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

<https://doi.org/10.1523/ENEURO.0461-21.2022>

Cite as: eNeuro 2022; 10.1523/ENEURO.0461-21.2022

Received: 28 October 2021

Revised: 26 January 2022

Accepted: 2 February 2022

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

Alerts: Sign up at www.eneuro.org/alerts to receive customized email alerts when the fully formatted version of this article is published.

Copyright © 2022 Dairaghi et al.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license, which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

1 **Manuscript Title Page**

2 **1. Manuscript Title (50 word maximum)**

3 The Dopamine D4 receptor regulates gonadotropin-releasing hormone neuron excitability in male mice.

4 **2. Abbreviated Title (50 character maximum)**

5 Dopamine D4 receptor-mediated inhibition of GnRH neurons

6 **3. List all Author Names and Affiliations in order as they would appear in the published article**

7 Leigh Dairaghi^{*1}, Stephanie Constantin^{*1,2}, Andy Oh¹, David Shostak¹ & Susan Wray¹

8 * Equal contribution

9 ¹, Cellular and Developmental Neurobiology Section, National Institute of Neurological Disorders and
10 Stroke/National Institutes of Health, Bethesda, Maryland 20892;

11 ², Current address: Section on Cellular Signaling, Eunice Kennedy Shriver NICHD/National Institutes of
12 Health, Bethesda MD 20892

13 **4. Author Contributions:**

14 LD, SC and SW Designed research, LD, AO and DS Performed research, LD SC and SW Analyzed data; LD,
15 SC and SW Wrote the paper.

16 **5. Correspondence should be addressed to (include email address)**

17 Dr. Susan Wray, Cellular and Developmental Neurobiology Section, National Institute of Neurological
18 Disorders and Stroke, National Institutes of Health, 35 Convent Drive MSC 3703, Building 35, Room
19 3A1012, Bethesda, Maryland 20892. E-mail: wrays@ninds.nih.gov

20 **6. Number of Figures:** 7

9. Number of words for Abstract: 196

21 **7. Number of Tables:** 3

10. Number of words for Significance Statement: 64

22 **8. Number of Multimedia:** 0

11. Number of words for Introduction: 431

23 **12. Number of words for Discussion:** 966

24 **13. Acknowledgements:** Thanks to Dr. David Sibley (NIH, NINDS) for generously providing us with
25 quinpirole and spiperone.

26 **14. Conflict of Interest:** "Authors report no conflict of interest"

27 **15. Funding sources:** This work was supported by the Intramural Research Program of the National
28 Institutes of Health, National Institute of Neurological Disorders and Stroke (ZIA NS002824).

29 **ABSTRACT**

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

47 **SIGNIFICANCE STATEMENT**

48 The convergence of information on neurons secreting gonadotropin-releasing hormone (GnRH)
49 shape their secretory profile and consequently, fertility. Dopamine inhibits GnRH neurons, yet
50 the specific dopamine receptor subtype(s) involved remain unclear. Using single RT-PCR,
51 immunofluorescence, and dopamine receptor-specific pharmacological tools, we show here that

52 D4R plays a role in the dopaminergic inhibition of GnRH neurons, adding a new tool for the
53 modulation of reproductive function.

54 INTRODUCTION

55 Dopamine is a regulator of reproductive function at many levels along the hypothalamic-
56 pituitary-gonadal axis. A female-specific dopaminergic inhibitory tone, observed in developing
57 rats (Becu-Villalobos and Libertun, 1995, p.; Lacau de Mengido et al., 1987), influences the
58 timing of puberty (de Mengido et al., 1989; Gerber et al., 1984; Lamberts and Wuttke, 1981; Ruf
59 and Holmes, 1974). In seasonal breeders, dopamine is a component of anestrus (Lehman et al.,
60 1996; Saxena et al., 2015). Ovarian dopamine receptors regulate ovulation (Venegas-Meneses
61 et al., 2015, p.) and dopaminergic neurons control anterior pituitary function (Gonzalez-Iglesias
62 et al., 2008; Hnasko et al., 2007). In the hypothalamus, tyrosine hydroxylase (TH)-containing
63 fibers contact gonadotropin-releasing hormone (GnRH) neuron soma in the preoptic area and
64 terminals in the median eminence (Jennes et al., 1983; Kuljis and Advis, 1989; Leranth et al.,
65 1988). Both the tubero-infundibular dopaminergic pathway (Leranth et al., 1988; Mitchell et al.,
66 2003) and the anteroventral periventricular nucleus (AVPV) (Horvath et al., 1993) contribute to
67 the TH innervation of GnRH secreting neurons. Notably, the kisspeptin neuron subpopulation in
68 the arcuate nucleus (ARC) acts on the tubero-infundibular dopaminergic pathway (Ribeiro et al.,
69 2015) and the sexually dimorphic kisspeptin neuron subpopulation in the AVPV coexpresses TH
70 (Clarkson and Herbison, 2011)(Simerly et al., 1985), indicating that dopamine may influence
71 GnRH neuronal activity both directly and indirectly. Throughout the brain, dopamine modulates
72 neuronal excitability(Beaulieu et al., 2015). However, dopamine hyperpolarizes GnRH neurons,
73 via the activation of potassium channels (Liu and Herbison, 2013).

74 Dopamine receptors, based on their structural, pharmacological, and signaling
75 properties, are divided into two families of G protein-coupled receptors: D1-like (D1 and D5
76 subtypes) and D2-like (D2, D3, and D4 subtypes) (Beaulieu et al., 2015). D1-like receptors

couple to $G\alpha_s$ protein, through which they activate adenylyl cyclase, inhibit potassium channels and increase neuronal excitability while D2-like receptors couple to $G\alpha_{i/o}$ protein and have the opposite effect (Beaulieu et al., 2015). Consistent with this, in fish, dopamine inhibits GnRH neurons via D2-like receptors (Bryant et al., 2016). However, in mouse, both D1- and D2-like receptors have been reported to contribute to the inhibition (Liu and Herbison, 2013). The current study aimed to identify dopamine receptor subtype(s) regulating GnRH neurons and to elucidate how $G\alpha_s$ protein-coupled D1-like receptors could possibly inhibit GnRH neurons. Our microarray dataset from GnRH neurons in explants revealed a high level of *Drd4* transcripts coding for D4R compared to other dopamine receptors, suggesting a potential preferential role in GnRH neurons. The present results demonstrate that, 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 tyrosine hydroxylase containing neurons, calcium imaging revealed that activation of D1-like receptors only increased GnRH neuronal activity, via G_s protein coupling. In contrast, activation of D2-like receptors, including D4R, decreased GnRH neuronal activity, via $G_{i/o}$ 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% CO₂ humidified incubator. Fresh media containing fluorodeoxyuridine (2.3 μM; Sigma-Aldrich, St. Louis, MO) 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 day 2-3 *in vitro*, GnRH neurons migrate out of the explant and can be recorded in the periphery.

107 ***Single GnRH cell isolation & microarray data***

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

111 cDNA libraires from 9 individual GnRH neurons 10 div (see below) were randomly
112 grouped into three samples. This material was amplified, processed for microarray data
113 generation and analysis. All cDNA were labeled and hybridized by the DIRP NIH microarray
114 core facility with GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA).
115 Custom R scripts, including covariance-based PCA, correlation heat maps, LOWESS analysis,
116 and clustering checked the dataset for noise and outliers. Normalization, i.e. the average
117 expression of genes, was performed identically throughout the dataset and log-transformed.
118 Gene expression values were calculated using Robust Multi-array Average (RMA) procedure
119 and compared with repeated-measurement one-way ANOVA, using data from one probe set
120 (Drd1_629) as a reference.

121 ***PCR on single cells and explants***

122 Poly(A) amplified cDNA libraries were previously created from whole explants and individual
123 GnRH cells in explants (Kramer, 2002). The quality of cDNA from each cell was verified by PCR
124 for GnRH, L19, and β-tubulin (Giacobini et al., 2004). Primers were designed in the 3'-
125 untranslated region of genes Drd1, Drd2, Drd3, Drd4, and Drd5 (Table 1). All primers were
126 screened with BLAST to ensure specificity. For each reaction, 1X PCR buffer, 2 mM MgCl₂,

250 μ M of each deoxynucleotide; Life Technologies, Carlsbad, CA), 250 nM forward primer, 250 nM reverse primer, and 2.5 U Ampli-Taq Gold (Life Technologies) were combined with 1 μ l template cDNA. PCR for Drd1 and Drd2 was performed as follows: initial 10 min denaturation (94°C), 40 cycles of 30 sec denaturation (94°C), 30 sec annealing (55°C), and 2 min extension (72°C), followed by a 10 min post-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.

Calcium Imaging

Calcium imaging recordings were performed on explants between 6 and 11 days *in vitro* (d) as previously described (Klenke and Taylor-Burds, 2012) (Figure 1A-B). Briefly, cells were loaded with 13.5 μ M 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, Hamden, CT) and continuously perfused with SFM at a rate of 300 μ l/min using a peristaltic pump (Instech; Plymouth Meeting, PA). All experiments had a control period (SFM 5 min), amino acid blocking period (AAB 5 min), 1 or 2 drug treatment periods (5 or 10 min each) and a final washout period (SFM 5 min). Time-lapse images piloted by imaging software (BioVision; Exton, PA) were taken of cells every 2 seconds for up to 30 minutes. Images were obtained through a 20x fluorescence objective using an inverted Nikon microscope

152 and a charge-coupled device camera (QImaging; Surrey, Canada) connected to a computer.
 153 Excitation wavelengths of 465–495 nm were provided through a medium-width excitation
 154 bandpass filter, and emission was monitored through a 40 nm bandpass centered on 535 nm.
 155 All recordings were completed with a 40 mM KCl stimulation to ensure viability of cells. Changes
 156 in levels of grey over time (optical density [OD], arbitrary unit) were quantified in single GnRH
 157 neurons *a posteriori* with iVision and calcium oscillations, reflecting neuronal activity (Constantin
 158 and Wray, 2008), detected with MATLAB (Mathworks; Natick, MA). The frequency of oscillations
 159 was expressed in peaks/minute. The phenotype of cells included in the results was confirmed
 160 by immunocytochemistry using anti-GnRH primary antibody previously described (Wray et al.,
 161 1989).

162 ***Electrophysiology***

163 Loose patch clamp was performed on GnRH neurons from acute brain slices. Since sex
 164 hormone (estradiol, progesterone) fluctuations during the estrous cycle in females are known to
 165 influence GnRH neuron excitability (Adams et al., 2018; Farkas et al., 2013; Liu and Herbison,
 166 2011; Silveira et al., 2017), male mice were used for slice recording (Constantin et al., 2021b,
 167 2021a; Constantin and Wray, 2018; Herde et al., 2013; Lee et al., 2012).
 168 GnRH-GFP mice (MGI:6158458) (Spergel et al., 1999) were killed at ~1030 h by cervical
 169 dislocation then decapitated. The brain was glued to a vibratome plate and submerged into ice-
 170 cold low $[Ca^{2+}]$ / high $[Mg^{2+}]$ (0.5 mM / 6 mM respectively) artificial cerebrospinal fluid (aCSF),
 171 bubbled with 95% O₂ / 5% CO₂. After vibratome sectioning (Leica VT1000S), coronal 200 μ m-
 172 slices were incubated at 30°C in normal aCSF containing: 118 mM NaCl, 3 mM KCl, 2.5 mM
 173 CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, 25 mM NaHCO₃, and 11 mM D-glucose (pH 7.3),
 174 bubbled with 95% O₂ / 5% CO₂.

175 Slices were transferred into a recording chamber mounted on an upright microscope
 176 (Nikon Eclipse FN1, Tokyo, Japan) and continuously perfused with oxygenated normal aCSF

177 maintained at 28–30°C, at a rate of approximately 2 ml/min (Constantin et al., 2012). GnRH
178 neurons were identified in slices under fluorescence (20 nm narrow bandpass EGFP filter
179 centered at 480 nm) using a 40X water immersion objective (Nikon 40X/0.80 W, WD 2.0).
180 GnRH neurons were patched under differential interference contrast through a charge-coupled
181 device camera (QImaging Retiga EXi Blue, Surrey, Canada) piloted by the open-source
182 software Micro-Manager version 1.4. The pipettes (3–5 MOhm) were backfilled with aCSF.
183 Electrophysiological recordings were acquired with a Multiclamp 700B amplifier (Molecular
184 Devices, San Jose, CA) using a lowpass filter at 10 kHz and digitized by a Digidata (1550)
185 analog-to-digital converter at 10 kHz (Molecular Devices).

186 ***Analysis***

187 For calcium imaging, nonparametric Friedman tests, followed by Dunn's multiple comparisons
188 test, were used to compare peaks/min of cells between treatment periods of an experiment (Fig.
189 1D, bar plot). The magnitude of the effect evoked by an agonist was calculated for each cell as
190 the difference (delta) between the frequency of calcium oscillations during the period before the
191 agonist and the frequency of calcium oscillations during the period with agonist. Nonparametric
192 Mann-Whitney rank tests were used to compare delta between two paradigms only and
193 nonparametric Kruskal-Wallis tests, followed by Dunn's multiple comparisons test, were used to
194 compare delta of the effects between >2 paradigms. A response, inhibition or excitation, to a
195 pharmacological challenge was defined as $\text{delta} < -1$ or $\text{delta} > 1$ peak/min, respectively.

196 For electrophysiology, action potentials (APs) were detected with Clampfit 10 on
197 continuous recordings. The average firing frequency was calculated over the last 3 minutes of
198 two consecutive recording periods. The firing rate during the second period (dopamine
199 application) was normalized to the firing rate during the first period and expressed as a
200 percentage. A response to dopamine was defined as a change in firing greater than 20%.
201 Individual cell changes in firing between 2 consecutive treatments were analyzed with paired t -

test. Changes in firing rate between 2 paradigms were analyzed with unpaired *t*-test. Significance was determined by $p < 0.05$, and data are presented as mean $\pm$ SEM (n & N, representing the number of cells and animals used, respectively).

Drugs

(-)-Bicuculline methochloride (BIC; GABA_A receptor antagonist), D-(-)-2-amino-5-phosphonopentanoic acid [AP5; N-methyl-D-aspartate (NMDA) glutamatergic receptor antagonist], CNQX disodium salt (AMPA/kainate receptor antagonist), A 68930 hydrochloride (D1/5R agonist), SCH-23390 hydrochloride (D1/5R antagonist), (S)-(-)-Sulpiride (D2/D3R antagonist), A 412997 dihydrochloride (D4R agonist), L-745,870 trihydrochloride and PD 168568 dihydrochloride (D4R antagonist) and (-)-Quinpirole hydrochloride (D2-like receptor agonist) were obtained from Tocris (Minneapolis, MN).

Cholera toxin (CTX; Gas protein uncoupling agent) and pertussis toxin (PTX; Gai/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 Dr. David Sibley (NIH, NINDS). All stock solutions were aliquoted and stored at -20°C in either DMSO or distilled water, and solutions were prepared immediately prior to each experiment by diluting 1:1,000 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 hours prior to recording.

Immunocytochemistry

After calcium imaging, explants (6-11 d) were fixed for 30 min with 0.1 M phosphate buffer saline (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-

227 100 + 0.1% NaAzide) for 1 h and washed several times in PBS. The explants were incubated at
 228 4°C overnight in the primary antibody (in PBS with 1% BSA + 0.1% NaAzide) (SW-1, (Wray et
 229 al., 1989); Table 2). The next day, explants were washed in PBS, incubated for 1 h with
 230 biotinylated secondary donkey anti-rabbit antibody (1:500 in PBS/0.3% Triton X-100; Vector
 231 Laboratories, Inc), washed in PBS, and processed for avidin-biotin horseradish peroxidase/3,3'-
 232 diaminobenzidine (Fig. 1A).

233 ***Immunofluorescence***

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

235 Explants were fixed for 1 h in 4% formaldehyde. After washing in PBS, explants were incubated
 236 in blocking solution (1 h; 10% normal horse serum + 0.3% Triton X-100), washed in PBS, and
 237 then incubated in primary antibody (anti-D4 or anti-D1; 2 nights at 4°C). The next day, explants
 238 were washed in PBS, incubated in either Alexa 555-conjugated secondary anti-rabbit antibody
 239 for anti-D4 antibody (1 h; 1:1,000; Life Technologies) or biotinylated secondary anti-mouse or
 240 anti-rabbit antibody, for anti-D4 clone 2B9 & HL7420 AP antibody respectively, or biotinylated
 241 secondary anti-rat antibody for anti-D1 antibody (1 h; 1:500 + 0.3% Triton X-100; Vector
 242 Laboratories, Inc). Explants treated with a biotinylated secondary antibody were washed then
 243 incubated with Alexa 555-conjugated avidin (1 h; 1:1,000; Life Technologies). After few washes
 244 in PBS, explants were briefly fixed, washed, and incubated in a second primary antibody raised
 245 in different species (anti-GnRH; 1-2 nights at 4°C). The next day explants were washed, treated
 246 with Alexa 488-conjugated anti-mouse or anti-rabbit antibody (1 h; 1:1000; Invitrogen) for anti-
 247 GnRH F1D3C5+SMI-41 or SW-1 antibody respectively, washed in PBS, and coverslipped with
 248 an antifade mounting solution (Electron Microscopy Sciences). No anti-D4R primary antibody
 249 controls were run for each antibody to determine non-specific background staining on GnRH
 250 labeled neurons (see Fig. 6).

251 **RESULTS**

252 ***All dopamine receptors are detected in prenatal GnRH neurons***

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

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

265 ***Exogenous dopamine directly reduces GnRH neuron activity***

266 Exogenous dopamine (1 μ M) was applied to explants and changes in frequency of
 267 intracellular calcium in GnRH neurons were determined using calcium imaging. Since
 268 GABAergic and glutamatergic inputs to GnRH neurons are robust in explants (Constantin et al.,
 269 2010), excitatory inputs were blocked by treatment with amino acid blockers (AAB; 20 μ M BIC,
 270 10 μ M CNQX, 10 μ M AP5). After a control period (SFM 5 min, followed by AAB 5 min),
 271 dopamine was added (Figure 1D top). Dopamine decreased the mean frequency of calcium
 272 oscillations in GnRH neurons, indicating mainly inhibition (Figure 1D, bottom left). Using ± 1
 273 peaks/min as cut-off values in changes in the frequency of calcium oscillations, ~25% of the
 274 GnRH cells were inhibited by dopamine ($\Delta < -1$) while ~5% were excited ($\Delta > 1$) (Figure 1D,
 275 bottom right).

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

277 Canonically, the D1-like subfamily of dopamine receptors couple to the stimulatory G α s protein
 278 subunit while the D2-like subfamily couple to the inhibitory G α i/o protein subunit. Thus, the
 279 decrease in GnRH neuronal activity after exposure to dopamine suggested the activation of D2-
 280 like receptors. Consistent with this observation, dopamine-mediated inhibition persisted in
 281 presence of the D1-like receptor antagonist SCH-23390 (10 nM; Figure 2A, traces) and the
 282 magnitudes of the dopamine inhibition, with or without SCH-23390, were the same (Figure 2A,
 283 beeswarm plot) suggesting a minimal role of D1-like receptors, if any. Conversely, dopamine-
 284 mediated inhibition was abolished in presence of D2-like receptor antagonist spiperone (50 nM;
 285 Figure 2B, beeswarm plot). Notably, while the numbers of cells showing inhibition decreased
 286 (~25% down to ~8%) by spiperone + dopamine (Figure 2B, top trace), the numbers of cells
 287 showing excitation increased (~5% up to ~16%) (Figure 2B, bottom trace). Consistent with the
 288 SCH-23390/dopamine experiment, the D2-like receptor agonist quinpirole (50 nM) decreased
 289 the frequency of calcium oscillations in GnRH neurons (Figure 2C, traces) and the magnitudes
 290 of the quinpirole and dopamine inhibition were the same (Figure 2C, beeswarm plot).

291 Since the three members of the D2-like receptor family were found by PCR, we used
 292 pharmacology to determine which receptor(s) transduced the dopamine-mediated inhibition. In
 293 presence of the D2/3R antagonist, sulpiride (20 nM) and SCH-23390 (D1/5R antagonist),
 294 dopamine still inhibited the activity of GnRH neurons (Figure 3A, traces) with the same
 295 magnitude as dopamine alone (Figure 3A, beeswarm plot), indicating a major role for D4R in the
 296 inhibition. Accordingly, the D4R agonist A 412997 (50 nM) alone decreased the frequency of
 297 calcium oscillations in GnRH neurons (Figure 3B, traces). About ~14% of the GnRH cells were
 298 inhibited by A 412997 while ~1% were excited. The magnitude of the inhibition with A 412997
 299 was similar to the one with dopamine (Figure 3B, beeswarm plot). To ensure the subtype
 300 specificity of A 412997, the D4R antagonists L-745,870 (100 nM) was co-applied with A 412997
 301 and the inhibition was prevented (Figure 3C, traces & beeswarm plot). Finally, to test the role of
 302 D2/3R in the dopamine-mediated inhibition, quinpirole (D2-like agonist) was applied in presence

303 of another D4R antagonist PD 168568 (50 nM), also effective upon the A 412997-mediated
 304 inhibition, to assess the contribution of D4R in the D2-like-mediated inhibition. The inhibition
 305 was partially prevented by the D4R antagonist (Figure 3D, traces & beeswarm plot). Together,
 306 the data indicate that dopaminergic inhibition of GnRH neurons required the activation of D4R.

307 ***The dopamine D1-like receptor activates GnRH neuronal activity***

308 The D1-like receptor agonist A 68930 (10 nM) was applied to test the function of D1/5R. The
 309 frequency of calcium oscillations in GnRH neurons increased (Figure 4A, traces). No GnRH
 310 cells were inhibited by A 68930 while ~21% were excited (Figure 4A, beeswarm plot). The co-
 311 application of A 68930 and SCH-23390 (D1/5R antagonist) prevented the increase of GnRH
 312 neuronal activity, (Figure 4B, traces & beeswarm plot) but co-application of A 68930 and L-
 313 745,870 (D4R antagonist) did not (Figure 4C, traces & beeswarm plot), validating a D1/5R-
 314 mediated excitation. These data indicate that dopaminergic inhibition of GnRH neurons did not
 315 require D1/5R activation and was entirely mediated through D2-like receptors, specifically D4R.

316 ***D4R triggered Gai/o protein signaling while D1-like receptors triggered Gas protein*** 317 ***signaling***

318 Canonically, the D2-like subfamily of dopamine receptors couples to the inhibitory Gai/o protein
 319 subunit (Beaulieu et al., 2015). Explants were treated with pertussis toxin (PTX; 250 ng/ml) for
 320 >4 h to uncouple Gai/o proteins from their receptors. The D4R was selectively activated using a
 321 cocktail of subtype specific antagonists (SCH-23390 for D1/5R and sulpiride for D2/3R) with
 322 dopamine. Compared to previous observation, the PTX pretreatment prevented the dopamine
 323 inhibition (Figure 5A, beeswarm plot), confirming the Gai/o-mediated inhibition. Since the D1-
 324 like subfamily of dopamine receptors couples to the excitatory Gas protein subunit (Beaulieu et
 325 al., 2015), another group of explants were treated with cholera toxin (CTX; 10 ng/ml) for >4 h to
 326 uncouple the Gas protein from their receptors. Compared to previous observation, CTX

327 pretreatment prevented the excitatory effect of the D1-like receptor agonist A 68930 (Figure 5B,
328 beeswarm plot), supporting G α s-mediated excitation.

329 ***The dopamine D4R is present in GnRH neurons***

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

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

336 D4R activation was next analyzed in GnRH cells in adult brain slices. Similar to that shown in
337 Figure 3A for GnRH cells maintained in explants, blocking D1/5R and D2/3R with SCH-
338 23390 (S; 5 μ M) and sulpiride (Su; 5 μ M), respectively, then applying dopamine (5 μ M) (Figure
339 7A) resulted in inhibition of neuronal activity in GnRH cells in brain slices. The effect of
340 dopamine, with D1/5 & D2/3R antagonism, was compared to the effect of dopamine alone
341 (Figure 7B). Six out of 8 cells (6 animals) showed inhibition with dopamine after D1/5R and
342 D2/3R antagonists. The firing rate went from 0.70 ± 0.25 to 0.26 ± 0.12 Hz. Four out of 5 cells (4
343 animals) showed inhibition with dopamine alone. The firing rate went from 0.37 ± 0.12 to
344 0.091 ± 0.07 Hz (Figure 7C). The degree of inhibition was similar with or without D1/5R and
345 D2/3R antagonists (Figure 7D).

346 **DISCUSSION**

347 This report establishes the presence of D4R in GnRH neurons, using RT-PCR and
348 immunocytochemistry, and its role in the dopamine-mediated inhibition of GnRH neurons, using
349 calcium imaging and electrophysiology. Calcium imaging experiments revealed that D4R-
350 mediated inhibition occurs downstream of G $\alpha_{i/o}$ protein. In contrast, activation of D1-like receptors

351 leads to an excitation that requires the G_s protein. Notably, although activation of D1-like or D2-
352 like receptor subfamilies with specific agonists consistently produced either activation or
353 inhibition of GnRH neurons, respectively, the net effect of dopamine was always inhibitory,
354 suggesting mainly activation of D2-like receptors. However, the exact mechanism of the action
355 of dopamine requires caution. Although the canonical pathway of D1-like receptors is excitatory
356 (Pivonello et al., 2007; Podda et al., 2010), the activation of D1-like receptor can be permissive,
357 whereby D1 receptor activation appears necessary for D2 receptor-mediated inhibition (Wachtel
358 et al., 1989; White, 1987). However, in the system used here, dopamine-mediated inhibition
359 persisted in presence of the D1-like receptor antagonist SCH-23390 and the magnitudes of the
360 dopamine inhibition, with or without SCH-23390, were the same suggesting a minimal role of
361 D1-like receptors, if any. Furthermore, as many G-protein coupled receptors, dopamine
362 receptors can exist as monomers, homo- & hetero- dimers and each assembly possesses
363 specific binding and coupling profiles according to occupancy, offering tissue-specific responses
364 (reviewed in: (Maggio et al., 2009; Perreault et al., 2014; Van Craenenbroeck et al., 2011)). In
365 our data, the D1-like receptor agonist produced only excitation while dopamine with D2-like
366 receptor antagonist produced both inhibition and excitation, leading to a lack of net effect. The
367 simplest explanation for the latter might be greater binding of dopamine for D2-like receptors
368 and/or a more efficient signaling pathway downstream of these receptors (Schoffemeer et al.,
369 1994; Skinbjerg et al., 2012). The more convoluted explanation might be that D1-like receptor
370 agonist activates D1-like dimers only, and that dopamine with D2-like receptor antagonist
371 activates both D1-like dimers and D1-like+D2-like dimers via transactivation (Terrillon and
372 Bouvier, 2004).

373 The complexity of dimerization might rationalize the “apparent” inhibition driven by D1-
374 like receptors in mouse, especially in the cells where the inhibition was equally sensitive to
375 raclopride and SCH-23390 (Liu and Herbison, 2013). In addition, the D2-like receptor mediated
376 inhibition was identified as dependent on activation of D4R and a $G_{i/o}$ downstream signaling

377 pathway. This observation provides another explanation to the D1-like receptor driven inhibition
 378 in mouse. Raclopride is ineffective on D4R (Boy et al., 1998) but SCH-23390 would likely inhibit
 379 D1-like+D4R dimers, accounting for the majority of cells reported whose inhibition was sensitive
 380 to SCH-23390, but not to raclopride (Liu and Herbison, 2013). Consistent with this, both
 381 transcript and protein for D4R were found in GnRH cells. Inhibition of GnRH cells via dopamine
 382 D4R is supported by the fact that this receptor, along with the D3R, are downregulated during
 383 proestrus (Vastagh et al., 2016), and that it canonically couples to $G_{i/o}$ protein inwardly rectifying
 384 potassium channels (Inanobe et al., 1999; Wedemeyer et al., 2007; Werner et al., 1996),
 385 effective inhibitors of GnRH neuronal activity (Constantin and Wray, 2018, 2016; Klenke et al.,
 386 2010).

387 Notably, the D2/3R have been commonly linked to reproductive function. For example, a
 388 reduction in the dopaminergic tone and the expression of D2R are observed in women suffering
 389 from polycystic ovary syndrome (Chaudhari et al., 2018) and the secretion of luteinizing
 390 hormone (LH) is sensitive to D2/3R antagonist in prepubertal females (Lacau de Mengido et al.,
 391 1987). However, since discriminative pharmacological tools are recent, the role of the D4R
 392 might have been underestimated. For example, D2R+D4R is a functional heteromer that would
 393 likely be antagonized by D2/3R antagonist. PCR analysis of cDNA libraries from GnRH neurons
 394 support the expression of multiple dopamine receptor subtypes. While the pharmacological data
 395 suggest a prominent role of D4R in the control of GnRH neuronal activity by dopamine, the role
 396 of the other receptors in the control of GnRH/LH secretion cannot be excluded, especially with
 397 dimerization and transactivation mechanisms.

398 *In vivo*, dopaminergic inputs could regulate GnRH neuronal activity since they have been
 399 found around their soma (Jennes et al., 1983; Leranth et al., 1988), i.e. near the site of initiation
 400 of action potentials (Iremonger and Herbison, 2012). In addition, dopamine may regulate GnRH
 401 secretion since GnRH neuron nerve terminals also receive dopaminergic inputs (Jennes et al.,
 402 1983; Kuljis and Advis, 1989). Dopaminergic receptors have been shown to exhibit a subcellular

403 regionalization (Hersch et al., 1995; Levey et al., 1993). Thus, excitatory D1-like receptors
 404 located on GnRH neuron nerve terminals (Fuxe et al., 1988) could be responsible for dopamine-
 405 stimulated GnRH secretion (Rasmussen et al., 1986, 1986) while D4R located on GnRH cell
 406 soma could be responsible for dopamine-inhibited GnRH secretion (Tasaka et al., 1985).
 407 Although not exclusively associated with dopaminergic neurotransmission, dopamine- and
 408 cAMP-regulated neuronal phosphoprotein, a major convergent point of signaling pathways, has
 409 been detected in GnRH nerve terminals (Meister et al., 1988). Finally, indirect effects of
 410 dopamine on GnRH secretion cannot be ruled out either (James et al., 1987; Jarjour et al.,
 411 1986; Rotsztein et al., 1976).

412 In addition to the complexity of GnRH neuron morphology and the dimerization of
 413 dopamine receptors, growing evidence supports the physiological role of dopamine receptor
 414 heterodimers with other receptors (reviewed in (Beaulieu et al., 2015)). The D4R is structurally
 415 a unique receptor, exhibiting a unique third intracellular loop favoring interactions with multiple
 416 signaling proteins (Woods, 2010). Notably, exon III which encodes the third loop of the D4R is
 417 highly polymorphic (Lichter et al., 1993) and determines signaling efficiency (Rondou et al.,
 418 2010). Consequently, D4R is an important player of neuromodulation (α 1 and β 1 adrenergic
 419 receptors: (Gonzalez et al., 2012); Glutamate receptor: (Price and Pittman, 2001); GABA_A
 420 receptor: (Azdad et al., 2003; Gasca-Martinez et al., 2010; Graziane et al., 2009; Shin et al.,
 421 2003; Wang et al., 2002); NMDA receptor: (Andersson et al., 2012; Wang et al., 2003); AMPA
 422 receptor: (Gu et al., 2006; Yuen et al., 2010; Yuen and Yan, 2011)), possibly via
 423 Ca²⁺/calmodulin dependent protein kinase II mechanism (Gu et al., 2006; Yuen et al., 2010;
 424 Yuen and Yan, 2011) and/or interfering with receptor trafficking (Graziane et al., 2009; Yuen et
 425 al., 2010; Yuen and Yan, 2011, 2009). Consistent with D4R being only a neuromodulator of
 426 fertility, mice lacking D4R exhibit behavioral changes (Thanos et al., 2015) but normal fertility
 427 (Rubinstein et al., 1997), similar to mice whose kisspeptin neurons exhibit TH deletion
 428 (Stephens and Rouse, 2017). Kisspeptin/TH neurons seem to provide a sex-specific

429 pheromonal input to GnRH neurons (Taziaux and Bakker, 2015). Notably, in human, the exon III
 430 polymorphism of D4R is linked to the etiology of attention-deficit/hyperactivity disorder (Leung et
 431 al., 2017) but has also been linked to normal behaviors such as higher novelty-seeking (Ebstein
 432 et al., 1996) and greater sex-specific affective knowledge (Ben-Israel et al., 2015).

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

437 REFERENCES

- 438 Adams C, Chen X, Moenter SM (2018) Changes in GABAergic Transmission to and Intrinsic Excitability
 439 of Gonadotropin-Releasing Hormone (GnRH) Neurons during the Estrous Cycle in Mice. *eNeuro*
 440 5.
- 441 Andersson R, Johnston A, Fisahn A (2012) Dopamine D4 receptor activation increases hippocampal
 442 gamma oscillations by enhancing synchronization of fast-spiking interneurons. *PloS one*
 443 7:e40906.
- 444 Azdad K, Piet R, Poulain DA, Oliet SH (2003) Dopamine D4 receptor-mediated presynaptic inhibition of
 445 GABAergic transmission in the rat supraoptic nucleus. *Journal of neurophysiology* 90:559–65.
- 446 Beaulieu JM, Espinoza S, Gainetdinov RR (2015) Dopamine receptors - IUPHAR Review 13. *British*
 447 *journal of pharmacology* 172:1–23.
- 448 Becu-Villalobos D, Libertun C (1995) Development of gonadotropin-releasing hormone (GnRH) neuron
 449 regulation in the female rat. *Cell Mol Neurobiol* 15:165–76.
- 450 Ben-Israel S, Uzefovsky F, Ebstein RP, Knafo-Noam A (2015) Dopamine D4 receptor polymorphism and
 451 sex interact to predict children's affective knowledge. *Frontiers in psychology* 6:846.
- 452 Boy C, Klimke A, Holschbach M, Herzog H, Muhlensiepen H, Rota Kops E, Sonnenberg F, Gaebel W,
 453 Stocklin G, Markstein R, Muller-Gartner HW (1998) Imaging dopamine D4 receptors in the living

454 primate brain: a positron emission tomography study using the novel D1/D4 antagonist [^{11}C]SDZ
 455 GLC 756. *Synapse* 30:341–350.

456 Bryant AS, Greenwood AK, Juntti SA, Byrne AE, Fernald RD (2016) Dopaminergic inhibition of
 457 gonadotropin-releasing hormone neurons in the cichlid fish *Astatotilapia burtoni*. *The Journal of*
 458 *experimental biology* 219:3861–3865.

459 Burger LL, Vanacker C, Phumsatitpong C, Wagenmaker ER, Wang L, Olson DP, Moenter SM (2018)
 460 Identification of genes enriched in GnRH neurons by translating ribosome affinity purification and
 461 RNASeq in mice. *Endocrinology* 159:1922–40.

462 Chaudhari N, Dawalbhakta M, Nampoothiri L (2018) GnRH dysregulation in polycystic ovarian syndrome
 463 (PCOS) is a manifestation of an altered neurotransmitter profile. *Reproductive biology and*
 464 *endocrinology : RB&E* 16:37.

465 Clarkson J, Herbison AE (2011) Dual phenotype kisspeptin-dopamine neurones of the rostral
 466 periventricular area of the third ventricle project to gonadotrophin-releasing hormone neurones.
 467 *Journal of neuroendocrinology* 23:293–301.

468 Constantin S, Caligioni CS, Stojilkovic S, Wray S (2009) Kisspeptin-10 facilitates a plasma membrane-
 469 driven calcium oscillator in GnRH-1 neurons. *Endocrinology* 150:1400–12.

470 Constantin S, Klenke U, Wray S (2016) BPA directly decreases GnRH neuronal activity via a non-
 471 canonical pathway. *Endocrinology* 157:1980–90.

472 Constantin S, Klenke U, Wray S (2010) The calcium oscillator of GnRH-1 neurons is developmentally
 473 regulated. *Endocrinology* 151:3863–73.

474 Constantin S, Piet R, Iremonger K, Yeo SH, Clarkson J, Porteous R, Herbison AE (2012) GnRH Neuron
 475 Firing and Response to GABA in Vitro Depend on Acute Brain Slice Thickness and Orientation.
 476 *Endocrinology* 153:3758–69.

477 Constantin S, Pizano K, Matson K, Shan Y, Reynolds D, Wray S (2021a) An Inhibitory Circuit From
 478 Brainstem to GnRH Neurons in Male Mice: A New Role for the RFRP Receptor. *Endocrinology*
 479 162.

- 480 Constantin S, Reynolds D, Oh A, Pizano K, Wray S (2021b) Nitric oxide resets kisspeptin-excited GnRH
481 neurons via PIP2 replenishment. *Proceedings of the National Academy of Sciences of the United*
482 *States of America* 118.
- 483 Constantin S, Wray S (2018) Nociceptin/Orphanin-FQ Inhibits Gonadotropin-Releasing Hormone
484 Neurons via G-Protein-Gated Inwardly Rectifying Potassium Channels. *eNeuro* 5:ENEURO.0161-
485 18.2018.
- 486 Constantin S, Wray S (2016) Galanin activates G-protein gated inwardly rectifying potassium channels
487 and suppresses kisspeptin-10 activation of GnRH neurons. *Endocrinology* 157:3197–212.
- 488 Constantin S, Wray S (2008) Gonadotropin-releasing hormone-1 neuronal activity is independent of cyclic
489 nucleotide-gated channels. *Endocrinology* 149:279–90.
- 490 de Mengido IM, Becu-Villalobos D, Diaz G, Libertun C (1989) Chronic activation of dopamine receptors in
491 the female infantile rat: effect on hypophyseal hormones and on the onset of puberty.
492 *Endocrinology* 124:746–53.
- 493 Ebstein RP, Novick O, Umansky R, Priel B, Osher Y, Blaine D, Bennett ER, Nemanov L, Katz M,
494 Belmaker RH (1996) Dopamine D4 receptor (D4DR) exon III polymorphism associated with the
495 human personality trait of Novelty Seeking. *Nature genetics* 12:78–80.
- 496 Farkas I, Vastagh C, Sárvári M, Liposits Z (2013) Ghrelin decreases firing activity of gonadotropin-
497 releasing hormone (GnRH) neurons in an estrous cycle and endocannabinoid signaling
498 dependent manner. *PloS One* 8:e78178.
- 499 Fuxe K, Agnati LF, Andersson K, Cintra A, Harfstrand A, Zoli M, Eneroth P, Goldstein M (1988) D1
500 receptor mechanisms in the median eminence and their inhibitory regulation of LHRH release.
501 *Neurochemistry international* 13:165–78.
- 502 Gasca-Martinez D, Hernandez A, Sierra A, Valdiosera R, Anaya-Martinez V, Floran B, Erlij D, Aceves J
503 (2010) Dopamine inhibits GABA transmission from the globus pallidus to the thalamic reticular
504 nucleus via presynaptic D4 receptors. *Neuroscience* 169:1672–81.
- 505 Gerber P, Docke F, Rohde W, Dorner G (1984) Evidence that inhibition of medial preoptic dopaminergic
506 activity may be involved in the prepubertal desensitization to the negative oestrogen feedback in
507 female rats. *Experimental and clinical endocrinology* 84:7–12.

- 508 Giacobini P, Kopin AS, Beart PM, Mercer LD, Fasolo A, Wray S (2004) Cholecystokinin modulates
509 migration of gonadotropin-releasing hormone-1 neurons. *J Neurosci* 24:4737–48.
- 510 Gonzalez S, Moreno-Delgado D, Moreno E, Perez-Capote K, Franco R, Mallol J, Cortes A, Casado V,
511 Lluís C, Ortiz J, Ferre S, Canela E, McCormick PJ (2012) Circadian-related heteromerization of
512 adrenergic and dopamine D(4) receptors modulates melatonin synthesis and release in the pineal
513 gland. *PLoS biology* 10:e1001347.
- 514 Gonzalez-Iglesias AE, Murano T, Li S, Tomic M, Stojilkovic SS (2008) Dopamine inhibits basal prolactin
515 release in pituitary lactotrophs through pertussis toxin-sensitive and -insensitive signaling
516 pathways. *Endocrinology* 149:1470–9.
- 517 Graziane NM, Yuen EY, Yan Z (2009) Dopamine D4 Receptors Regulate GABAA Receptor Trafficking via
518 an Actin/Cofilin/Myosin-dependent Mechanism. *The Journal of biological chemistry* 284:8329–36.
- 519 Gu Z, Jiang Q, Yuen EY, Yan Z (2006) Activation of dopamine D4 receptors induces synaptic
520 translocation of Ca²⁺/calmodulin-dependent protein kinase II in cultured prefrontal cortical
521 neurons. *Molecular pharmacology* 69:813–22.
- 522 Herde M, Iremonger K, Constantin S, Herbison A (2013) GnRH Neurons Elaborate a Long-Range
523 Projection with Shared Axonal and Dendritic Functions. *J Neurosci* 33:12689–97.
- 524 Hersch SM, Ciliax BJ, Gutekunst CA, Rees HD, Heilman CJ, Yung KK, Bolam JP, Ince E, Yi H, Levey AI
525 (1995) Electron microscopic analysis of D1 and D2 dopamine receptor proteins in the dorsal
526 striatum and their synaptic relationships with motor corticostriatal afferents. *The Journal of*
527 *neuroscience : the official journal of the Society for Neuroscience* 15:5222–37.
- 528 Hnasko TS, Hnasko RM, Sotak BN, Kapur RP, Palmiter RD (2007) Genetic disruption of dopamine
529 production results in pituitary adenomas and severe prolactinemia. *Neuroendocrinology* 86:48–
530 57.
- 531 Horvath TL, Naftolin F, Leranth C (1993) Luteinizing hormone-releasing hormone and gamma-
532 aminobutyric acid neurons in the medial preoptic area are synaptic targets of dopamine axons
533 originating in anterior periventricular areas. *Journal of neuroendocrinology* 5:71–9.
- 534 Inanobe A, Yoshimoto Y, Horio Y, Morishige KI, Hibino H, Matsumoto S, Tokunaga Y, Maeda T, Hata Y,
535 Takai Y, Kurachi Y (1999) Characterization of G-protein-gated K⁺ channels composed of Kir3.2

- subunits in dopaminergic neurons of the substantia nigra. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19:1006–17.
- Iremonger KJ, Herbison AE (2012) Initiation and propagation of action potentials in gonadotropin-releasing hormone neuron dendrites. *J Neurosci* 32:151–8.
- James MD, MacKenzie FJ, Tuohy-Jones PA, Wilson CA (1987) Dopaminergic neurones in the zona incerta exert a stimulatory control on gonadotrophin release via D1 dopamine receptors. *Neuroendocrinology* 45:348–55.
- Jarjour LT, Handelsman DJ, Raum WJ, Swerdloff RS (1986) Mechanism of action of dopamine on the in vitro release of gonadotropin-releasing hormone. *Endocrinology* 119:1726–32.
- Jennes L, Stumpf WE, Tappaz ML (1983) Anatomical relationships of dopaminergic and GABAergic systems with the GnRH-systems in the septo-hypothalamic area. *Immunohistochemical studies. Experimental brain research* 50:91–9.
- Klenke U, Constantin S, Wray S (2010) Neuropeptide Y directly inhibits neuronal activity in a subpopulation of gonadotropin-releasing hormone-1 neurons via Y1 receptors. *Endocrinology* 151:2736–46.
- Klenke U, Taylor-Burds C (2012) Culturing embryonic nasal explants for developmental and physiological study. *Current protocols in neuroscience / editorial board, Jacqueline N Crawley . [et al]* Chapter 3:Unit 3.25.1-16.
- Kramer PR (2002) cDNA library construction from single cells. *Current protocols in neuroscience* Chapter 4:Unit 4.27.
- Kuljis RO, Advis JP (1989) Immunocytochemical and physiological evidence of a synapse between dopamine- and luteinizing hormone releasing hormone-containing neurons in the ewe median eminence. *Endocrinology* 124:1579–81.
- Lacau de Mengido I, Becu-Villalobos D, Libertun C (1987) Sexual differences in the dopaminergic control of luteinizing hormone secretion in the developing rat. *Brain research* 432:91–5.
- Lamberts R, Wuttke W (1981) Puberty of female rats may in part be explained by decreased hypothalamic dopamine receptor sensitivity. *Brain research* 215:375–81.

- 563 Lee K, Liu X, Herbison AE (2012) Burst firing in gonadotrophin-releasing hormone neurones does not
564 require ionotropic GABA or glutamate receptor activation. *J Neuroendocrinol* 24:1476–83.
- 565 Lehman MN, Durham DM, Jansen HT, Adrian B, Goodman RL (1996) Dopaminergic A14/A15 neurons
566 are activated during estradiol negative feedback in anestrus, but not breeding season, ewes.
567 *Endocrinology* 137:4443–50.
- 568 Leranth C, MacLusky NJ, Shanabrough M, Naftolin F (1988) Catecholaminergic innervation of luteinizing
569 hormone-releasing hormone and glutamic acid decarboxylase immunopositive neurons in the rat
570 medial preoptic area. An electron-microscopic double immunostaining and degeneration study.
571 *Neuroendocrinology* 48:591–602.
- 572 Leung PW, Chan JK, Chen LH, Lee CC, Hung SF, Ho TP, Tang CP, Moyzis RK, Swanson JM (2017)
573 Family-based association study of DRD4 gene in methylphenidate-responded Attention
574 Deficit/Hyperactivity Disorder. *PloS one* 12:e0173748.
- 575 Levey AI, Hersch SM, Rye DB, Sunahara RK, Niznik HB, Kitt CA, Price DL, Maggio R, Brann MR, Ciliax
576 BJ (1993) Localization of D1 and D2 dopamine receptors in brain with subtype-specific
577 antibodies. *Proceedings of the National Academy of Sciences of the United States of America*
578 90:8861–5.
- 579 Lichter JB, Barr CL, Kennedy JL, Van Tol HH, Kidd KK, Livak KJ (1993) A hypervariable segment in the
580 human dopamine receptor D4 (DRD4) gene. *Human molecular genetics* 2:767–73.
- 581 Liu X, Herbison AE (2013) Dopamine regulation of gonadotropin-releasing hormone neuron excitability in
582 male and female mice. *Endocrinology* 154:340–50.
- 583 Liu X, Herbison AE (2011) Estrous cycle- and sex-dependent changes in pre- and postsynaptic GABAB
584 control of GnRH neuron excitability. *Endocrinology* 152:4856–64.
- 585 Maggio R, Aloisi G, Silvano E, Rossi M, Millan MJ (2009) Heterodimerization of dopamine receptors: new
586 insights into functional and therapeutic significance. *Parkinsonism Relat Disord* 15 Suppl 4:S2-7.
- 587 Meister B, Hokfelt T, Tsuruo Y, Hemmings H, Ouimet C, Greengard P, Goldstein M (1988) DARPP-32, a
588 dopamine- and cyclic AMP-regulated phosphoprotein in tanycytes of the mediobasal
589 hypothalamus: distribution and relation to dopamine and luteinizing hormone-releasing hormone
590 neurons and other glial elements. *Neuroscience* 27:607–22.

- 591 Messina A, Ferraris N, Wray S, Cagnoni G, Donohue DE, Casoni F, Kramer PR, Derijck AA, Adolfs Y,
592 Fasolo A, Pasterkamp RJ, Giacobini P (2011) Dysregulation of Semaphorin7A/beta1-integrin
593 signaling leads to defective GnRH-1 cell migration, abnormal gonadal development and altered
594 fertility. *Human molecular genetics* 20:4759–74.
- 595 Mitchell V, Loyens A, Spergel DJ, Flactif M, Poulain P, Tramu G, Beauvillain JC (2003) A confocal
596 microscopic study of gonadotropin-releasing hormone (GnRH) neuron inputs to dopaminergic
597 neurons containing estrogen receptor alpha in the arcuate nucleus of GnRH-green fluorescent
598 protein transgenic mice. *Neuroendocrinology* 77:198–207.
- 599 Perreault ML, Hasbi A, O'Dowd BF, George SR (2014) Heteromeric dopamine receptor signaling
600 complexes: emerging neurobiology and disease relevance. *Neuropsychopharmacology* 39:156–
601 168.
- 602 Pivonello R, Ferone D, Lombardi G, Colao A, Lamberts SW, Hofland LJ (2007) Novel insights in
603 dopamine receptor physiology. *European journal of endocrinology* 156 Suppl 1:S13-21.
- 604 Podda MV, Riccardi E, D'Ascenzo M, Azzena GB, Grassi C (2010) Dopamine D1-like receptor activation
605 depolarizes medium spiny neurons of the mouse nucleus accumbens by inhibiting inwardly
606 rectifying K⁺ currents through a cAMP-dependent protein kinase A-independent mechanism.
607 *Neuroscience* 167:678–90.
- 608 Price CJ, Pittman QJ (2001) Dopamine D4 receptor activation inhibits presynaptically glutamatergic
609 neurotransmission in the rat supraoptic nucleus. *Journal of neurophysiology* 86:1149–55.
- 610 Rasmussen DD, Liu JH, Wolf PL, Yen SS (1986) Gonadotropin-releasing hormone neurosecretion in the
611 human hypothalamus: in vitro regulation by dopamine. *J Clin Endocrinol Metab* 62:479–83.
- 612 Ribeiro AB, Leite CM, Kalil B, Franci CR, Anselmo-Franci JA, Szawka RE (2015) Kisspeptin regulates
613 tuberoinfundibular dopaminergic neurones and prolactin secretion in an oestradiol-dependent
614 manner in male and female rats. *Journal of neuroendocrinology* 27:88–99.
- 615 Rondou P, Haegeman G, Van Craenenbroeck K (2010) The dopamine D4 receptor: biochemical and
616 signalling properties. *Cellular and molecular life sciences : CMLS* 67:1971–86.

- 617 Rotsztein WH, Charli JL, Pattou E, Epelbaum J, Kordon C (1976) In vitro release of luteinizing hormone-
 618 releasing hormone (LHRH) from rat mediobasal hypothalamus: effects of potassium, calcium and
 619 dopamine. *Endocrinology* 99:1663–6.
- 620 Rubinstein M, Phillips TJ, Bunzow JR, Falzone TL, Dziewczapolski G, Zhang G, Fang Y, Larson JL,
 621 McDougall JA, Chester JA, Saez C, Pugsley TA, Gershanik O, Low MJ, Grandy DK (1997) Mice
 622 lacking dopamine D4 receptors are supersensitive to ethanol, cocaine, and methamphetamine.
 623 *Cell* 90:991–1001.
- 624 Ruf KB, Holmes MJ (1974) Delayed vaginal opening in rats after an intraventricular injection of 6-
 625 hydroxydopamine. *The Journal of endocrinology* 60:383–4.
- 626 Saxena VK, De K, Kumar D, Naqvi SM, Krishnaswamy N, Tiwari AK (2015) Induction of ovulation in
 627 anestrus ewes using a dopamine receptor antagonist. *Theriogenology* 84:1362–6.
- 628 Schoffemeer AN, Hogenboom F, Mulder AH, Ronken E, Stoof JC, Drukarch B (1994) Dopamine displays
 629 an identical apparent affinity towards functional dopamine D1 and D2 receptors in rat striatal
 630 slices: possible implications for the regulatory role of D2 receptors. *Synapse (New York, NY)*
 631 17:190–5.
- 632 Sharifi N, Reuss AE, Wray S (2002) Prenatal LHRH neurons in nasal explant cultures express estrogen
 633 receptor beta transcript. *Endocrinology* 143:2503–7.
- 634 Shin RM, Masuda M, Miura M, Sano H, Shirasawa T, Song WJ, Kobayashi K, Aosaki T (2003) Dopamine
 635 D4 receptor-induced postsynaptic inhibition of GABAergic currents in mouse globus pallidus
 636 neurons. *The Journal of neuroscience: the official journal of the Society for Neuroscience*
 637 23:11662–72.
- 638 Silveira MA, Burger LL, DeFazio RA, Wagenmaker ER, Moenter SM (2017) GnRH Neuron Activity and
 639 Pituitary Response in Estradiol-Induced vs Proestrous Luteinizing Hormone Surges in Female
 640 Mice. *Endocrinology* 158:356–366.
- 641 Simerly RB, Swanson LW, Gorski RA (1985) The distribution of monoaminergic cells and fibers in a
 642 periventricular preoptic nucleus involved in the control of gonadotropin release:
 643 immunohistochemical evidence for a dopaminergic sexual dimorphism. *Brain research* 330:55–
 644 64.

- 645 Skinbjerg M, Sibley DR, Javitch JA, Abi-Dargham A (2012) Imaging the high-affinity state of the dopamine
646 D2 receptor in vivo: fact or fiction? *Biochemical pharmacology* 83:193–8.
- 647 Spergel DJ, Kruth U, Hanley DF, Sprengel R, Seeburg PH (1999) GABA- and glutamate-activated
648 channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in
649 transgenic mice. *J Neurosci* 19:2037–50.
- 650 Stephens SBZ, Rouse ML (2017) Effects of Selective Deletion of Tyrosine Hydroxylase from Kisspeptin
651 Cells on Puberty and Reproduction in Male and Female Mice. *eNeuro* 4.
- 652 Tasaka K, Miyake A, Sakumoto T, Aono T (1985) Dopamine decreases release of luteinizing hormone
653 releasing hormone from superfused rat mediobasal hypothalamus. *J Endocrinol Invest* 8:373–6.
- 654 Taziaux M, Bakker J (2015) Absence of Female-Typical Pheromone-Induced Hypothalamic Neural
655 Responses and Kisspeptin Neuronal Activity in alpha-Fetoprotein Knockout Female Mice.
656 *Endocrinology* 156:2595–607.
- 657 Terrillon S, Bouvier M (2004) Roles of G-protein-coupled receptor dimerization. *EMBO Rep* 5:30–34.
- 658 Thanos PK, Roushdy K, Sarwar Z, Rice O, Ashby CR Jr, Grandy DK (2015) The effect of dopamine D4
659 receptor density on novelty seeking, activity, social interaction, and alcohol binge drinking in adult
660 mice. *Synapse (New York, NY)* 69:356–64.
- 661 Todman MG, Han SK, Herbison AE (2005) Profiling neurotransmitter receptor expression in mouse
662 gonadotropin-releasing hormone neurons using green fluorescent protein-promoter transgenics
663 and microarrays. *Neuroscience* 132:703–12.
- 664 Van Craenenbroeck K, Borroto-Escuela DO, Romero-Fernandez W, Skierska K, Rondou P, Lintermans
665 B, Vanhoenacker P, Fuxe K, Ciruela F, Haegeman G (2011) Dopamine D4 receptor
666 oligomerization--contribution to receptor biogenesis. *FEBS J* 278:1333–1344.
- 667 Vastagh C, Rodolosse A, Solymosi N, Liposits Z (2016) Altered Expression of Genes Encoding
668 Neurotransmitter Receptors in GnRH Neurons of Proestrous Mice. *Frontiers in cellular*
669 *neuroscience* 10:230.
- 670 Venegas-Meneses B, Padilla JF, Juarez CE, Moran JL, Moran C, Rosas-Murrieta NH, Handal A,
671 Dominguez R (2015) Effects of ovarian dopaminergic receptors on ovulation. *Endocrine* 50:783–
672 96.

- 673 Wachtel SR, Hu XT, Galloway MP, White FJ (1989) D1 dopamine receptor stimulation enables the
 674 postsynaptic, but not autoreceptor, effects of D2 dopamine agonists in nigrostriatal and
 675 mesoaccumbens dopamine systems. *Synapse* 4:327–346.
- 676 Wang X, Zhong P, Gu Z, Yan Z (2003) Regulation of NMDA receptors by dopamine D4 signaling in
 677 prefrontal cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience*
 678 23:9852–61.
- 679 Wang X, Zhong P, Yan Z (2002) Dopamine D4 receptors modulate GABAergic signaling in pyramidal
 680 neurons of prefrontal cortex. *The Journal of neuroscience : the official journal of the Society for*
 681 *Neuroscience* 22:9185–93.
- 682 Wedemeyer C, Goutman JD, Avale ME, Franchini LF, Rubinstein M, Calvo DJ (2007) Functional
 683 activation by central monoamines of human dopamine D(4) receptor polymorphic variants
 684 coupled to GIRK channels in *Xenopus* oocytes. *European journal of pharmacology* 562:165–73.
- 685 Werner P, Hussy N, Buell G, Jones KA, North RA (1996) D2, D3, and D4 dopamine receptors couple to G
 686 protein-regulated potassium channels in *Xenopus* oocytes. *Molecular pharmacology* 49:656–61.
- 687 White FJ (1987) D-1 dopamine receptor stimulation enables the inhibition of nucleus accumbens neurons
 688 by a D-2 receptor agonist. *Eur J Pharmacol* 135:101–105.
- 689 Woods AS (2010) The dopamine D(4) receptor, the ultimate disordered protein. *Journal of receptor and*
 690 *signal transduction research* 30:331–6.
- 691 Wray S, Nieburgs A, Elkabes S (1989) Spatiotemporal cell expression of luteinizing hormone-releasing
 692 hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. *Brain*
 693 *Res Dev Brain Res* 46:309–18.
- 694 Yuen EY, Yan Z (2011) Cellular mechanisms for dopamine D4 receptor-induced homeostatic regulation
 695 of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. *The Journal of*
 696 *biological chemistry* 286:24957–65.
- 697 Yuen EY, Yan Z (2009) Dopamine D4 receptors regulate AMPA receptor trafficking and glutamatergic
 698 transmission in GABAergic interneurons of prefrontal cortex. *The Journal of neuroscience : the*
 699 *official journal of the Society for Neuroscience* 29:550–62.

700 Yuen EY, Zhong P, Yan Z (2010) Homeostatic regulation of glutamatergic transmission by dopamine D4
 701 receptors. *Proceedings of the National Academy of Sciences of the United States of America*
 702 107:22308–13.

704 LEGENDS

705 **Figure 1. GnRH neurons respond to dopamine.** **A**, GnRH neurons obtained from an E11.5
 706 mouse and maintained in culture for 7 days recorded for calcium imaging. GnRH cells (arrows)
 707 were identified under brightfield by their bipolar morphology (left panel), recorded after loading
 708 with Calcium Green 1-AM (middle panel), and then stained for GnRH after recording (right
 709 panel). Scale bar 50 μ m. **B**, Representative calcium imaging recording of two single GnRH
 710 neurons showing spontaneous intracellular calcium oscillations (peaks) in SFM. Recordings
 711 were terminated with 40 mM KCl to ensure viability of cells. Y-axis, arbitrary optical density units
 712 [OD]; x-axis, 2 min. **C**, Analysis of single GnRH neuron cDNA libraries show all five dopamine
 713 receptors, dopamine D1-like receptors (D1 and D5) and dopamine D2-like dopamine receptors
 714 (D2, D3, and D4), within the GnRH neuronal population. **D**, Representative calcium imaging
 715 recordings of two GnRH neurons in AAB (20 μ M BIC, 10 μ M CNQX, and 10 μ M AP5) plus
 716 dopamine (1 μ M). The rate of spontaneous calcium oscillations in GnRH neurons was reduced
 717 after blocking the GABAergic and glutamatergic excitatory inputs, then further inhibited with
 718 dopamine, indicating a direct effect of dopamine on GnRH neuronal activity. Bar graph (mean $\pm$
 719 SEM) indicates the quantification in peaks/min from all cells (n=162, N=6). Nonparametric
 720 Friedman tests, followed by Dunn's multiple comparisons test with significance shown using
 721 GraphPad style: *, 0.01<p<0.05; **, 0.001<p<0.01; ***, 0.0001<p<0.001; ****, p<0.0001.
 722 Beeswarm plot indicates the spread of individual cell changes in the frequency of calcium
 723 oscillations in response to dopamine (DA) compared to the spread of individual cell
 724 spontaneous changes when maintained in AAB (mean $\pm$ SD). The delta values are calculated at

725 the drug switch highlighted on the bar graph. Nonparametric Kruskal-Wallis, followed by
 726 Dunn's multiple comparisons test, was used with DA as the reference.

727

728 **Figure 2. The inhibitory effect of dopamine is mediated through the dopamine D2-like**
 729 **receptor subfamily. A-C**, Representative calcium imaging recordings (left, optical density units
 730 [OD]) and calculated delta values for all GnRH cells in that experimental group in beeswarm plot
 731 (mean $\pm$ SD) (right). **A**, Blocking D1/5R (SCH-23390 10 nM) did not prevent the inhibitory effect
 732 of dopamine (1 μ M) (n=111, N=3). **B**, In contrast, a D2-like receptor antagonist (spiperone
 733 50 nM) blocked the inhibitory effect of dopamine. Notably, while the numbers of cells showing
 734 inhibition decreased (~25% down to ~8%) by spiperone + dopamine (left, top trace), the
 735 numbers of cells showing excitation increased (~5% up to ~16%, right, bottom trace &
 736 beeswarm plot; n=64, N=3). **C**, The inhibition could be evoked with a dopamine D2-like receptor
 737 agonist (quinpirole 50 nM) (n=238, N=6). Combined, these data suggest that the inhibitory effect
 738 of dopamine is mediated via the dopamine D2-like receptor subfamily. Beeswarm plot indicates
 739 the spread of individual cell changes in the frequency of calcium oscillations in response to
 740 dopamine (DA, from Fig. 1D) compared to the spread of individual cell changes when
 741 challenged with antagonist/DA or agonist. Nonparametric Kruskal-Wallis, followed by Dunn's
 742 multiple comparisons test, was used with DA as the reference.

743

744 **Figure 3. Dopamine-mediated inhibition of GnRH neurons is dependent on dopamine D4**
 745 **receptor. A-D**, Representative calcium imaging recordings (left, optical density units [OD]) and
 746 calculated delta values for all GnRH cells in that experimental group in beeswarm plot (mean $\pm$
 747 SD) (right). **A**, Blocking D2/D3R (sulpiride 20 nM) did not abolish the dopamine-induced
 748 inhibition of GnRH neurons (n=120, N=4). **B**, Activation of D4R using a specific agonist
 749 (A 412997 50 nM) inhibited GnRH neuronal activity (n=99, N=3). **C**, Co-application of a D4R
 750 antagonist (L-745,870 100 nM) prevented the effect of the D4R agonist, demonstrating the

specificity of A412997 (n=93, N=3). **D**, Application of another D4R antagonist (PD 168568 50 nM) also prevented the inhibitory effect of the D2-like receptor agonist (quinpirole 50 nM) in GnRH neurons, indicating that the inhibitory effect of quinpirole is mostly mediated through D4R (n=89, N=3). Beeswarm plot indicates the spread of individual cell changes in the frequency of calcium oscillations in response to dopamine (DA, from Fig. 1D) compared to the spread of individual cell changes when challenged with antagonist/DA or agonist. Nonparametric Kruskal-Wallis, followed by Dunn's multiple comparisons test, was used with DA as the reference (A-B) or nonparametric Mann-Whitney rank test when only using 2 samples were compared (C-D).

Figure 4. Dopamine D1-like receptor activation increases the activity of GnRH neurons.

A-C, Representative calcium imaging recordings (left, optical density units [OD]) and calculated delta values for all GnRH cells in that experimental group in beeswarm plot (mean $\pm$ 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-745,870 100 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 to the spread of individual cell changes when maintained in AAB or challenged with antagonist/ A 68930. Nonparametric Kruskal-Wallis, followed by Dunn's multiple comparisons test, was used with A 68930 as the reference.

Figure 5. The D1-like and D2-like subfamilies have opposite effects on GnRH neuronal activity via different signaling pathways.

A-B, Calculated delta values for all GnRH cells in that experimental group in beeswarm plot (mean $\pm$ SD). Control experiments (right), perturbation experiments (left). Control from Fig. 3A in (A) showing D4R activation (dopamine

777 [1 μ M] + D1/5R antagonist [SCH-23390 50 nM] + D2/3R antagonist [Sulpiride 20 nM]) and
778 control from Fig. 4A in (B) showing D1-like receptor activation (D1/5R agonist [A 68930 1 nM]).
779 **A**, D4R activation failed to inhibit GnRH neurons incubated in pertussis toxin (PTX >4 hours,
780 250 ng/ml, wrench symbolizes uncoupling Gai/o), demonstrating Gai/o-mediated D4R inhibition
781 (n=84, N=3). **B**, D1/5R activation failed to excite GnRH neurons incubated in cholera toxin (CTX
782 >4 hours, 10 ng/ml, wrench symbolizes uncoupling Gas)), demonstrating Gas-mediated D1/5R
783 inhibition (n=70, N=3). Beeswarm plot indicates the spread of individual cell changes in the
784 frequency of calcium oscillations in response to Sulpiride+SCH-23390/DA or A 68930 compared
785 to the spread of individual cell changes when pretreated with PTX or CTX respectively.
786 Nonparametric Mann-Whitney rank test was used to compare 2 samples (A). Nonparametric
787 Kruskal-Wallis, followed by Dunn's multiple comparisons test, was used with A 68930 as the
788 reference (B).

790 **Figure 6. GnRH neurons are immunoreactive for dopamine D4 receptor and dopamine D1**
791 **receptor. A**, Single confocal plans showing GnRH neurons (green), colabeled with D4R (red;
792 scale bar, 10 μ m) in 1-week-old explants (N=3), using three different primary antibodies. **B**,
793 Single confocal plans showing GnRH neurons (green), colabeled with D1R (red; scale bar,
794 10 μ m) in 1-week-old explants (N=3). **C**, Example of control for no primary antibody 2B9 (left) vs
795 primary antibody 2B9 (right) during double label immunofluorescent staining (images from
796 conventional fluorescent microscope, 40X; scale bar, 20 μ m).

798 **Figure 7. Dopamine D4 receptor activation inhibits GnRH neuron firing.**
799 Electrophysiological recording of adult GFP-tagged GnRH neurons and corresponding
800 periodograms showing that dopamine (5 μ M) inhibits GnRH neuron firing rate, with or without
801 D1/5R & D2/3R antagonists (**A-B**). The gray bars on the periodograms indicate where the mean
802 firing rates (pre and during dopamine application) were calculated for statistical analysis. **C**,

803 Summary data showing quantification of firing rate (in Hz) with dopamine (DA) in individual
804 GnRH neurons, with (closed circles) or without (opened circles) D1/5R & D2/3R antagonists.
805 The values represent the average firing rate over the last 3 min of each period. Individual cell
806 changes in firing between 2 consecutive treatments were analyzed with paired *t*-test. **D**,
807 Summary data showing respective changes in firing rate, with (closed circles) or without
808 (opened circles) D1/5R & D2/3R antagonists. Changes in firing rate between 2 paradigms were
809 analyzed with unpaired *t*-test. Significance is shown using GraphPad style: *, 0.01<*p*<0.05; **,
810 0.001<*p*<0.01; ***, 0.0001<*p*<0.001; ****, *p*<0.0001.
811

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

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: GTACCATCAAGTCCCCTCGG	55 C (40x)	120 bp
	R: CAGCCCTTCCTTCAGTTCTATC		
D2 dopamine receptor (Drd2) (XM_006509996.3)	F: GCTGAAGTTGGAGGTGGTAA	55 C (40x)	145 bp
	R: CCAGACCCAATGGTATCAGCA		
D3 dopamine receptor (Drd3) (NM_007877.2, XM_006521777.2) *Nested: F2/R2	F1: GGCCCTTCATTGTCTGTTGGC	57.5 → 59.5 C (20x, +0.1 C/cycle) + 59.5 C (30x)	228 bp
	R1: AAGTGGGTAAGGGAATGTCTC		
	F2: CCTTCTTCTTGACTCACGTTCT	58 → 60 C (20x, +0.1 C/cycle) + 60 C (30x)	158 bp
	R2: CTTGAGGAAGGCTTTGCGGA		
D4 dopamine receptor (Drd4) (XM_006536156.3, XM_017321972.1)	F: TCTCTGGAAGCTTGGGAAACT	55 → 57 C (20x, +0.1 C/cycle) + 57 C (30x)	284 bp
	R: GGCAGGAAACAAGACCAAA		
D5 dopamine receptor (Drd5) (NM_013503.3) *Nested: F1/R2	F1: GAGTACGGTGAAGTGCCTTTAT	55 → 57 C (20x, +0.1 C/cycle) + 57 C (30x)	245 bp
	R1: GAGTACGGTGAAGTTCCTTTAT		
	R2: CGGCTGTTCAGAAGACTCATAA	55 → 57 C (20x, +0.1 C/cycle) + 57 C (30x)	202 bp

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	1:15000 (<i>in vivo</i>) 1:5000 (<i>in vitro</i>)
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	1:24000
	DRD4 clone 2B9	Millipore, MABN125	Mouse; monoclonal	10 µg/ml
	D4 HL7420 AP	A. Buonanno	Rabbit; polyclonal	2 µg/ml
Dopamine Receptor D1	clone 1-1-F11	Sigma, D187	Rat; monoclonal	1:100

Table 3. Affymetrix RMA Table

Affymetrix Probe set ID	Gene Name	Full Gene Name	RMA value 10 d <i>in vitro</i>		P value	Mouse	
			mean	SEM		Adult brain slice	Prenatal explant
Dopamine receptors							
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		
1422829_at	Drd4	dopamine receptor 4	6.34	0.21	0.0067		
1422830_s_at	Drd4	dopamine receptor 4	5.42	0.07	0.0019		
1455629_at	Drd1a	dopamine receptor D1A	4.30	0.09	Ref	PMID: 29522155	
1456051_at	Drd1a	dopamine receptor D1A	4.31	0.20	>0.9999	PMID: 29522155	
Neuropeptide / steroid / opioid receptors							
1450493_at	Kiss1r	Kiss1 receptor	7.39	0.22		PMID: 29522155	PMID: 18948403
1422099_a_at	Oprl1	opioid receptor-like 1	4.21	0.27			PMID: 30627649
1426103_a_at	Esr2	estrogen receptor 2 (beta)	5.91	0.09		PMID: 29522155	PMID: 12072381
1428250_at	Gper	G protein-coupled estrogen receptor 1	4.88	0.15		PMID: 29522155	PMID: 26934298
1422266_at	Sstr2	somatostatin receptor 2	4.23	0.12		PMID: 15837132	
1441603_at	Sstr3	somatostatin receptor 3	5.03	0.26		PMID: 15837132	
1422281_at	Sstr4	somatostatin receptor 4	4.19	0.21		PMID: 15837132	
1449572_at	Tshr	thyrotropin releasing hormone receptor	3.34	0.10		PMID: 15837132	
1418810_at	Crrh1	corticotropin releasing hormone receptor 1	4.96	0.06		PMID: 15837132	
1422204_at	Avpr1b	arginine vasopressin receptor 1B	5.05	0.28		PMID: 15837132	
1427704_a_at	Galr1	galanin receptor 1	3.67	0.18		PMID: 15837132, PMID: 29522155	PMID: 27359210
1422942_at	Galr2	galanin receptor 2	3.39	0.03		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	
1450260_at	Grpr	gastrin releasing peptide receptor	3.08	0.17		PMID: 15837132	
1422265_at	Brs3	bombesin-like receptor 3	3.72	0.13		PMID: 15837132	
1421667_at	Nmur1	neuromedin U receptor 1	5.66	0.12		PMID: 15837132	
1422121_at	Oprd1	opioid receptor, delta 1	5.15	0.28		PMID: 15837132	
1417151_a_at	Ntsr2	neurotensin receptor 2	4.87	0.10		PMID: 15837132	
1450278_at	Tacr3	tachykinin receptor 3	4.27	0.11		PMID: 15837132	
1422263_at	Bdkrb2	bradykinin receptor, beta 2	5.56	0.09		PMID: 15837132	
1449160_at	Npr1	natriuretic peptide receptor 1	4.28	0.17		PMID: 15837132	
1427191_at	Npr2	natriuretic peptide receptor 2	5.01	0.24		PMID: 15837132	
Glutamate (Glu) receptors							
1457003_at	Grin2b	Glu receptor, ionotropic, NMDA2B, epsilon 1	5.42	0.16		PMID: 15837132	
1421393_at	Grin2d	Glu receptor, ionotropic, NMDA2D, epsilon 4	6.99	0.18		PMID: 15837132	
1458285_at	Gria1	Glu receptor, ionotropic, AMPA1, alpha 1	4.34	0.10		PMID: 15837132	
1420563_at	Gria3	Glu receptor, ionotropic, AMPA3, alpha 3	5.35	0.07		PMID: 15837132	
1421569_at	Grid1	Glu receptor, ionotropic, delta 1	4.67	0.01		PMID: 15837132	
1435487_at	Grid2	Glu receptor, ionotropic, delta 2	2.65	0.22		PMID: 15837132	
1456119_at	Grm5	Glu receptor, metabotropic 5	3.13	0.31		PMID: 15837132	
1421530_a_at	Grm8	Glu receptor, metabotropic 8	3.23	0.24		PMID: 15837132	
Gamma-aminobutyric acid (GABA) receptors							
1443865_at	Gabra2	GABA receptor A, alpha 2	4.24	0.26		PMID: 15837132	
1419719_at	Gabrb1	GABA receptor A, beta 1	4.63	0.24		PMID: 15837132	
1450319_at	Gabrb2	GABA receptor A, beta 2	3.28	0.20		PMID: 15837132	
1450300_at	Gabbr1	GABA receptor C, rho 1	3.07	0.07		PMID: 15837132	
Other amine receptors							
1450003_at	Adra2b	adrenergic receptor, alpha 2b	5.13	0.10		PMID: 15837132	
1423420_at	Adrb1	adrenergic receptor, beta 1	2.90	0.13		PMID: 15837132	
1438710_at	Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	4.63	0.29		PMID: 15837132	
1418268_at	Htr3a	5-hydroxytryptamine (serotonin) receptor 3A	3.99	0.22		PMID: 15837132	
1427654_a_at	Htr4	5 hydroxytryptamine (serotonin) receptor 4	4.68	0.15		PMID: 15837132	
1419210_at	Hrh1	histamine receptor H1	4.12	0.25		PMID: 15837132	
1423639_at	Hrh2	histamine receptor H2	6.07	0.10		PMID: 15837132	
Cholinergic receptors							
1439611_at	Chrm1	cholinergic receptor, muscarinic 1, CNS	4.69	0.11		PMID: 15837132	
1420682_at	Chnb1	cholinergic receptor, nicotinic, beta 1	2.43	0.10		PMID: 15837132	

1420744_at	Chmb2	cholinergic receptor, nicotinic, beta 2	5.16	0.04		PMID: 15837132	
1449532_at	Chmg	cholinergic receptor, nicotinic, gamma	6.54	0.15		PMID: 15837132	