig. 7A) resulted in inhibition of neuronal activity in GnRH cells in brain slices. The effect of dopamine, with D1/5 and D2/3R antagonism, was compared with the effect of dopamine alone (Fig. 7B). Six out of 8 cells (six animals) showed inhibition with dopamine after D1/5R and D2/3R antagonists. The firing rate went from 0.70 ± 0.25 to 0.26 ± 0.12 Hz. Four out of five cells (four animals) showed inhibition with dopamine alone. The firing rate went from 0.37 ± 0.12 to 0.091 ± 0.07 Hz (Fig. 7C). The degree of inhibition was similar with or without D1/5R and D2/3R antagonists (Fig. 7D).

Figure 4. Dopamine D1-like receptor activation increases the activity of GnRH neurons. A–C, Representative calcium imaging recordings (left, OD units) and calculated Δ values for all GnRH cells in that experimental group in beeswarm plot (mean ± SD; right). A, Contrary to the inhibitory effect of dopamine, application of a D1-like receptor agonist (A 68930 1 nM) increased the rate of spontaneous calcium oscillations in GnRH neurons (n = 86, N = 3). B, The stimulatory effect of A 68930 was blocked by the D1-like receptor antagonist (SCH-23390 50 nM), demonstrating the specificity of A 68930 (n = 77, N = 3). C, Blocking the D4R (L-745870 100 nM) did not prevent the excitatory effect of SCH-23390 (n = 77, N = 3). Beeswarm plot indicates the spread of individual cell changes in the frequency of calcium oscillations in response to A 68930 compared with the spread of individual cell changes when maintained in AAB or challenged with antagonist/A 68930. Nonparametric Kruskall–Wallis, followed by Dunn’s multiple comparisons test, was used with A 68930 as the reference.
Discussion

This report establishes the presence of D4R in GnRH neurons, using RT-PCR and immunocytochemistry, and its role in the dopamine-mediated inhibition of GnRH neurons, using calcium imaging and electrophysiology. Calcium imaging experiments revealed that D4R-mediated inhibition occurs downstream of G_{a_{i/o}} protein. In contrast, activation of D1-like receptors leads to an excitation that requires the G_{a_s} protein. Notably, although activation of D1-like or D2-like receptor subfamilies with specific agonists consistently produced either activation or inhibition of GnRH neurons, respectively, the net effect of dopamine was always inhibitory, suggesting mainly activation of D2-like receptors. However, the exact mechanism of the action of dopamine requires caution. Although the canonical pathway of D1-like receptors is excitatory (Pivonello et al., 2007; Podda et al., 2010), the activation of D1-like receptor can be permissive, whereby D1 receptor activation appears necessary for D2 receptor-mediated inhibition (White, 1987; Wachtel et al., 1989).

However, in the system used here, dopamine-mediated inhibition persisted in the presence of the D1-like receptor antagonist SCH-23390 and the magnitudes of the dopamine inhibition, with or without SCH-23390, were the same suggesting a minimal role of D1-like receptors, if any. Furthermore, as many G-protein-coupled receptors, dopamine receptors can exist as monomers, homodimers and heterodimers and each assembly possesses specific binding and coupling profiles according to occupancy, offering tissue-specific responses (for review, see Maggio et al., 2009; Van Craenenbroeck et al., 2011; Perreault et al., 2014). In our data, the D1-like receptor agonist produced only excitation while dopamine with D2-like receptor antagonist produced both inhibition and excitation, leading to a lack of net effect. The simplest explanation for the latter might be greater binding of dopamine for D2-like receptors and/or a more efficient signaling pathway downstream of these receptors (Schoffelmeer et al.,

Figure 5. The D1-like and D2-like subfamilies have opposite effects on GnRH neuronal activity via different signaling pathways. A, B, Calculated δ values for all GnRH cells in that experimental group in beeswarm plot (mean ± SD). Control experiments (right), perturbation experiments (left). Control from Figure 3A in A showing D4R activation [dopamine (1 μM) + D1/5R antagonist (SCH-23390 50 nM) + D2/3R antagonist (sulpiride 20 nM)] and control from Figure 4A in B showing D1-like receptor activation [D1/5R agonist (A 68930 1 nM)]. A, D4R activation failed to inhibit GnRH neurons incubated in PTX (>4 h, 250 ng/ml, wrench symbolizes uncoupling G_{a_{i/o}}), demonstrating G_{a_{i/o}}-mediated D4R inhibition (n = 84, N = 3). B, D1/5R activation failed to excite GnRH neurons incubated in CTX (>4 h, 10 ng/ml, wrench symbolizes uncoupling G_{a_s}, demonstrating G_{a_s}-mediated D1/5R inhibition (n = 70, N = 3). Beeswarm plot indicates the spread of individual cell changes in the frequency of calcium oscillations in response to sulpiride+SCH-23390/DA or A 68930 compared with the spread of individual cell changes when pretreated with PTX or CTX, respectively. Nonparametric Mann–Whitney rank test was used to compare two samples (A). Nonparametric Kruskall–Wallis, followed by Dunn’s multiple comparisons test, was used with A 68930 as the reference (B).

Figure 6. GnRH neurons are immunoreactive for dopamine D4 receptor and dopamine D1 receptor. A, Single confocal plans showing GnRH neurons (green), colabeled with D4R (red; scale bar: 10 μm) in one-week-old explants (N = 3), using three different primary antibodies. B, Single confocal plans showing GnRH neurons (green), colabeled with D1R (red; scale bar: 10 μm) in one-week-old explants (N = 3). C, Example of control for no primary antibody 2B9 (left) versus primary antibody 2B9 (right) during double label immunofluorescent staining (images from conventional fluorescent microscope, 40×; scale bar: 20 μm).
The complexity of dimerization might rationalize the “apparent” inhibition driven by D1-like receptors in mice, especially in the cells where the inhibition was equally sensitive to raclopride and SCH-23390 (Liu and Herbison, 2013). In addition, the D2-like receptor mediated inhibition was identified as dependent on activation of D4R and a G<sub>ai/o</sub> downstream signaling pathway. This observation provides another explanation to the D1-like receptor driven inhibition in mice. Raclopride is ineffective on D4R (Boy et al., 1998) but SCH-23390 would likely inhibit D1-like+D4R dimers, accounting for the majority of cells reported whose inhibition was sensitive to SCH-23390, but not to raclopride (Liu and Herbison, 2013). Consistent with this, both transcript and protein for D4R were found in GnRH cells. Inhibition of GnRH cells via dopamine D4R is supported by the fact that this receptor, along with the D3R, are downregulated during proestrus (Vastagh et al., 2016), and that it canonically couples to G<sub>ai/o</sub> protein inwardly rectifying potassium channels (Werner et al., 1996; Inanobe et al., 1999; Wedemeyer et al., 2007), effective inhibitors of GnRH neuronal activity (Klenke et al., 2010; Constantin and Wray, 2016, 2018).

Notably, the D2/3R have been commonly linked to reproductive function. For example, a reduction in the dopaminergic tone and the expression of D2R are observed in women suffering from polycystic ovary syndrome (Chaudhari et al., 2018) and the secretion of luteinizing hormone (LH) is sensitive to D2/3R antagonist in prepubertal females (Lacau de Mengido et al., 1987). However, since discriminative pharmacological tools are recent, the role of the D4R might have been underestimated. For example, D2R+D4R is a functional heteromer that would likely be antagonized by D2/3R antagonist. PCR analysis of cDNA libraries from GnRH neurons support the expression of multiple dopamine receptor subtypes. While the pharmacological data suggest a prominent role of D4R in the control of GnRH neuronal activity by dopamine, the role of the other receptors in the control of GnRH/LH secretion cannot be excluded, especially with dimerization and transactivation mechanisms.

In vivo, dopaminergic inputs could regulate GnRH neuronal activity since they have been found around their vicinity.
| Affymetrix probe set ID | Gene name          | Full gene name | RMA value | SEM  | P value | Adult brain slice | Prenatal explant |
|------------------------|-------------------|----------------|-----------|------|---------|-------------------|------------------|
| 1418950_at             | Drd2              | Dopamine receptor 2 | 3.71      | 0.15 | 0.0220  | PMID: 15837132,  | PMID: 29522155   |
| 1422278_at             | Drd3              | Dopamine receptor 3 | 2.71      | 0.21 | 0.0371  | MQTT: 12072381   | PMID: 29522155   |
| 1422829_at             | Drd4              | Dopamine receptor 4 | 6.34      | 0.21 | 0.0067  | MQTT: 15837132   | PMID: 29522155   |
| 1422830_s_at           | Drd4              | Dopamine receptor 4 | 5.42      | 0.07 | 0.0019  | MQTT: 15837132   | PMID: 29522155   |
| 1455629_at             | Drd1a             | Dopamine receptor D1A | 4.30     | 0.09 | Ref     | PMID: 29522155   | PMID: 29522155   |
| 1456051_at             | Drd1a             | Dopamine receptor D1A | 4.31      | 0.20 | >0.9999 | PMID: 29522155   | PMID: 29522155   |
| 1450493_at             | Kiss1r            | Kiss1 receptor   | 7.39      | 0.22 |         | PMID: 29522155   | PMID: 27359210   |
| 1422099_s_at           | Oprf              | Opioid receptor-like 1 | 4.21     | 0.27 |         | PMID: 30627649   | PMID: 29522155   |
| 1426103_at             | Esr2              | Estrogen receptor 2 (β) | 5.91      | 0.09 |         | PMID: 29522155   | PMID: 12072381   |
| 1422256_at             | Sst2              | Somatostatin receptor 2 | 4.23      | 0.12 |         | PMID: 15837132   | PMID: 29522155   |
| 1441603_at             | Sst3              | Somatostatin receptor 3 | 5.03      | 0.26 |         | PMID: 15837132   | PMID: 29522155   |
| 1422811_at             | Sstr4             | Somatostatin receptor 4 | 4.19      | 0.21 |         | PMID: 15837132   | PMID: 29522155   |
| 1448572_at             | Thhr              | Thyrotropin-releasing hormone receptor | 3.34 | 0.10 |         | PMID: 15837132   | PMID: 15837132   |
| 1418810_at             | Ctrh1             | Corticotropin-releasing hormone receptor 1 | 4.96 | 0.06 |         | PMID: 15837132   | PMID: 15837132   |
| 1422204_at             | Avpr1b            | Arginine vasopressin receptor 1B | 5.05 | 0.28 |         | PMID: 15837132   | PMID: 29522155   |
| 1427704_at             | Galr1             | Galanin receptor 1 | 3.67      | 0.18 |         | PMID: 15837132   | PMID: 29522155   |
| 1422942_at             | Galr2             | Galanin receptor 2 | 3.39      | 0.03 |         | PMID: 15837132   | PMID: 15837132   |
| 1426054_at             | Npy1r             | Neuropeptide Y receptor Y1 | 2.93 | 0.08 |         | PMID: 29522155   | PMID: 20351316   |
| 1417489_at             | Npy2r             | Neuropeptide Y receptor Y2 | 2.16 | 0.08 |         | PMID: 15837132   | PMID: 29522155   |
| 1422342_at             | Nmbr              | Neuromedin B receptor | 3.78      | 0.11 |         | PMID: 15837132   | PMID: 15837132   |
| 1450260_at             | Grpr              | Gastrin releasing peptide receptor | 3.08 | 0.17 |         | PMID: 15837132   | PMID: 15837132   |
| 1422265_at             | Bsr3              | Bombesin-like receptor 3 | 3.72 | 0.13 |         | PMID: 15837132   | PMID: 15837132   |
| 1421667_at             | Nmur1             | Neuromedin U receptor 1 | 5.66 | 0.12 |         | PMID: 15837132   | PMID: 15837132   |
| 1422121_at             | Oprd1             | Opioid receptor, δ 1 | 5.15 | 0.28 |         | PMID: 15837132   | PMID: 15837132   |
| 1417151_at             | Ntr2              | Neurotensin receptor 2 | 4.87 | 0.10 |         | PMID: 15837132   | PMID: 15837132   |
| 1450278_at             | Tacr3             | Tachykinin receptor 3 | 4.27 | 0.11 |         | PMID: 15837132   | PMID: 15837132   |
| 1422263_at             | Bdkrb2            | Bradykinin receptor, β 2 | 5.56 | 0.09 |         | PMID: 15837132   | PMID: 15837132   |
| 1449160_at             | Npr1              | Natriuretic peptide receptor 1 | 4.28 | 0.17 |         | PMID: 15837132   | PMID: 15837132   |
| 1427191_at             | Npr2              | Natriuretic peptide receptor 2 | 5.01 | 0.24 |         | PMID: 15837132   | PMID: 15837132   |
| 1457003_at             | Gria1             | Glu receptor, ionotropic, AMPA1, α 1 | 5.35 | 0.07 |         | PMID: 15837132   | PMID: 15837132   |
| 1421530_s_at           | Gria2             | Glu receptor, ionotropic, AMPA2, α 2 | 5.42 | 0.16 |         | PMID: 15837132   | PMID: 15837132   |
| 1422265_at             | Chrnb1            | Cholinergic receptor, nicotinic, β 1 | 2.43 | 0.10 |         | PMID: 15837132   | PMID: 15837132   |
| 1422265_at             | Chrnb2            | Cholinergic receptor, nicotinic, β 2 | 5.16 | 0.04 |         | PMID: 15837132   | PMID: 15837132   |
| 1449322_at             | Chnrg             | Cholinergic receptor, nicotinic, γ | 6.54 | 0.15 |         | PMID: 15837132   | PMID: 15837132   |
soma (Jennes et al., 1983; Leranth et al., 1988), i.e., near the site of initiation of APs (Iremonger and Herbison, 2012). In addition, dopamine may regulate GnRH secretion since GnRH neuron nerve terminals also receive dopaminergic inputs (Jennes et al., 1983; Kuljis and Advis, 1989). Dopaminergic receptors have been shown to exhibit a subcellular regionalization (Levey et al., 1993; Hersch et al., 1995). Thus, excitatory D1-like receptors located on GnRH neuron nerve terminals (Fuxe et al., 1988) could be responsible for dopamine-stimulated GnRH secretion (Rasmussen et al., 1986), while D4R located on GnRH cell soma could be responsible for dopamine-inhibited GnRH secretion (Tasaka et al., 1985). Although not exclusively associated with dopaminergic neurotransmission, dopamine-regulated and cAMP-regulated neuronal phosphoprotein, a major convergent point of signaling pathways, has been detected in GnRH nerve terminals (Meister et al., 1988). Finally, indirect effects of dopamine on GnRH secretion cannot be ruled out either (Rotsztejn et al., 1976; Jarjour et al., 1986; James et al., 1987).

In addition to the complexity of GnRH neuron morphology and the dimerization of dopamine receptors, growing evidence supports the physiological role of dopamine receptor heterodimers with other receptors (for review, see Beaulieu et al., 2015). The D4R is structurally a unique receptor, exhibiting a unique third intracellular loop favoring interactions with multiple signaling proteins (Woods, 2010). Notably, exon III which encodes the third loop of the D4R is highly polymorphic (Lichter et al., 1993) and determines signaling efficiency (Rondou et al., 2010). Consequently, D4R is an important player of neuromodulation [α1 and β1 adrenergic receptors (González et al., 2012), glutamate receptor (Price and Pittman, 2001), GABA_α receptor (Wang et al., 2002; Azdad et al., 2003; Shin et al., 2003; Graziane et al., 2009; Gasca-Martinez et al., 2010), NMDA receptor (Wang et al., 2003; Andersson et al., 2012), AMPA receptor (Gu et al., 2006; Yuen et al., 2010; Yuen and Yan, 2011)] possibly via Ca^2+/-calmodulin-dependent protein kinase II mechanism (Gu et al., 2006; Yuen et al., 2010; Yuen and Yan, 2011) and/or interfering with receptor trafficking (Graziane et al., 2009; Yuen and Yan, 2009, 2011; Yuen et al., 2010). Consistent with D4R being only a neuromodulator of fertility, mice lacking D4R exhibit behavioral changes (Thanos et al., 2015) but normal fertility (Rubinstein et al., 1997), similar to mice whose kisspeptin neurons exhibit TH deletion (Stephens et al., 2017). Kisspeptin/TH neurons seem to provide a sex-specific pheromonal input to GnRH neurons (Taziax and Bakker, 2015). Notably, in human, the exon III polymorphism of D4R is linked to the etiology of attention-deficit/hyperactivity disorder (Leung et al., 2017) but has also been linked to normal behaviors such as higher novelty-seeking (Ebstein et al., 1996) and greater sex-specific affective knowledge (Ben-Israel et al., 2015).

Thus, although many nuances remain unclear on the overall action of dopamine and its receptors on reproductive function, our data confirm dopamine as a robust inhibitor of GnRH neuronal activity and pinpoints the D4R as the main integrator of dopamine signal to GnRH neurons, via the activation of a G_s/o protein-dependent signaling pathway.

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