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ORIGINAL ARTICLE

Functional Characterisation of Eel Dopamine D_2 Receptors and Involvement in the Direct Inhibition of Pituitary Gonadotrophins

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In various vertebrate species, dopamine (DA) exerts an inhibitory action on reproduction. In the European eel, DA plays a pivotal role in the inhibitory control of gonadotroph function and the blockade of puberty. *In vivo* studies have suggested that this effect is mediated by receptors pharmacologically related to the D_2 family. In the European eel, two distinct D_2 receptor (D2-R) paralogous genes have been identified (D2A-R and D2B-R) and both were shown to be expressed in the pituitary. We investigated the potential role of each paralogue in the control of gonadotroph function in this species. Eel recombinant D2A-R or D2B-R were expressed in HEK 293 cells, with a universal Gα subunit, and receptor activation was followed by inositol phosphate production. Recombinant D2-Rs exhibited a comparable affinity for DA, although they had differential affinities for mammalian D2-R agonists and antagonists, supporting subtle structure/activity differences. Furthermore, using eel pituitary cell primary cultures, the expression by gonadotroph cells of both native eel D2-R paralogues was examined by *in situ* hybridisation of D2A-R or D2B-R transcripts, coupled with immunofluorescence of luteinising hormone (LH)β or follicle-stimulating (FSH)β. LH and to a lesser extent, FSH cells expressed both D2-R transcripts but with a clear predominance of D2B-R. Notably, D2B-R transcripts were detected for the majority of LH cells. Accordingly, using these cultures, we showed that DA potently inhibited basal and testosterone-stimulated LHβ expression and less potently basal and activin-stimulated FSHβ expression. We also tested some D2-R antagonists, aiming to select the most adequate one to be used in innovative protocols for induction of eel sexual maturation. We identified eticlopride as the most potent inhibitor of DA action on basal and stimulated LH expression *in vitro*. Our data suggest a differential functionalisation of the duplicated receptor genes and demonstrate that mainly D2B-R is involved in the dopaminergic inhibitory control of eel gonadotroph function.

Key words: dopamine receptors, LH, FSH, pituitary cells, teleost, Anguilla

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In teleosts, as in all vertebrates, the brain–pituitary axis controls gonad maturation, thus regulating the onset of puberty and reproductive function. External and internal signals are integrated at the brain level, mainly by preoptic-hypothalamic neurones that produce gonadotrophin-releasing hormone (GnRH) and that, in teleosts, directly innervate the *proximalis pars distalis* of the adenohypophysis, where gonadotroph cells are located. In response to GnRH stimulation, gonadotroph cells produce and release the gonadotrophins, follicule-stimulating hormone (FSH) and luteinising hormone (LH), which stimulate gonadal functions (steroidogenesis and gametogenesis).

In some teleosts species (1,2), as well as in various other vertebrates, such as amphibians (3), birds (4) and mammals (5–10), in addition to the stimulatory effect of GnRH, dopamine (DA), a neurotransmitter of the central and peripheral nervous systems, has been identified as an inhibitory factor of reproductive function. This has been extensively studied in sheep, in which DA participates in the inhibition of gonadotrophic activity before puberty, as well as in the adult during seasonal anoestrus, by acting on GnRH neurones (5–7). More recently, in the mouse, DA has been shown as the most potent inhibitor of GnRH neurone excitability (10). In humans, numerous observations suggest that a direct inhibition by DA on LH release may occur under physiological and pathological conditions (8). In mammals, DA cells located in the preoptic/hypothalamic areas regulate anterior pituitary functions mainly by acting on GnRH terminals, at the median eminence level, whereas, in teleosts, hypophysiotropic neurones do not end at the median eminence but directly innervate the adenohypophysis [goldfish: (11,12); electric fish: (13); atlantic salmon: (14); trout: (15); European eel: (16,17) and zebrafish: (18)]. In teleosts, the DA neurones involved in the inhibitory control of reproduction constitute a specific nucleus in the most ventral part of the anterior preoptic area [goldfish: (11,12); European eel: (16); zebrafish: (18)].

The recruitment of nonhomologous DA cell populations for exerting similar functions in different vertebrates suggests that the nature of DA neurotransmission may be especially suitable for the neuroendocrine inhibitory control of reproduction, although the role, target and intensity of DA inhibition largely differs among vertebrate classes, as well as within each class. For example, in teleosts, the intensity of the dopaminergic inhibition varies according to the species, from a drastic barrier in cyprinids (1,19), to a milder control in salmonids (20).

Initial observations in fish suggested that DA was an adult-specific regulator of the last stages of gametogenesis, as a result of inhibiting basal and GnRH-induced LH secretion, in both females and males. The potential role of DA on the regulation of puberty onset in juveniles has received less attention. To date, a DA inhibitory control of puberty onset has been demonstrated only in a few species: the European eel (16,21), the grey mullet (22) and, recently, the sablefish, *Anoplopoma fimbria* (23).

The European eel, *Anguilla anguilla*, is a particularly relevant model for studying the factors and mechanisms controlling the onset of puberty as a result of its complex life cycle. Juvenile eels grow in the continental waters for several years and remain blocked at a prepubertal stage as long as the reproductive oceanic migration has not been completed. It has been demonstrated that this blockade is partly the result of a strong dopaminergic inhibitory effect on both basal and GnRH-induced gonadotrophin synthesis and release (16,17,21,24,25).

DA effects are mediated through proteins with seven transmembrane spanning domains that belong to the G-protein-coupled receptors (GPCR) superfamily. The DA receptors characterised in mammals have been originally segregated into two major classes termed D_1-family (D_1 and D_5) and D_2 family (D_2, D_3 and D_4) receptors according to their ability to stimulate or inhibit, respectively, adenylate cyclase activity (26). *In vitro* and *in vivo* experiments have demonstrated the involvement of receptors that are pharmacologically related to the D_2 but not D_1 receptor family in the dopaminergic inhibition of gonadotrophin secretion, in goldfish (27), European eel (16), tilapia (28,29) catfish (30), grey mullet (22) and zebrafish (31). In mammals and birds, dopamine D_2 receptors are encoded by a single gene and alternative splicing gives rise to two proteic isoforms: D2l (long) and D2s (short) (32–34). In the European eel, two cDNA sequences corresponding to two distinct DA D_2 receptor genes have been characterised (35). The deduced protein sequences, D2A-R and D2B-R, possess the characteristics of the D_2 family of DA receptors. These protein sequences share a 100% identity within transmembrane domains. However, they exhibit major divergence within the long third intracellular loop sequence, which is involved in the subcellular localisation of the receptor and in the regulatory interactions with intracellular signalling molecules. In addition, real-time quantitative polymerase chain reaction (PCR) analysis revealed a differential tissue distribution of D2A-R and D2B-R (35). This differential tissue distribution, as well as potential differential coupling with intracellular signalling molecules, strongly suggests a differential functionalisation of the two D_2 DA receptors in eel.

The focus of the present study is to assess whether both D2-R paralogues (D2A-R and D2B-R) characterised in the European eel give rise to two functional proteins displaying the pharmacological properties of a D_2 receptor and which of them is involved in the dopaminergic blockade of the gonadotroph function and puberty in this species. Accordingly, we first established the pharmacological profile of each of the recombinant eel D2-Rs transiently transfected in the human embryonic kidney cell line, HEK 293. Next, the detection of D2A-R or D2B-R mRNA by *in situ* hybridisation (ISH) and of the gonadotrophin β-subunits (LHβ and FSHβ) by immunofluorescence (IF) in primary cultures of eel pituitary cells, allowed us to determine the respective expression of the two native D2-Rs in gonadotroph cells. In this eel cell culture system, we also investigated the regulation by DA of basal and stimulated LHβ and FSHβ mRNA expression. Finally, in this *in vitro* sytem, we tested various D2-R antagonists and identified the best selective molecules for use in the development of future innovative protocols that are able to induce eel sexual maturation.

Materials and methods

Plasmid construction

The full sequences of D2A-R and D2B-R (Genbank accession numbers ABH06893 and ABH06894), previously cloned and identified in the European eel (*A. anguilla)* (35), were inserted between *Mlu*I and *Hind*III into a pRK5-tagged plasmid containing the mGluR5 signal peptide to allow their expression and targeting to the cell surface. The expressed recombinant receptors thus consisted of the signal peptide sequence of mGluR5, haemagglutinin (HA) epitope followed by the D2A-R or D2B-R sequence. The resulting plasmids, D2A and D2B receptors, were confirmed by sequencing. An enzyme-linked immunosorbent assay (ELISA) confirmed the protein expression of both eel DA recombinant receptors, whereas luminescence (BRET) and fluorescence (IPone) assays confirmed their G-coupling capacity in HEK 293 cells. A mammalian D_2 receptor (human D2-R: Genbank accession number M29066) was also used for the sake of comparison.

Transfection of HEK 293 cells

HEK 293 cells (ATCC, LGC Standards, Molsheim, France) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Villebon sur Yvette, France) supplemented with 10% foetal bovine serum (FBS) and antibiotics (penicillin and streptomycin, 50 U/ml final; both from Life Technologies, Grand Island, NY, USA) in sterile plastic Petri dishes at 37 °C under 5% CO_2 and saturated humidity. For transfection experiments, the cells were first rinsed with phosphate-buffered saline (PBS) and incubated for 5 min at 37 °C with trypsin-ethylenediaminetetraacetic acid 0.05% without $CaCl_2$/$MgSO_4$/$MgCl_2$ (Gibco). The trypsin reaction was stopped by adding 5 ml of FBS. Cell numbers were counted on a haemocytometer. Two different methods were used for HEK cell transfection: lipofection (for the BRET assays) and electroporation (for anti-HA ELISA and IPone assays).

For lipofection, cells were seeded to be 70–90% confluents (100 000 cells) at the time of transfection. The lipofectamine reagent (25%) was prepared with lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific), diluted in OptiMEM medium (Life Technologies). The eel D2A, D2B or mammalian D_2 receptor plasmids (30 ng/μl DNA per well) were diluted in combination with the α (alphaoRLuc or alphaiRLuc, β (FlagBeta2) and γ (Gamma2Venus) subunits (36) of the G protein (25 ng/well) in OptiMEM medium. The DNA vector solutions were mixed with lipofectamine reagent (1 : 1 ratio) and incubated for 20 min at room temperature (RT). The DNA–lipid complex was then applied to the cells and the cells were incubated at 37 °C for another 24 h.

For electroporation, ten million cells and 1 μg carrier DNA (D2A or D2B or mammalian D_2 receptor and Gαq or Gαqi9 plasmids) were mixed in a total volume of 150 μl of electroporation buffer (K_2HPO_4, 50 mM; CH_3COOK, 20 mM; KOH, 20 mM, pH 7.4). After electroporation (260 V, 1000 μF, Gene Pulser Electroporator; Bio-Rad, Hercules, CA, USA), the cells were resuspended in DMEM supplemented with serum and antibiotics. HEK cells were then plated in 96-well culture plates coated with poly-L-ornithine (15 μg/ml; Sigma, St Louis, MO, USA) at a density of 150 000 cells per well and incubated at 37 °C, under 5% CO_2 and satured humidity for 24 h.

Anti-HA ELISA

The expression levels of the HA-tagged receptors were determined in two independent experiments, using an ELISA assay, as described previously (37). Twenty-four hours after electroporation and incubation at 37 °C, the transfected cells were fixed with 3.7% paraformaldehyde (Sigma) for 5 min at RT and permeabilised or not for 5 min in blocking solution (BS: 1% FBS in PBS) with 0.05% Triton X-100 (Sigma). Permeabilised and nonpermeabilised cells were then blocked twice with BS and incubated in fresh BS for 30 min at RT. The cells were then incubated in anti-HA (Roche Diagnostics, Meylan, France) antibody diluted 1 : 200 (0.5 μg/ml) in BS for 30 min followed by 3 × 5 min wash in BS and with AffiniPure F(ab′)2 Fragment Goat anti-rat immunoglobulin G (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1 : 1600 (0.5 μg/ml) in BS for 30 min. The cells were rinsed 2 × 5 min in BS and 2 × 5 min in PBS. Finally, 90 μl PBS and 10 μl SuperSignal solution (Pierce, Rockford, IL, USA) were applied successively to the cells and the chemiluminescence generated by the bound antibody was quantified using a Wallac Victor plate reader (Molecular Devices, St Grégoire, France).

BRET assay

Twenty-four hours after lipofection, cells were washed twice with PBS and incubated in 100 μl of Krebs-glucose buffer. Coelentezarine (Molecular Probes, Carlsbad, CA, USA; dilution 1 : 40) and DA (Sigma) working solutions were prepared in Krebs-glucose buffer just prior to BRET measurement. The Krebs-glucose buffer was removed from the culture plate and replaced by 60 μl/well of fresh Krebs-glucose buffer and 20 μl/well coelentezarine working solution. The cell were incubated for 1 min at 37 °C before applying DA (20 μl of a fresh solution per well). BRET measurements were performed after 150 s of ligand incubation (i.e. after signal stabilisation). The BRET signal was calculated as the difference of emissions at 535 and 485 nm as detected with an INFINITE 500 microplate reader (Tecan, Lyon, France). Ligand-promoted BRET signal was calculated by subtracting value obtained in the absence of ligand. Background signal was measured in cells transfected with the empty pRK5 plasmid.

Inositol phosphate measurements (IPone assay)

Twenty-four hours after electroporation, the activation of eel recombinant D2A and D2B receptors upon cell treatment with DA, or dopaminergic ligands, was quantified by the production of inositol phosphate. This method is based on the coupling of Gαq with phospholipase C (PLC) signalling. Activation of Gαq-PLC leads to the production of inositol trisphosphate (IP3) and diacylglycerol. IP3 is degraded into IP2 and then IP1 (IPone). Li^+ ions block the degradation of IP1 into inositol, which allows the measurement of accumulated IP1 in cells, reflecting the initial production of IP3 upon PLC stimulation.

Preliminary IPone assays showed that D2A-R and D2B-R activation upon DA treatment does not result in the coupling of the receptors with Gαq protein. Therefore, to establish the pharmacological profile for D2A and D2B receptors, HEK cells were co-transfected by electroporation with D2A-R or D2B-R plasmids and the Gαqi-9 expression vector: Gαqi9 is a chimeric G protein α subunit, in which the last nine amino acids of Gαq are exchanged for those of Gαi (38). Thus, receptors usually coupled with Gi/o may recognise the C-terminal of the chimeric α Gqi9 and activate a Gq type, generating a IP3-Ca^{2+} signal.

DA receptor agonists and antagonists (Sigma) stock solutions were prepared in ethanol (10^{-2} M) and further diluted in stimulation buffer [SB; 10 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose and 50 mM LiCl (pH 7.4); IP-One HTRF® Assay kit; CisBio International, Paris, France] on the day of use. After 24 h of incubation at 37 °C, the culture medium was removed and cells were treated. In a first series of experiments, cells were treated with serial concentrations (from 10^{-9} to 10^{-5} M) of either DA or of specific agonists of the mammalian D2-R, apomorphine and quinpirole, or of the specific mammalian D1-R agonist, S143, also named (±)-6-chloro-PB hydrobromide. In a second series of experiments, the cells were treated with serial concentrations (from 10^{-9} to 10^{-5} M) of specific antagonists of the mammalian D2-R, domperidone and pimozide, or of the specific antagonist of the mammalian D1-R, SCH 23390. After 10 min of pre-incubation at 37 °C with the antagonists, DA was added to each well (final concentration: 10^{-7} M) and the cells were incubated for another 30 min at 37 °C.

The accumulation of inositol monophosphate (IPone), upon receptor activation by agonists or antagonists, was measured by a competitive immunoassay using the IP-One HTRF® Assay kit. Receptor activation was stopped by adding 50 mM phosphate buffer (pH 7.0), 1 M KF and 1% Triton X-100 containing HTRF assay reagents (the Europium cryptate-labelled anti-IP1 antibody and d2-labelled IP1). The plates were incubated for 1 h at RT and the fluorescence ratio (665 nm/620 nm) readout performed on a RUBYstar microplate reader (BMG Labtech, Ortenberg, Germany).

Primary culture of eel pituitary cells and treatments

Animals

Female European eels at the prepubertal silver stage (sexually immature; body weight between 200 and 400 g) were acquired from Gebr. Dil import-export BV (Akersloot, The Netherlands). Eels were maintained in aerated freshwater. They were not fed because they are naturally fasting at the silver stage. Animal manipulations were performed in accordance with the guidelines of the French Ethical Committee and under the supervision of authorised investigators.

Cell culture

Pituitaries from 25 eels were used for each pituitary cell culture. Dispersion and primary culture of pituitary cells were performed in accordance with a previously described protocol (39). Pituitaries were cut into 1-mm slices and incubated at 25 °C with porcine type II trypsin (400 μg/ml) (Sigma) in dispersion buffer (DB: calcium and magnesium free PBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml fungizone; Gibco). After 1 h of incubation, the trypsin solution was replaced by a solution of soya bean trypsin inhibitor (500 μg/ml) (Sigma) and DNAse (0.6 μg/ml) (Sigma) in DB for 10 min. Pituitary slices were then washed and mechanically dispersed by repeated passages through a plastic transfer pipette (Falcon; Dutscher, Brumath, France) in DB. The number of viable cells was estimated by trypan blue coloration (Sigma) using a Malassez cell/haemocytometer. The cells were then resuspended in culture medium (CM: Medium 199 with Earle's salt, sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml fungizone; Gibco) and plated either on 96-well plates at a density of 65 000 cells/well (five replicates per treatment) for quantitative real-time PCR (qPCR) experiments or at a density of 125 000 cells/well on glass coverslips deposited in 24-well plates for *in situ* hybridisation. Both 96-well culture plates and glass coverslips were pre-coated with poly-L-lysine (100 μg/ml; Sigma) to ensure cell attachment. Cultures were performed in serum-free CM at 18 °C under 3% CO_2 and saturated humidity.

Treatments

To allow cell attachment, the treatments were started 24 h (day 1) after the plates were seeded (day 0). All chemicals were purchased from Sigma. The CM was renewed and treatments applied to the cells every 2 days. Chemical stock solutions (10^{-2} M) were prepared in ethanol (testosterone and D2-R antagonist, butaclamol; Sigma), PBS (activin; R&D Systems, Lille, France), sterile water (DA and D2-R antagonist, eticlopride, Sigma) or dimethyl sulphoxide (DMSO) (D2-R antagonists, domperidone, haloperidol and D4-R antagonist, Fauc 213; Sigma) and further diluted in CM before use. The final concentration of ethanol or DMSO in the culture wells never exceeded 0.02% of the total volume of CM. Control wells were treated with a similar final concentration of ethanol or DMSO when required. Cultures were stopped at days 4, 7 and 10. Three independent cell culture experiments were conducted for each treatment and representative experiments are reported.

ISH and IF

Cell fixation and storage

Coverslips with attached eel pituitary cells were washed with PBS and fixed in 3.7% paraformaldehyde for 10 min at RT. The cells were then washed 3 × 10 min with PBS and dehydrated in increasing concentrations of methanol (50%, 75%, 80%, 96% and 100%). The coverslips were stored in methanol 100% at −20 °C until ISH and IF.

Probe synthesis

DA receptor (D2A-R and D2B-R (35) inserts of 1200 bp (D2A-R) and 1350 bp (D2B-R) in the pCRII vector were amplified and isolated from overnight culture using the Mini prep kit (Qiagen, Hilden, Germany). Using M13 forward and reverse primers, a PCR was performed and PCR amplification products were used as matrices for the synthesis of antisense (Sp6 RNA polymerase) and sense (T7 RNA polymerase) digoxigenin (DIG)-labelled cRNA probes (DIG RNA labelling kit; Roche Diagnostics), as described previously (35).

In situ *hybridisation*

Expression of DA receptor (D2A-R and D2B-R) mRNA in the eel pituitary cells was visualised by ISH. Fixated cells were rehydrated in decreasing concentrations of methanol (96%, 80%, 70% and 50%) and in PBS (3 × 10 min) at RT. After 2 h of prehybridisation at RT in hybridisation buffer (HB: 10% dextran sulphate; 50% formamide; 5 × SSC buffer: 0.75 mol/l NaCl, 0.075 mol/l Na_3-citrate, pH 7; 5 × Denhardt's solution, 500 μg/ml salmon sperm DNA; Sigma), the buffer was removed, and 300 ng/ml probe in fresh HB was applied to the cells. Hybridisation was performed for 12–16 h at 60 °C and followed by post-hybridisation rinses in 5 × SSC for 30 min at RT, 30% formamide in 5 × SSC for 15 min at 60 °C, 0.2 × SSC for 2 × 15 min at 60 °C and 0.2 × SSC at RT. The cells were then rinsed 3 × 10 min in wash buffer (WB: 0.1 mol/l Tris-HCl, 0.15 mol/l NaCl, pH 7.5) and incubated for 30 min at RT with 3% H_2O_2 in WB to remove endogenous peroxidase activity. After 3 × 10 min in WB, the cells were incubated for 1 h in the blocking solution (blocking reagent 1% in maleic acid buffer; Roche Diagnostics) and then incubated overnight at 4 °C with a peroxidase-conjugated sheep anti-DIG antibody (1 : 500; Roche Diagnostics). The cells were rinsed 10 × 10 min in PBS containing 0.1% Tween20 (PBS-TW) followed by incubation with a solution of tyramide hydrochloride (1 mg/ml; Sigma) coupled with NHS-fluoresceine (4 mg/ml; Pierce) in darkness at RT. After 20 min of incubation, H_2O_2 was added to the tyramide-fluorescein isothiocyanate solution (0.001% final concentration) and the cells were incubated for another 45 min in darkness at RT. Finally, they were rinsed in PBS-TW 10 × 10 min.

Immunofluorescence

After hybridisation, the cells were first incubated in a blocking solution (BS: PBS-TW with goat serum 10%; Sigma) for 1 h at RT and then incubated overnight at 4 °C with a rabbit antibody raised against carp LHβ by E. Burzawa-Gérard and S. Dufour, as previously validated in the eel (16,39) or a rabbit antibody against eel FSHβ (obtained by J. Tomkiewicz and P. Ravn; Bioneer A/S, Hørsholm,, Denmark), both diluted 1 : 1200 (final concentration in BS). The next day, the cells were rinsed 3 × 15 min in PBS-TW and incubated for 1 h at RT with goat anti-rabbit Alexafluor 594 (10 μg/ml) (Invitrogen) diluted in PBS-TW. The cells were then rinsed 3 × 15 min in PBS and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml; Sigma). Cell preparations were rinsed 3 × 5 min in PBS, followed by 3 × 5 min in distilled water and mounted in Mowiol (Sigma). Observations were made on an epifluorescent microscope (Leica Microsystems, Wetzlar, Germany). Wavelengths of 425 nm (blue/DAPI); 527/30 nm (green/D2-R) and 610/75 nm (red/LHβ and FSHβ) were used. Images were taken and analysed using ImagePro (Media Cybernetics, Inc., Bethesda, MD, USA) and ImageJ (NIH, Bethesda, MD, USA).

For each independent experiment, stained cells and colocalisation of D2A or D2B receptor transcripts with LHβ or FSHβ subunit were estimated in at least 10 different fields (magnification × 40).

qPCR

The levels of mRNA for gonadotrophin β-subunits were quantified in primary cultures of eel pituitary cells by qPCR.

Primers and reference genes

The specific gene primers (Eurofins, Les Ulis, France) for the European eel FSHβ, LHβ and β-actin (reference gene) cDNAs were designed previously (40).

Cell lysis and cDNA synthesis

Total RNA was directly extracted in each culture well, using the Cell-to-cDNA™ II kit (Ambion, ThermoFisher Scientific) in accordance with the manufacturer's instructions. Briefly, cells were washed with cold sterile PBS (Gibco) and lysed with 80 µl/well Cell Lysis II buffer. The lysates were then submitted to treatment with the deoxyribonuclease I (Ambion). cDNA synthesis was performed in a final reaction volume of 20 µl. Five microlitres of the cell lysate were preincubated with 100 ng of random primers and 10 mM dNTP (Invitrogen). The RT reaction was then set up using 0.1 M dithiothreitol, 40 U of ribonuclease inhibitor and 200 U of reverse transcriptase Superscript III (Invitrogen), as described previously (40).

SYBR green assay

The quantitative assays of the expression of eel gonadotrophin subunit expression and actin were performed using the Light Cycler system with SYBR Green I sequence-unspecific detection (Roche, Meylan, France). The qPCRs were prepared with 4 µl of diluted cDNA template and 6 µl of SYBR Green Master Mix (1 µl of forward and reverse primers at a final concentration of 5 nM; 2 µl SYBR green; 2 µl H_2O). The PCRs were run using the conditions: initial activation of the Taq polymerase at 95 °C for 10 min, followed by 41 cycles of PCR: 95 °C for 10 s, 60 °C for 5 s and 72 °C for 10 s.

Serial dilutions of a cDNA pool from pituitary cell cultures were run in duplicate for the target (LHβ, FSHβ) and the reference (β-actin) genes to assess PCR efficiency and for use as the standard curve for each gene. PCRs for target and reference gene expression in unknown samples were run in duplicate from the same cDNA dilution taken from the same RT reaction. The cDNA pool used for the standard curve was also included in each run as a calibrator. Each PCR run contained a negative nontemplate control (water was substituted for cDNA) and a calibrator to assess inter-assay variations. The specificity of each reaction was checked by melting curve analysis to ensure the presence of only one amplification product. Relative expression levels were calculated as described previously (40).

Statistical analysis

The results are given as the mean ± SEM. Means were compared by Student's *t*-test or one-way ANOVA followed by Dunn's multiple comparison test, using Instat (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Recombinant eel D2A and D2B receptor coupling and pharmacology

Eel D2A and D2B receptor expression in transfected HEK 293 cells (ELISA anti-HA)

Human embryonic kidney (HEK 293) cells were transfected with the HA-tagged eel D2A-R or D2B-R, or the empty vector pRK5-HA for controls. The level of expression of the recombinant receptors was quantified by chemoluminescence (Fig. 1A). Assays were conducted on nonpermeabilised cells (Non Perm) to assess the level of receptor targeted at the plasma membrane, and on permeabilised cells (Perm) to measure the total receptor expression level. As assessed both in nonpermeabilised and in permeabilised cells (Fig. 1A), the level of D2A and D2B receptor expression was similar.

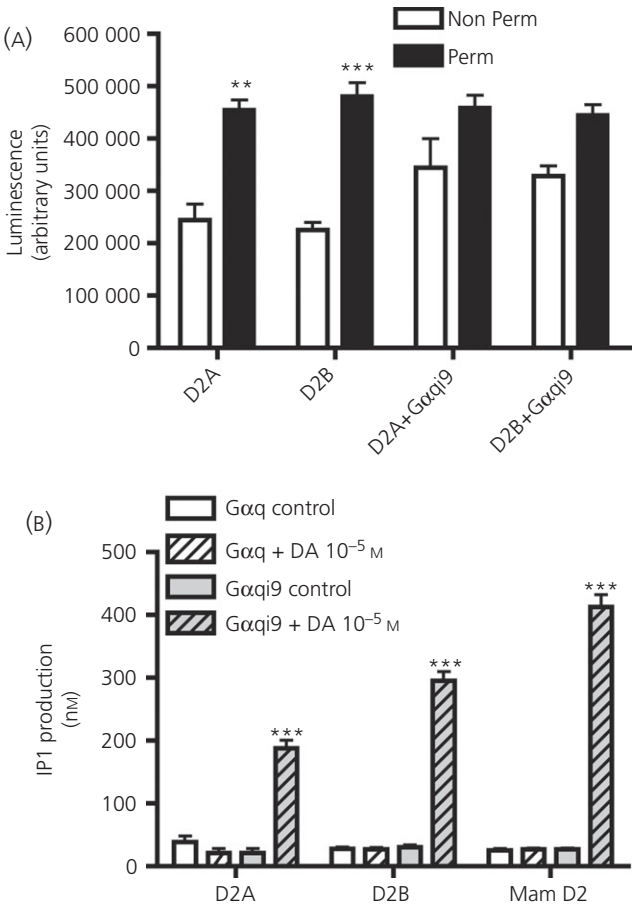

Fig. 1. Expression and activation of recombinant eel D2A and D2B receptors in HEK293 cells. (A) Cell surface and total cell expression of recombinant eel D2A and D2B receptors in HEK 293 cells. Cells were transfected with plasmids coding either pRK5-D2A-HA (D2A) or pRK5-D2B-HA (D2B), co-transfected or not with the universal chimeric Gqαi9 protein, and analysed 24 h later by an enzyme-linked immunosorbent assay using anti-haemagglutinin antibodies. Assays were conducted on nonpermeabilised cells (Non Perm) to assess the level of receptors located at the cell surface, and on permeabilised cells (Perm) to measure the total receptor expression level. The level of receptors targeted to the cell membane was half the total number of receptors expressed (**$P < 0.01$; ***$P < 0.001$, for D2A and D2B, respectively). Cotransfection of either D2A or D2B with Gqαi9, increased the percentage of translocation of D2A-R and D2B-R at the cell surface. Values are the mean ± SEM (n = 3 cell culture wells) from a typical experiment replicated twice with similar results. (B) Activation by dopamine (DA) of recombinant eel D2A and D2B receptors. HEK 293 cells were transfected with plasmids coding either pRK5-D2A-HA (D2A) or pRK5-D2B-HA (D2B), co-transfected with Gαq, or with universal Gαqi9, and analysed 24 h later by the IPone assay. As for mammalian D_2 receptor (Mam D2), DA stimulation of recombinant eel D2A or D2B receptors increased phospholipase C-mediated IPone (IP1) production, when co-expressed with the universal Gαqi9 (***$P < 0.001$). No DA effect was observed when D_2 receptors were co-expressed with Gαq, showing that eel D2-Rs, just as mammalian D2-R, are not coupled with Gαq protein. Values are the mean ± SEM (n = 3 cell culture wells) from a typical experiment replicated twice with similar results.

Approximately half of the expressed receptors were targeted to the cell surface: 53 ± 12% of the total amount of D2A-R ($P < 0.01$) and 47 ± 6% of the total amount of D2B-R ($P < 0.001$) were

found at the plasma membrane level (as quantified in nonpermeabilised cells versus permeabilised cells) (Fig. 1A).

Cotransfection of either D2A-R or D2B-R with the chimeric Gαqi9 protein, a 'universal' Gα subunit (see Materials and Methods), did not modify the total amount of receptor expression, although it increased the percentage of D2A and D2B receptors translocated at the cell membrane. When coexpressed with Gαqi9, up to three quarters of the totally expressed receptors were targeted to the cell surface: 75 ± 16% for D2A-R and 74 ± 6% for D2B-R (Fig. 1A).

Coupling of D2A and D2B receptors to G protein subunits (BRET and IPone assays)

Some preliminary results using the BRET technique (see Supporting information, Fig. S1) showed that both recombinant D2A and D2B receptors were activated upon cell exposure to DA when coexpressed with either Gαi or Gαo subunits: a specific BRET signal could be measured between DA-activated D2A-R and D2B-R and both the Gαi and Gαo proteins, as also observed for GABAb receptor, which is known to be coupled with Gαi and Gαo, and used as a positive control. In all cases, the BRET signal rapidly decreased after few minutes. Interestingly, the BRET signal measured for D2A-R was higher than that observed for D2B-R. By contrast, no BRET signal was obtained with vasopressin V2 receptors, which are known not to be coupled with Gαi/o, and used as a negative control. These results suggested that eel D2A and D2B receptors can couple to both Gαi and Gαo subunits.

Next, the inositol phosphate (IPone) accumulation assay, a highly quantitative and reproducible assay, which allows a large number of experiments to be performed, was used to measure receptor activation upon cell treatment with DA. This assay is based on the coupling of Gαq with PLC signalling: activation of Gαq-PLC leads to the production of IP3 and diacylglycerol. IP3 is degraded into IP2 and then IP1 (IPone). Li^+ ions block the degradation of IP1 into inositol, and allow the measurement of accumulated IP1 in cells, reflecting the initial production of IP3 upon PLC stimulation. However, the IPone assay first showed (Fig. 1B) that eel D2-Rs are not coupled with Gαq protein: DA stimulation of recombinant eel D2A-R or D2B-R did not activate PLC and IPone production when co-expressed with Gαq (Fig. 1B). Similarly, mammalian D2-R is not coupled with Gαq (Fig. 1B). By contrast, both eel D2Rs, as did the mammalian D2-R, highly activated IPone production when co-expressed with the chimeric Gαqi9 subunit ($P < 0.001$) (Fig. 1B). Indeed, Gαqi9 is a 'universal' Gα protein (see Material and Methods). Receptors usually coupled with Gαi/o may recognise the C-terminal of the chimeric Gαqi9 and activate an IP3-Ca^{2+} signal. Therefore, to be able to use IPone assay for studying activation of D2A-R and D2B-R, we co-transfected D2A-R or D2B-R plasmids with Gαqi-9.

D2A and D2B receptor activation upon agonists and antagonists treatments (IPone assay)

To establish the pharmacological profiles of both eel D_2 receptors in HEK cells cotransfected with Gαqi9, we quantified the production of inositol phosphate in the presence of various DA agonists or antagonists.

The transfected cells were exposed to increasing concentrations (10^{-9} to 10^{-5} M) of DA or specific agonists of the mammalian D2-R (Fig. 2A,B). A comparable affinity (EC_{50}:60–80 nM) of recombinant D2A and D2B receptors for their natural ligand, DA was observed. By contrast, D2A-R and D2B-R showed a differential affinity towards D2-R specific agonists. For D2A-R, the rank of potency for agonists was: DA (EC_{50}: 60 nM) > quinpirole (EC_{50}: 90 nM) and apomorphine (EC_{50}: 95 nM). For D2B-R, the rank of potency for agonists was: apomorphine (EC_{50}: 20 nM) > DA (EC_{50}: 80 nM) > quinpirole (EC_{50}: 120 nM). Both D2A-R and D2B-R were weakly activable (EC_{50} > 10 000 and 1300 nM, respectively) by S143, an agonist of D1-R.

The transfected cells were also exposed to DA (10^{-7} M), together with increasing concentrations of specific antagonists of the mammalian D2-R (10^{-9} to 10^{-5} M) (Fig. 2C,D). Mammalian D2-R specific antagonists were able to antagonise the effect of DA on D2A-R and D2B-R, with a differential affinity, although the rank of potency was the same (domperidone IC_{50}: 10–60 nM) > pimozide (IC_{50}: 100–130 nM). SCH 23390, a specific antagonist of mammalian D1-R, weakly antagonised the effect of DA on D2A-R and D2B-R (in both cases, IC_{50} > 1000 nM).

D2A-R, D2B-R and gonadotrophin localisation in eel pituitary cell primary cultures

The expression of the native D2A and D2B receptor transcripts in the gonadotroph cells was studied in dispersed eel pituitary cells by means of fluorescent ISH coupled with IF (Fig. 3). For each experiment, the average of stained cells was calculated and the results were expressed as percentages. At the prepubertal silver stage, 13.9 ± 1.2% of all pituitary cells were shown to express LHβ and 16.7 ± 1.3% to express FSHβ (n = 4 independent cell cultures). Furthermore, 21.7 ± 1.7% of all pituitary cells expressed D2A-R transcripts and 31.4 ± 3.6% expressed D2B-R transcripts (n = 7 independent cell cultures).

Both D2A and D2B receptor transcripts could be detected in LH, as well as in FSH pituitary cells (Fig. 3). Among the cells expressing the LHβ subunit, 11.5 ± 3.2% were shown to express D2A-R, whereas 69.5 ± 8% expressed D2B-R transcripts (n = 4 independent cell cultures). This revealed that approximately six-fold more LH cells expressed D2B-R than D2A-R. It is also interesting to note that, in some eel batches, up to 88% of LH cells expressed D2B-R transcripts. Among the cells expressing the FSHβ subunit, 11.6 ± 2.6% expressed D2A-R transcripts and 27.7 ± 12% expressed D2B-R transcripts (n = 4 independent cell cultures). Thus, at least twice as many FSH cells expressed D2B-R than D2A-R transcripts. These results indicate that LH cells, and to a lesser extent FSH cells, predominantly express D2B-R.

Dopaminergic inhibition of gonadotrophin expression in eel pituitary cell primary cultures

Effect of DA on basal and stimulated LHβ expression

Incubation of eel pituitary cells with DA (10^{-6} M) induced a decrease in the basal LHβ expression level of 71% after 4 days ($P < 0.05$), 82% after 7 days ($P < 0.001$) and 80% after 10 days

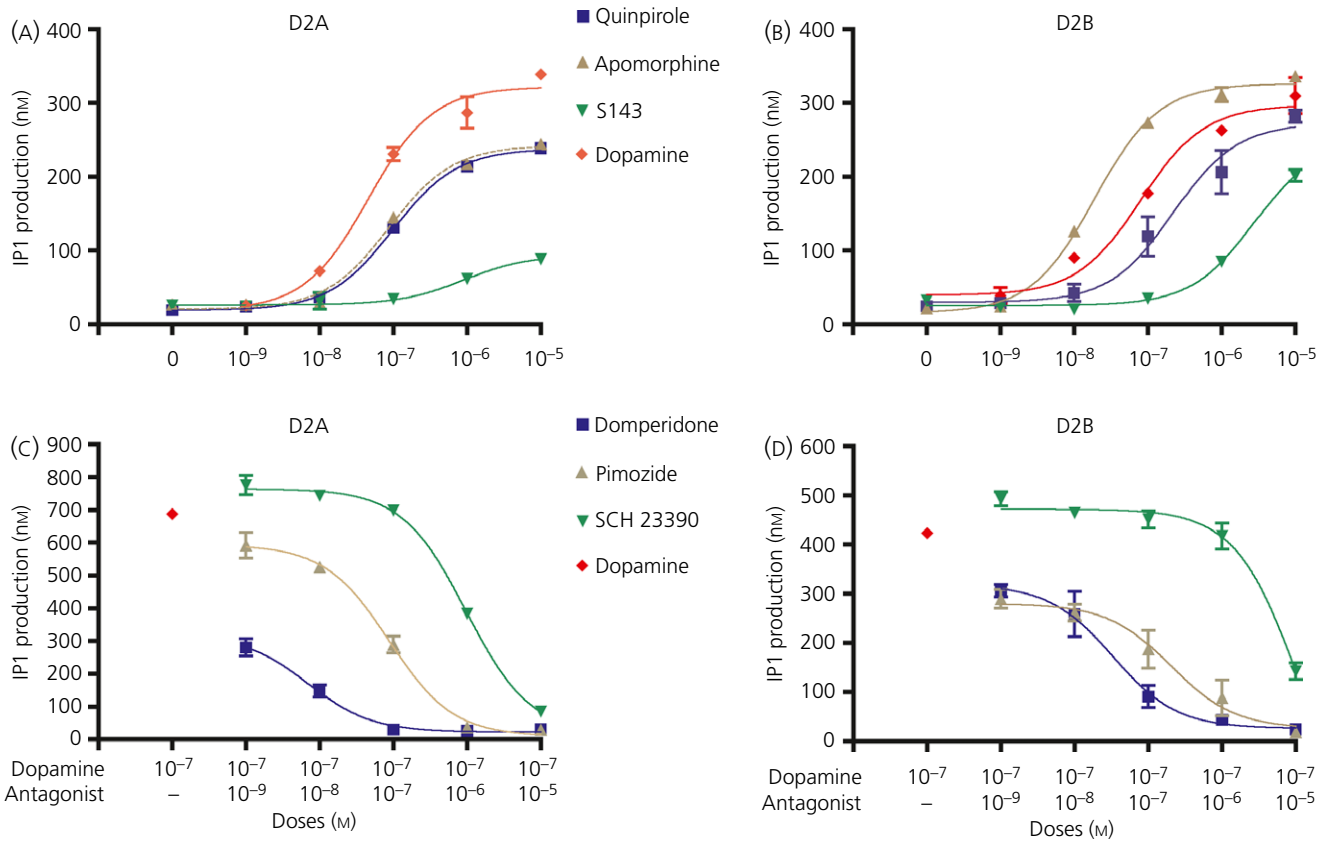

Fig. 2. Pharmacology of recombinant eel D2A and D2B receptors. (A,B) Recombinant eel D2A-R and D2B-R are activated by mammalian D2-R selective agonists. Dose–response stimulation curves for dopamine (DA) agonist-mediated inositol phosphate (IP1) production. HEK 293 cells co-expressing D2A- or D2B-R with Gαqi9 were treated with increasing concentrations of D2-R selective agonists (DA, quinpirole, apomorphine) or of a D1-R selective agonist (S143), and analysed 24 h later by the IPone assay. (C,D) Recombinant eel D2A-R and D2B-R are inhibited by mammalian D2-R selective antagonists. Dose–response inhibition curves for D2-R antagonist-mediated inositol phosphate (IP1) production. HEK 293 cells co-expressing D2A- or D2B-R with Gαqi9 were treated with DA and increasing concentrations of D2-R selective antagonists (domperidone, pimozide) or of a D1-R selective antagonist (SCH 23390) and analysed 24 h later by the IPone assay. The curves were fitted with the 'three-parameter logistic equation' from PRISM (GraphPad Software Inc., San Diego, CA, USA). (A, B) and (C, D) are the mean ± SEM (n = 3 cell culture wells) from a typical experiment replicated twice with similar results.

($P < 0.05$) of treatment compared to nontreated cells (data not shown). Subsequent treatments were thus carried on for 7 days. However, because the basal expression level of LHβ was low at the silver stage, a precise dose–effect curve was difficult to plot. Consequently, we further studied DA effects on LHβ expression stimulated by testosterone (41). After 7 days (Fig. 4A), and as described above, DA (10^{-6} M) induced a significant decrease in basal LHβ expression (86%; $P < 0.001$), whereas testosterone (10^{-8} M) alone induced a significant increase in LHβ expression (×10; $P < 0.001$), compared to nontreated cells. Eel pituitary cells were also treated with testosterone (10^{-8} M) and increasing concentrations of DA (10^{-8}, 10^{-7} and 10^{-6} M) (Fig. 4A). The testosterone stimulatory effect was significantly and dose-dependently inhibited by DA (45% at 10^{-7} M and 81% at 10^{-6} M; $P < 0.001$).

Effect of DA on basal and stimulated FSHβ expression

Incubation of eel pituitary cells with DA (10^{-6} M) for 7 days induced a significant (35%) decrease in the basal FSHβ expression level ($P < 0.001$) compared to nontreated cells (Fig. 4B). As for LHβ, the basal FSHβ expression level was low in the eel pituitary, at the silver stage. Thus, to further analyse the inhibitory effect of DA on FSH, FSHβ expression was stimulated by activin. Eel pituitary cells were treated for 7 days with DA alone (10^{-6} M), or with activin (10^{-9} M) and increasing concentrations of DA (10^{-8}, 10^{-7} and 10^{-6} M) (Fig. 4B). As reported previously (42), activin (10^{-9} M) induced a significant increase in FSHβ expression (× 6.2; $P < 0.001$ compared to the control cells). This activin stimulatory effect was significantly inhibited only by the highest dose (10^{-6} M) of DA (41% inhibition; $P < 0.001$).

Screening of DA D2-R antagonists on LH expression in eel pituitary cell primary cultures

The effects on LHβ expression of D2-R antagonists were tested using eel pituitary cells treated with testosterone and DA. Cells were incubated with testosterone (10^{-8} M), DA (10^{-7} M) and increasing concentrations of DA-R antagonists (10^{-8}, 10^{-7} and

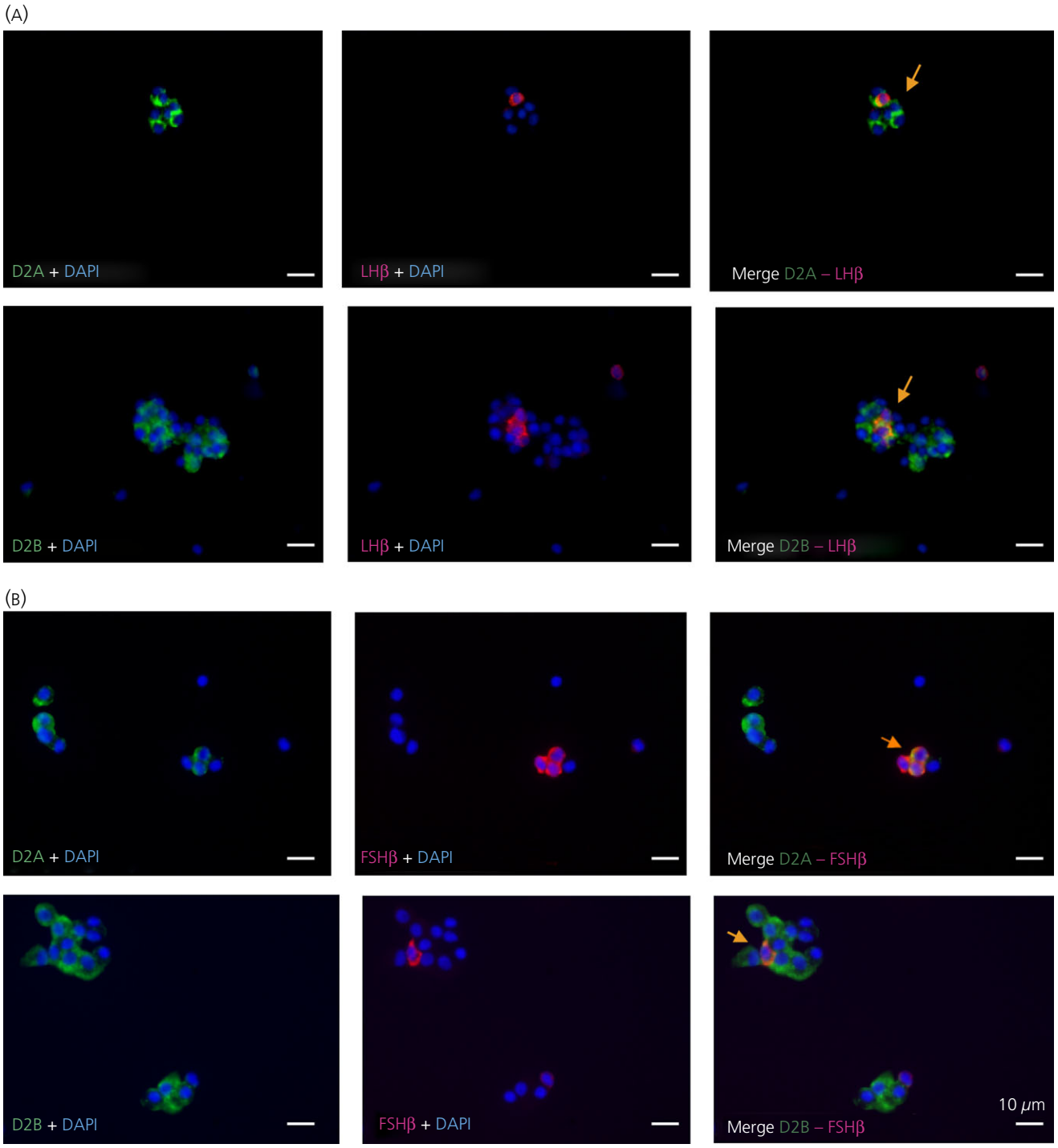

Fig. 3. Localisation of native D2A-R and D2B receptors on eel pituitary luteinising hormone (LH) and follicle-stimulating hormone (FSH) cells. D2-R transcripts in the gonadotroph cells were revealed in dispersed eel pituitary cells by means of fluorescent *in situ* hybridisation (in green), coupled with immunofluorescence to LH or FSHβ subunits (in red). Cells co-expressing D2-R and gonadotrophin β subunit appear in orange (arrow). (A) Localisation of D2A-R and D2B-Rs on eel pituitary LH cells. (B) Localisation of D2A-R and D2B-Rs on eel pituitary FSH cells. Cell nuclei were stained with DAPI, 4′,6-diamidino-2-phenylindole (in blue).

10^{-6} M) for 7 days (Fig. 5). The testosterone-induced LH expression was significantly inhibited by 10^{-7} M DA (Fig. 5). Four different selective D2-R antagonists were tested: eticlopride, domperidone, haloperidol and butaclamol. Eticlopride had no significant effect at 10^{-8} M. However, at 10^{-7}, and 10^{-6} M, it fully antagonised the inhibitory effect of DA on testosterone-stimulated LHβ mRNA levels

($P < 0.05$; $P < 0.01$, respectively, compared to testosterone + DA treated cells,). This resulted in LHβ mRNA levels similar to that measured in cells exposed to testosterone alone (Fig. 5).

Domperidone and haloperidol were also able to antagonise the inhibitory effect of DA on testosterone-stimulated LHβ mRNA expression, although with a lower potency than eticlopride. At a concentration of 10^{-7} M, a slight but nonsignificant effect was observed. At 10^{-6} M, both domperidone and haloperidol significantly antagonised the inhibitory effect of DA. This resulted in LHβ mRNA levels that were significantly higher than those in cells treated with testosterone and DA ($P < 0.05$ for domperidone; $P < 0.01$ for haloperidol) and similar to the levels in cells treated with testosterone alone (Fig. 5).

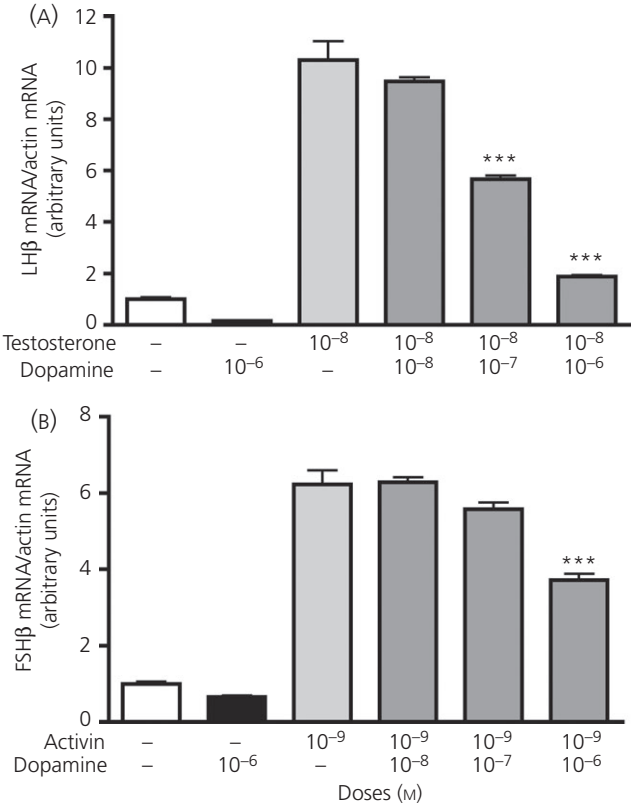

Fig. 4. Dose-dependent inhibitory effect of dopamine (DA) on luteinising hormone (LH)β and follicle-stimulating hormone (FSH)β expression by primary cultures of eel pituitary cells. (A) Dose-dependent effects of DA on testosterone-stimulated LHβ expression, as measured by a quantitative real-time polymerase chain reaction (qPCR). Dispersed eel pituitary cells were treated for 7 days with 10^{-6} M DA alone, 10^{-8} M testosterone alone, or the combination of testosterone and increasing concentrations of DA (10^{-8}, 10^{-7} and 10^{-6} M). (B) Dose-dependent effects of DA on activin-stimulated FSHβ expression, as measured by qPCR. Dispersed eel pituitary cells were treated for 7 days with DA (10^{-6} M) alone, activin (10^{-9} M) alone, or the combination of activin and increasing concentrations of DA (10^{-8}, 10^{-7} and 10^{-6} M). Data are normalised to β-actin and expressed as arbitrary units; values the are mean ± SEM (n = 5 cell culture wells) from one experiment representative of three independent cell culture experiments. ***$P < 0.001$: significant difference compared to cells treated with testosterone alone (A), or with activin (B) alone.

By contrast, butaclamol, which is also a selective DA D2-R antagonist, had no significant effects on 10^{-7} M DA-induced inhibition of LHβ expression, when applied to cells at concentrations of up to 10^{-6} M (Fig. 5) and for different incubation periods (4, 7 and 10 days; data not shown).

As a control, we also tested Fauc 213, a selective D4 receptor antagonist, and observed that it had no effect on DA-induced inhibition of LHβ mRNA at any of the doses tested (Fig. 5).

Because FSHβ expression was much less sensitive to the inhibitory effect of DA than LHβ expression, a high concentration (10^{-6} M) of DA was necessary to decrease FSHβ mRNA levels. Under these conditions, the D2-R antagonists tested (eticlopride, domperidone, haloperidol, butaclamol), and also used at 10^{-6} M, were unable to significantly counteract the effects of DA on FSHβ expression (data not shown). Higher D2-R antagonist concentrations are toxic to the cells and so their actions on FSHβ expression could not be investigated.

Discussion

The present work aimed to determine whether the two paralogue genes D2A and D2B previously cloned in the European eel give rise to two functional proteins displaying the pharmacological properties of a D2 receptor, to localise their expression in pituitary gonadotroph cells, and to further investigate their role in the dopaminergic prepubertal blockade of gonadotroph function demonstrated in this species (16,21).

Recombinant eel D2A and D2B receptors are functional GPCRs

Similar to all the known vertebrate DA receptors, the D2 receptor belongs to the large superfamily of GPCRs, which are seven-transmembrane-helix proteins that interact with and activate intracellular heterotrimeric G proteins. Depending on the subset of G-proteins activated, GPCRs modulate specific signalling pathways. The Gα subunit has a decisive role in discriminating between different receptor types (43,44) and also between different functional states of the receptor (45), and thereby determines the downstream signalling cascades.

Mammalian D2-Rs are known to be coupled with the inhibitory G proteins Gαi/αo, which, upon receptor activation, not only reduce intracellular cAMP levels, but also may inhibit phosphatidyl inositol metabolism and Ca^{2+} channels, activate K^+ channels, or modulate Na^+/H^+ antiporters (46–50).

To characterise the functional properties of eel recombinant D2A-R and D2B-R, we expressed them in heterologous (HEK 293) cells. Using an epitope-tagging technique, we first showed that both paralogues were consistently and equally targeted at the plasma membrane. The fact that only a subset (approximately 50%) of the transfected receptors are found at the plasma membrane has been also observed with the murine D2-R in other cell lines by transient expression studies (51,52), although the ratio of intracellularly located to surface D2 receptors expressed in heterologous cells may vary with the cell line. This may reflect the fact that D2-

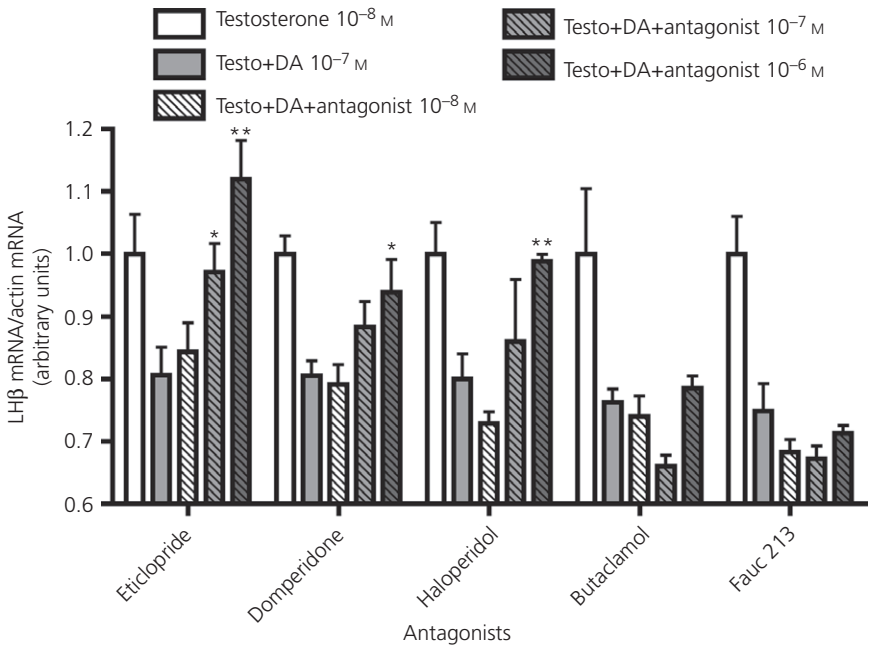

Fig. 5. Dose-dependent effects of D2-R antagonists on luteinising hormone (LH)β expression by primary cultures of eel pituitary cells. Pituitary cells were treated with either testosterone (10^{-8} M), testosterone + dopamine (DA) (10^{-7} M), or testosterone + DA in combination with increasing concentrations (10^{-8}, 10^{-7} and 10^{-6} M) of D2-R antagonists (eticlopride, domperidone, haloperidol and butaclamol) or of D4-R antagonist (Fauc 213). Data are normalised to β-actin and expressed as arbitrary units; values are the mean ± SEM (n = 5 culture wells) from one experiment representative of three independent cell culture experiments. *P < 0.05; **P < 0.01 significant difference compared to cells treated with testosterone + DA.

Rs are retained intracellularly because heterologous cells do not express the co-proteins required for proper targeting to the plasma membrane or that intracellular receptors could serve as a reserve pool for rapid delivery to the plasma membrane under certain physiological conditions. Previous studies on the subcellular localisation of mammalian membrane receptors by immunoelectron microscopy have also shown that the β_2 adrenergic receptor (53), the α_2c adrenergic receptor (54) and the D_2 DA receptor were detected not only in the plasma membrane region, but also in intracellular compartments (55–58). These studies suggested the coexistence of two functional populations of receptors, and also that their ratio may differ according to the brain region, the tissue or the physiological situation. We also showed that, when cotransfected with a universal G protein (Gαqi9), both D2-R paralogues can be further (approximately 75%) targeted to the plasma membrane. To our knowledge, this comprises the first evidence indicating that a G protein may target a receptor to the cell surface in a non-mammalian vertebrate.

Eel D2A-R and D2B-R protein sequences were shown to exhibit large divergence within the third intracellular loop, suggesting that their coupling to G proteins or other partners might be different and that they might modulate different intracellular signalling pathways. To identify the potential G-protein coupling of eel D2-R subtypes, we first used a BRET technique. Our results showed that a specific BRET signal can be measured between DA-activated eel D2A-R and D2B-R and both the Gαi and Gαo proteins, suggesting that, as for the mammalian D2-R, both eel D2A-R and D2B-R were coupled with Gαi and Gαo. The higher BRET signal measured for D2A-R may suggest that D2A-R would be more tightly coupled with these G proteins than D2B-R.

Using an inositol phosphate accumulation assay, we then showed that, although in the presence of the universal chimeric Gαqi9, DA can elicit a dose-dependent increase in IPone accumulation, it cannot do so in the presence of Gαq. Co-expression of D2A-R and D2B-R with Gαqi9 conferred both receptors the ability to stimulate IP production, which strongly suggests that stimulation of the eel D2-Rs with DA triggers Gαi/αo-mediated, but not Gαq-mediated, signalling. Therefore, eel D2A-R and D2B-R, similar to their mammalian homologue, may couple with a member of the Gαi/αo-protein family: it is very well documented that DA inhibits adenylate cyclase activity in the rat anterior pituitary *in vivo* and in cultured lactotrophs *in vitro* by coupling to pertussis toxin (PTX)-sensitive signalling pathways, implicating its association with the Gαi/αo family (59,60). Similarly, a previous study in a teleost showed that recombinant tilapia D2-R transiently transfected in COS7 cells was sensitive to PTX, indicating that Gαi/αo are also involved in its signal transduction (61). Taken together, our results show that the G-coupling properties of the eel recombinant D2-Rs are comparable to those of mammalian or piscine recombinant D2-Rs expressed in various cell lines.

Both eel D2-Rs are pharmacologically related to mammalian DA receptor of the D_2 family

Functional assessment of the recombinant eel D2A-R and D2B-R in HEK 293 cells using the IPone assay in the presence of the

universal Gαqi9 revealed that these receptors display pharmacological properties characteristic of DA receptors of the D_2 family. We showed that, in transfected cells, both D2-Rs displayed a similar affinity for DA, their natural ligand. Both recombinant receptors were also activated by specific agonists of the mammalian D2-R (apomorphine and quinpirole), whereas only a weak response was observed with a D1-R agonist (S143). Similarly, both eel D2A-R and D2B-R were inhibited by specific antagonists of the mammalian D2-R (domperidone and pimozide), whereas they were poorly inhibited by a D1-R antagonist (SCH 23390). Therefore, the present results further corroborate the classification of the eel D2A-R and D2B-R among the D_2 family of receptors, as previously based on their sequence identity with that of D2-Rs from other vertebrate species. Our previous radioligand binding studies, performed on brain or pituitary membranes, also showed the existence of binding sites exhibiting the typical pharmacological profile of D_2-like receptors. However, both receptors were analysed at the same time (35). In the present study, by contrast, we could compare recombinant D2A-R and D2B-R. The present pharmacological studies revealed that they displayed differential affinities for agonists and antagonists of the mammalian D2-Rs, further supporting differences in their binding properties. Differences in G-coupling affinity were also suggested by our BRET experiments: D2A-R appears to be more efficiently coupled with Gαi/αo than D2B-R, suggesting differences in their signalling pathway and effector systems. In the same line, the fact that there is no desensitisation of the gonadotroph cells to DA inhibitory treatment over the long term implies that the paralogue (D2B-R), which may play the major role in this neuroendocrine control, does not exhibit desensitisation properties. The potential differences in signalling pathways downstream of G proteins should now be explored; they may have also contributed to the conservation of duplicated receptors.

The data obtained in the present study also highlight that, throughout the evolution of vertebrates, different means of modulating the cellular response to the dopaminergic signal have been recruited. In mammals and birds, a single D_2 receptor gene encodes two proteic isoforms (D2s and D2l) generated by an alternative splicing at the level of the third intracellular loop (32–34). Spliced isoforms were not found in the eel D_2 gene, instead the two D_2 receptors are encoded by two distinct genes (35). The presence of two D2-R paralogues in the eel probably results from the teleost specific whole genome duplication (3R) (62). Teleosts are known to possess more DA receptor genes compared to tetrapods (63). In the goldfish, three D2-R genes were reported (64), although phylogenetical analysis suggests that the additional one, compared to the eel, may result from the specific tetraploidisation of this group (65). In zebrafish, three D2-R genes were also isolated and initially named D2a, D2b and D2c (66), a nomenclature that we used in a previous study (31). However, they were renamed D2a, D2like and D2b, respectively, by Ensembl/ZFIN (63). Recent phylogenetical studies further showed that the zebrafish D2a and D2b are 3R-duplicated genes, orthologous to the amniote D_2, whereas the D2like would be an additional gene lost in tetrapods (renamed D8) (63). Based on the phylogenetic analysis, it was confirmed that that the D2A and D2B in eels are orthologous to the amniote D_2, with most probably eel D2A being orthologous to the zebrafish D2a and eel D2B being orthologous to the zebrafish D2b (63). The present study is the first one to compare the functional and pharmacological properties of 3R-duplicated D2-R paralogues.

D2A-R and D2B-R are expressed in eel pituitary gonadotroph cells

We had previously shown, using both qPCR and *in situ* hybridisation, that D2A-R and D2B-R transcripts were both expressed in the eel pituitary (35). To examine their respective roles in the control of gonadotroph cells, we thus searched for which D2-R paralogue was expressed by LH- and FSH-producing cells and thus potentially involved in the transduction of the dopaminergic inhibition of gonadotrophins. By contrast to the situation in mammals, in teleosts, LH and FSH are mainly produced by distinct pituitary cells (67,68). By combining the detection of D2A-R or D2B-R mRNA transcripts and of the gonadotrophin β-subunits (LHβ and FSHβ) in eel pituitary primary cell culture, we were able to demonstrate that a low percentage of LH- and FSH-producing cells expressed D2A-R. By contrast, a larger number of FSH- and a majority of LH-producing cells expressed D2B-R, indicating that it may play a prominent role in mediating the dopaminergic inhibitory control of gonadotrophins. The expression of D_2 receptors by the gonadotroph cells demonstrates that DA may act directly at the pituitary level to control gonadotroph function, further supporting our previous *in vivo* results (16,21).

DA regulates eel LH cells

In good agreement with the expression of D2-Rs by a large percentage of LH cells, our *in vitro* experiments in eel pituitary primary cell cultures showed a strong inhibitory effect of DA on both basal and testosterone-stimulated LHβ expression. Because of the very low gonadotrophin levels of prepubertal eels, using qPCR, an extremely sensitive assay to determine LHβ mRNA levels was especially relevant.

With prepubertal eels, we addressed chronic regulations in these animals undergoing a complete and long-lasting blockade of reproduction. We previously showed that weeks of treatment with GnRH agonist and DA antagonist were mandatory to remove this blockade and significantly increase LH secretion and gonadal activity *in vivo* (16). Therefore, in the present study, we also applied relatively long-term DA treatments to eel pituitary cells *in vitro*. As *in vivo*, no desentitisation to DA was observed *in vitro*, in agreement with the ability of DA to exert a chronic neuroendocrine blockade of eel puberty.

The present results demonstrate a direct effect of DA at the pituitary level, which is well correlated with the D2-R expression. To date, the dopaminergic inhibition of LH had only been investigated *in vivo* in the eel (16,21). Although we did not measure LHβ protein in the present study, we did so in the previous *in vivo* investigation using a radioimmunoassay (69) and

demonstrated that DA exerted an inhibitory effect both on the LH pituitary content and its plasma release (16). Using the same radioimmunoassay, we also previously showed parallel regulations of pituitary LHβ mRNA and protein levels in various *in vivo* and *in vitro* experiments (40,41). Although we cannot exclude some paracrine effects of DA in that not only LH cells, but also some other eel pituitary cell types express D2-Rs, the expression of D2Rs by a majority of LH cells supports a main direct effect of DA in the inhibitory control of LH.

Our cell culture experiments also showed that DA inhibitory effect on LHβ expression could be counteracted by pharmacological D2-R antagonists, such as domperidone, haloperidol and eticlopride, whereas a selective D4-R antagonist (Fauc 213) had no effect on the DA-induced inhibition of LHβ expression.

The predominant expression of D2B-R, as compared to D2A-R, in eel LH cells suggests a major role for this paralogue in the control of LH. This is in good agreement with recent results obtained in the Japanese eel showing that D2B-R expression is reduced in the forebrain and pituitary during the prepubertal metamorphosis (silvering), precisely when LH expression increases, whereas no change was observed in D2A-R transcript levels (70).

ISH and IF studies also revealed that, even if eel LH cells mostly expressed D2B-R, a small percentage might also express the other paralogue, D2A-R. In zebrafish, recent studies of our group demonstrated that the two D_2 paralogues D2a and D2b (former D2c) were highly expressed in LH-producing cells, indicating that they both may be involved in mediating the inhibitory neuroendocrine control of DA (31). These data suggest a differential involvement of D_2 paralogues in the regulation of LH cells, according to species or life stages.

DA also regulates eel FSH cells

We next showed that some FSH cells also expressed DA D2-Rs, and thus that DA is also a potential direct regulator of FSH cells in the eel. As for LH cells, FSH cells mainly expressed the D2B-R paralogue. However, it is worth noting that a much lower percentage of FSH cells expressed D2-Rs, compared to LH cells. Accordingly, our eel pituitary primary cell culture experiments showed that the inhibitory effects of DA on basal or activin-stimulated FSHβ expression were much less pronounced than on LHβ expression. Indeed, the maximal inhibition on FSHβ expression was approximately 40%, whereas that on LHβ expression might reach more than 85%. Furthermore, the dose of DA that is able to significantly inhibit FSHβ expression was approximately 10-fold higher than that for LHβ expression.

The literature on FSH regulation in teleosts is scarce and controversial. One study in rainbow trout reported that treatment with α-methyl-*p*-tyrosine, an inhibitor of catecholamine synthesis, as well as with pimozide, a D2-R antagonist, increased blood LH levels but failed to modify blood FSH levels, regardless of the stage of the fish (71). In the same line, one review concluded that DA did not regulate FSH secretion in teleosts (29). Yet, α-methyl-*p*-tyrosine was later shown to increase FSH release in mature rainbow trout (72), suggesting the control of FSH by DA in mature fish. Concerning immature fish, the present study showed that DA may exert an inhibitory effect on basal and activin-stimulated FSH expression in pituitary primary cell cultures from prepubertal female eels. In agreement, in the immature male striped bass, pimozide is able to suppress the stimulatory effect of GnRHa and T on both LHβ and FSHβ mRNA levels *in vivo* (73). Moreover, in sablefish, long-term *in vivo* treatment with the D2-R antagonist metoclopramide stimulates pituitary FSH expression in prepubertal females (23). This suggests that DA may exert an inhibitory control on FSH in immature fish from various species.

Research perspectives

From an applied point of view, considering the dramatic decline of wild populations of eels (74), one of the major challenges is to identify the effectors controlling their puberty and reproduction. The development of alternative methods either for the stimulation of gonadal maturation or for the activation of final oocyte maturation and ovulation in experimentally matured eels is also of particular interest. Given that, in our primary cultures of eel pituitary cells, LH cells respond quite well to DA, this *in vitro* model of homologous cells expressing native receptors and appropriate G-proteins may be reliably used to screen various dopaminergic antagonists, prior to injecting them *in vivo* to induce a preovulatory surge. In our cellular system, we could test various D2-R antagonists. In addition to domperidone, which was already known and widely used for inducing spawning in various cyprinids in aquaculture (LINPE method) (75), we could also identify more potent molecules, especially eticlopride, which could now be tested *in vivo* on eels and other teleosts.

From a basic point of view, the present study also opens new research avenues. In particular, the presence and possible coexpression of two D2-Rs paralogues by the same gonadotroph cells in teleosts is worthy of future investigations concerning their potential interactions. For example, mammalian D2/D3 receptors may form heterologous dimers (76,77) with specific properties compared to monomers or homodimers. D2A-R and D2B-R heterodimerisation would thus provide another route for further diversifying their binding, coupling profiles and intracellular signals.

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Disclosure

The authors declare that they have no competing financial interests.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Gαo- and Gαi-coupling to recombinant eel D2A and D2B receptors.

