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Novel Identification of Peripheral Dopaminergic D2 Receptor in Male Germ Cells

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Abstract Dopamine is a recognized modulator in the central nervous system (CNS) and peripheral organ functions. The presence of peripheral dopamine receptors outside the CNS has suggested an intriguing interaction between the nervous system and other functional systems, such as the reproductive system. In the present study we analyzed the expression of D2R receptors in rat testis, rat spermatogenic cells and spermatozoa, in different mammals. The RT-PCR analysis of rat testis mRNA showed specific bands corresponding to the two dopamine receptor D2R (L and S) isoforms previously described in the brain. Using Western blot analysis, we confirmed that the protein is present in rat testis, isolated spermatogenic cells and also in spermatozoa of a range of different mammals, such as rat, mouse, bull, and human. The immunohistochemistry analysis of rat adult testis showed that the receptor was expressed in all germ cells (pre- and post-meiotic phase) of the tubule with staining predominant in spermatogonia. Confocal analysis by indirect immunofluorescence revealed that in non-capacitated spermatozoa of rat, mouse, bull, and human, D2R is mainly localized in the flagellum, and is also observed in the acrosomal region of the sperm head (except in human spermatozoa). Our findings demonstrate that the two D2 receptor isoforms are expressed in rat testis and that the receptor protein is present in different mammalian spermatozoa. The presence of D2R receptors in male germ cells implies new and unsuspected roles for dopamine signaling in testicular and sperm physiology. J. Cell. Biochem. 100: 141–150, 2007.

Key words: D2 receptors; spermatozoa; male germ cells

Dopamine is the predominant catecholamine neurotransmitter in the central nervous system (CNS) and is involved in neurological and psychiatric disorders, including Parkinson's disease and schizophrenia [Missale et al., 1998]. The D2-like dopamine receptors (including D2, D3, and D4) share a high affinity for antipsychotic drugs and couple through pertussis toxin-sensitive G proteins ($G_{i/o}$). Two isoforms of the D2 receptor [the long form (D2L) and the short form (D2S)] have been identified [Dal Toso et al., 1989; Giros et al., 1989; Monsma et al., 1989; Wolfe et al., 1999]. The D2L and D2S receptors are generated from the same gene by alternative splicing. The D2L receptor has a 29 amino acid insertion in the third cytoplasmic loop of the protein. This insertion is absent in the D2S receptor. The third intracellular loop of G-protein-coupled receptors (including D2R) has been shown to be critical for interaction with intracellular effectors [O'Dowd et al., 1988; Missale et al., 1998].

Dopamine is also a recognized modulator of peripheral organ function. The peripheral location of dopamine receptors and significant physiological effects of dopaminergic agents on renal, adrenal, and cardiovascular function have been documented [Missale et al., 1998]. The presence of dopamine receptor subtypes

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outside the CNS has been shown by pharmacological and biochemical techniques [Missale et al., 1998; Hyun et al., 2002]. Dopamine receptors have been identified in kidney, heart, and lymphocytes [Ozono et al., 1996; Barili et al., 1998; Caronti et al., 1998; Missale et al., 1998; Amenta et al., 1999].

The mammalian ovary and testis are regulated by gonadotropic hormones and are also subject to regulatory influences by locally produced factors, including neurotransmitters [Skinner, 1991; Mayerhofer et al., 1996, 1999a; Ojeda et al., 1996; Gnessi et al., 1997]. Catecholamines are likely to play a special part in this "neuroendocrinotrophic stimulatory complex" [Gnessi et al., 1997]. The catecholamines found in the gonads may be derived from sympathetic innervation or from the adrenal gland via the blood stream [Campos et al., 1990; Prince, 1992, 1996; Rauchenwald et al., 1995; Stjernquist, 1996]. In human and non-human primate species, neuron-like cells identified as novel member of the interstitial cell compartment expressing neuron-specific intermediate filament (NF-200) and tyrosine hydroxylase (TH) could be another potential source of catecholamines present in the ovary and testis [Dees et al., 1995; Mayerhofer et al., 1996, 1998, 1999b, Frungieri et al., 2000]. Studies done in testis, showed TH immunoreactivity in Leydig cells but did not show only in the morphologically distinct neuron-like cells. However, in a recent study, the catecholaminergic phenotype of human Leydig cells was suggested [Romeo et al., 2004]. These new putative intratesticular source of catecholamines together with innervation and through blood stream may be involved in spermatogenic regulation. Additionally, it has been demonstrated that equine ovarian tissues express D1 and D2 dopamine receptors, suggesting that dopamine can act directly on the female reproductive organ [King et al., 2005]. The presence of dopamine receptors in ovarian tissues could indicate that dopamine may be modulating cellular function possibly through mechanisms also present in the male reproductive tissues.

Dopamine also plays a critical role in promoting sexual drive and penile erection. In a previous study, dopamine D1 and D2 receptor mRNAs and their corresponding proteins were detected in rat corpus cavernosum [Hyun et al., 2002]. Dopamine and apomorphine (a dopamine agonist) induce penile erection in humans and rats, and a stretching-yawning syndrome in normal rats. Hull et al. [1992] reported that in male rats, low levels of dopaminergic stimulation (via the D1 receptor) increased erection, and higher levels of prolonged stimulation produced seminal emission (through D2 receptors).

In an attempt to define the role of dopamine and its receptors in rat gonads, we have here evaluated the expression and anatomical location of peripheral dopamine D2 receptor in rat testis germ cells and in mammalian spermatozoa.

MATERIALS AND METHODS

Sample Collection

Male Sprague–Dawley rats (body weight 300–350 g) were anaesthetized by an intraperitoneal injection with pentobarbital sodium (30 mg/kg). The testis and other control tissues were excised and washed in an ice-cold 0.9% NaCl solution to remove blood traces. The extracted tissues were used for RNA and protein extraction; or fixed by immersion in 4% paraformaldehyde (24 h) for immunohistochemistry. Rat and mouse spermatozoa were isolated from the epididymis of adult male and human semen was collected in sterile plastic containers from healthy young men [Angulo et al., 1998; Zambrano et al., 2001]. Ethical approval was obtained by written consent according to the regulations of the Ethics Committee from the Universidad Austral de Chile. Bovine spermatozoa ejaculates were obtained from the Centro de Inseminación Artificial, Universidad Austral de Chile.

Spermatogenic Cell Preparation

Rat spermatogenic cell populations were prepared from Sprague–Dawley rat testis as described by Romrell et al. [1976]. The pachytene spermatocyte ($85 \pm 5\%$ purity) and round spermatid fractions ($92 \pm 4\%$ purity) were identified both by their size as well as by the typical aspect of their nucleus stained with H33342 [Reyes et al., 1997]. The rat spermatid fraction contained cells between stages 1 and 7. Our method of vital cell identification does not allow for classification of rat spermatids at these stages of development.

Neuronal Primary Cultures

Neurons were obtained from 17-day-old embryos from Sprague–Dawley rats. The forebrains

were removed and neocortex was dissected. Tissue was digested for 15 min with 0.12% trypsin (wt/vol, Gibco-BRL, Rockville, MD) in 0.1 M phosphate buffer (PBS, pH 7.4, 320 mOsm) and triturated to homogeneity with a fire-polished Pasteur pipette. Neurons were plated at 0.3×10^6 cells/cm^2 in plates coated with poli-L-lysine (molecular weight > 350 kDa, Sigma, St. Louis, MO). After 20 min, floating cells were removed and attached cells were cultured for 5 days in Neurobasal Medium (Gibco-BRL) supplemented with B27 (Gibco-BRL) and 293 mg/ml L-glutamine (Nalgene, Rochester, NY).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were isolated by the method of Chomczynski and Sacchi [1987] from rat testis, neuronal primary cultures, and total brain. Only RNA samples that yielded intact 18S and 28S bands with the expected band ratio were included in subsequent experiments. The RT reaction was performed in a reaction mixture of 20 μl total volume containing 2 μg total RNA of each sample, 200 U Moloney murine leukemia virus reverse transcriptase (BioLabs, New England), 1 mmol/L each of the dNTPs (dATP, dCTP, dTTP, and dGTP), 20 U of ribonuclease inhibitor, 0.5 μg oligo (dT) primer, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCL, 10 mmol/L dithiothreitol, and 3 mmol/L $MgCl_2$. The RT mix was incubated in a thermal cycler at 42°C for 50 min, followed by enzyme inactivation at 70°C for 15 min. Subsequent PCRs were carried out in the presence of 1 μmol/L sense and antisense primer, 3 mmol/L $MgCl_2$, 0.4 μl Taq DNA polymerase (Invitrogen, Los Angeles, CA), 1 μl of each dNTP (10 mmol/L), 2.5 μl of 10× buffer and 5 μl of template cDNA, in a total volume of 25 μl. The primers used in this study were obtained from Wang et al. [2000] and sequences used for PCR amplification are sense primer complementary to exon 5 [5′-(GAG TGT ATC ATT GCC AAC CCT GCC)-3′] and antisense primer complementary to exon 7 [5′-(TGG TGC TTG ACA GCA TCT CC)-3′] with 100% homology on the rat D2 gene; primers for rat β-actin were used as a positive control in each sample (data not shown). Conditions for the PCR were denaturation at 94°C (45 s), annealing at 60°C (45 s), and extension at 72°C (45 s) for 35 cycles for rat dopamine receptor D2. The PCR products were analyzed on 1.5% agarose gels, which were subsequently stained with ethidium bromide and visualized under ultraviolet light.

Immunoprecipitation

Isolated spermatogenic cells and spermatozoa were washed three times with a solution containing 150 mM NaCl, 10 mM sodium phosphate (PBS), pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. Tissues and cells were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and protease inhibitors). The cell lysate was pre-cleared with protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) for 60 min at 4°C. For immunoprecipitation, equal amounts of protein (400 μg of total cell lysate) were incubated overnight at 4°C with 2 μg of rabbit polyclonal antibodies anti-D2R (D2R11-A; Alpha Diagnostic Intl., Inc., San Antonio, TX), followed by addition of Protein A-Sepharose beads and incubated for a further 2 h at 4°C. Bound immune complexes were washed three times with lysis buffer containing protease inhibitors and detergents. The pellet was eluted by boiling for 5 min with 2X Laemmli sample buffer [Laemmli, 1970]. Supernatant proteins were separated by SDS–PAGE as below, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA), and immunoblotted with mouse monoclonal antibodies anti-D2R (sc-5303/B-10; Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoblotting

For the Western blot analysis, rat testis and brain tissues were homogenized separately in a buffer containing 0.32 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, and 10 μl/ml protease inhibitor. The homogenate was centrifuged at 900*g* for 10 min, and the resulting supernatant centrifuged at 100,000*g* for 1 h. The final pellet was resuspended in a buffer containing 50 mmol/L Tris-HCl, 10 mmol/L EDTA, 100 mmol/L NaCl, and 8 mmol/L $MgCl_2$, pH 7.4 and protein concentration was determined. After denaturing extracted membrane protein at 95°C for 3 min in sample buffer, membrane proteins (100 μg/lane) were separated using 10% discontinuous SDS–PAGE. The resolved membrane proteins were transferred to a PVDF membrane and then soaked in

5% non-fat dried milk in Tris-buffered saline containing Tween-20 (TBS-T; 10 mmol/L Tris-HCl, pH 7.2, 250 mmol/L NaCl, 0.05% Tween-20) at 4°C overnight. The membrane was incubated with rabbit anti-D2 receptor polyclonal primary antibody (1:1,000 dilution in PBS-T) at room temperature for 1 h, then incubated with a biotinylated secondary antibody (1:5,000 dilution in PBS-T) at room temperature for 1 h, and reacted with peroxidase-conjugated streptavidin at room temperature for 1 h. Specific bands were visualized by ECL® (enhanced chemiluminescence; Amersham Biosciences, Arlington Heights, IL) [Rauch et al., 2004]. As controls, membranes were incubated with antibodies pre-absorbed with the respective peptide used to generate the antibodies. Specifically, primary antibody was pre-incubated overnight at 4°C with a tenfold molar excess of peptide synthetic (Alpha Diagnostic Intl., Inc.), and the pre-adsorbed antibody was used to probe Western blots.

Immunostaining Procedures

For immunoperoxidase localization, rat testis and isolated germ cells were treated with 0.3% H_2O_2 for 5 min and incubated for 60 min at room temperature in 5% BSA-PBS pH 7.4, followed by incubation overnight at 4°C with anti-D2 receptor polyclonal primary antibody (1:50 dilution) in 1% BSA-PBS pH 7.4 and 0.3% Triton X-100. Tissues and cells were washed and incubated with anti-rabbit IgG-horseradish peroxidase (1:100, Amersham Biosciences) for 2.5 h at room temperature. Immunostaining was developed using 0.05% diaminobenzidine and 0.03% H_2O_2. Cells and tissues were counterstained with hematoxylin. As controls, cells and sections were incubated with antibodies pre-absorbed with the respective peptide used to generate the antibodies. Stained sections were examined with a Zeiss Axioskope II microscope equipped with a digital video camera (NikonDXM1200). Spermatocytes, spermatids, and spermatozoa were washed three times with 1X PBS pH 7.4, 1 mM PMSF at 4°C and analyzed by confocal laser scanning fluorescence microscopy using an anti-rabbit IgG-Alexa 488 (1:300, Invitrogen) and subsequently washed and mounted. Stained cells were examined with an Olympus Fluoview FV1000 laser scanning confocal microscope. The images obtained were processed with Adobe Photoshop 6.0.

Other Procedures

Protein concentration was determined by the method of Bradford [1976] using a commercial kit (Bio-Rad Laboratories; Hercules, CA).

RESULTS

Expression of Dopaminergic D2 Receptor in Rat Testis

The effectiveness of D2 receptor specific primers was checked by performing RT-PCR studies with total RNA extracted from rat brain and cultured neurons as positive controls (Fig. 1A, lanes 1 and 2). When RT-PCR was performed with poly (A) RNA from brain, cultured neurons, and testis, an amplification of two specific products differing by 87 base pairs and corresponding to the small (234 bp) and large (321 bp) D2 receptor transcripts were detected (Fig. 1A, lanes 1, 2, and 4, respectively). No products were detected when reverse transcriptase was omitted from the RT-PCR reaction, demonstrating that amplified products are indeed from cDNA and not from genomic DNA contamination (data not shown). Figure 1B shows the Western blot analysis of membrane proteins extracted from rat brain and testis (lanes 1 and 2, respectively). The D2 receptor antibody reacted with two bands that migrated with an apparent Mr of 47–50 kDa and bands with a higher molecular mass which could correspond to oligomerizations described previously for D2 receptor in other cell systems [Zawarynski et al., 1998; Lee et al., 2000]. The peptide pre-adsorbed antiserum control showed no stain for D2R testis membrane proteins (lane 3).

Localization of Dopaminergic D2 Receptor Protein in Spermatogenic Cells

Immunohistochemical staining was used to localize the peripheral dopamine D2 receptor in adult rat testis. Immunoreactive staining showed that the receptor was localized in spermatogonia as well as other germ cells in the tubule (Fig. 2A). D2 receptor detection was confirmed by immunocytochemical staining and immunofluorescence of isolated pachytene spermatocytes and spermatids, showing an intense immunoreaction at the membrane and intracellularly (Fig. 2C,E,G). Negative results were obtained when testis sections and cells

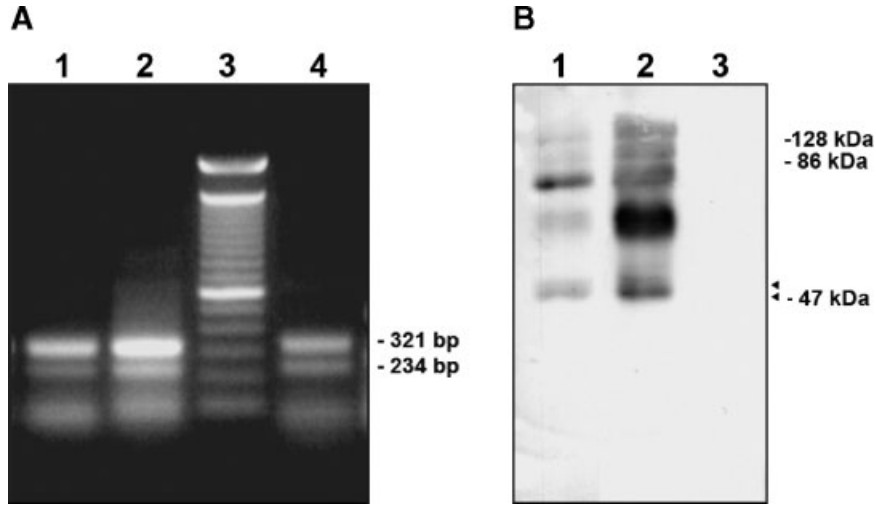


Fig. 1. Expression of D2 receptor in rat male germ cells. **A**: Total RNA extracted from brain (**lane 1**), cultured neurons (**lane 2**), and testis (**lane 4**) was subjected to RT-PCR using primers specific for D2 receptor. PCR products corresponding to the small (234 bp) and large (321 bp) D2 receptor transcripts are shown. The migration in the agarose gel of a series of DNA 100-mer size standards is shown in **lane 3**. **B**: Membrane proteins isolated from rat brain (**lane 1**), and rat testis (**lanes 2** and **3**) were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, transferred to PVDF membranes, and probed with anti-D2 receptor antibody (**lanes 1** and **2**) and pre-adsorbed anti-D2 receptor antibody (**lane 3**), followed by incubation with a secondary antibody coupled to peroxidase. ECL sizes on the right are kDa and indicate the migration of molecular mass standards.

were incubated with the pre-adsorbed antibody (Fig. 2B,D,F,H).

Immunofluorescent Localization of D2 Receptor in Spermatozoa

Fluorescent immunolocalization of D2 receptors in rat, mouse, bull, and human spermatozoa demonstrated that they express dopamine receptor D2 showing a unique pattern along the tail and acrosomal region (Fig. 3B–E, respectively). Rat neuronal primary cultures were used as positive controls (Fig. 3A). No immunoreactive material was seen in samples treated with the pre-adsorbed antibody (Fig. 3A′–E′).

Immunoprecipitation of Dopaminergic D2 Receptor

Immunoprecipitation and immunoblotting of protein extract from rat, mouse, human, and bovine spermatozoa demonstrated a strong stained protein band (Fig. 4, lanes 5, 7–9, respectively). The D2 receptor antibody also reacted with a unique broad band that migrated with an apparent Mr of 50 kDa in proteins extracted from isolated spermatocytes and spermatids, as well as testis and rat and mouse brain, included as positive controls (Fig. 4, lanes 3, 4, 2, 1 and 6, respectively). No immunoreactive bands were detected when pre-absorbed antibodies were used (Fig. 4, lane 10).

DISCUSSION

Dopamine is the predominant catecholamine neurotransmitter in the CNS and is involved in neurological and psychiatric disorders, including Parkinson's disease and schizophrenia. This catecholamine is also a recognized modulator of peripheral organ function [Missale et al., 1998]. The mammalian ovary and testis are regulated by gonadotropic hormones which include neurotransmitters [Skinner, 1991; Mayerhofer et al., 1996, 1999a; Ojeda et al., 1996; Gnessi et al., 1997], such as catecholamines. This may be derived from sympathetic innervation [Gnessi et al., 1997] or from the adrenal via the blood stream. However, considering that the testis shows TH activity, synthesis of dopamine cannot be ruled out at the present time [Mayerhofer et al., 1999b; Romeo et al., 2004]. The actions of dopamine on target tissues are mediated by cell surface receptors, the dopamine receptor family. To date, there are five genes encoding functional dopamine receptors on the human genome. These dopamine receptors have been subdivided into two subclasses referred to as D1 and D2 on the basis of various biophysical and pharmacological characteristics [Missale et al., 1998]. The D2-like dopamine receptors (including D2, D3, and D4) share a high affinity for antipsychotic drugs

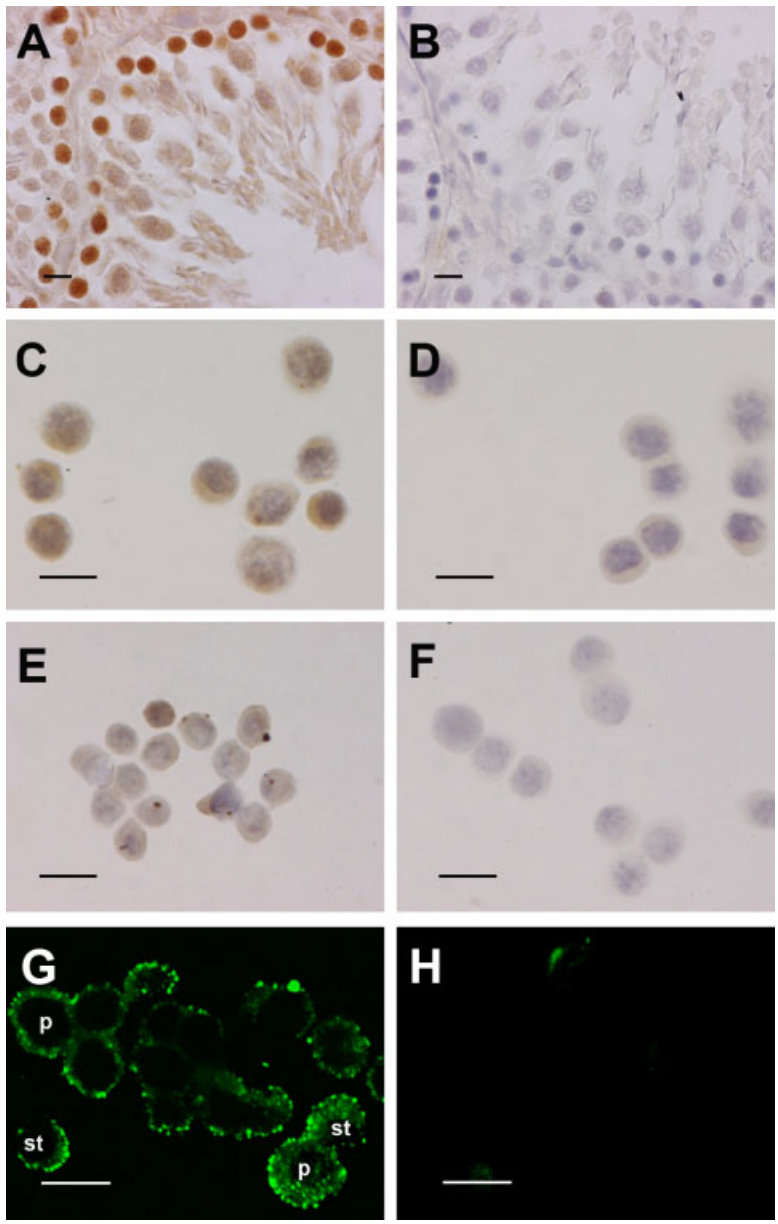
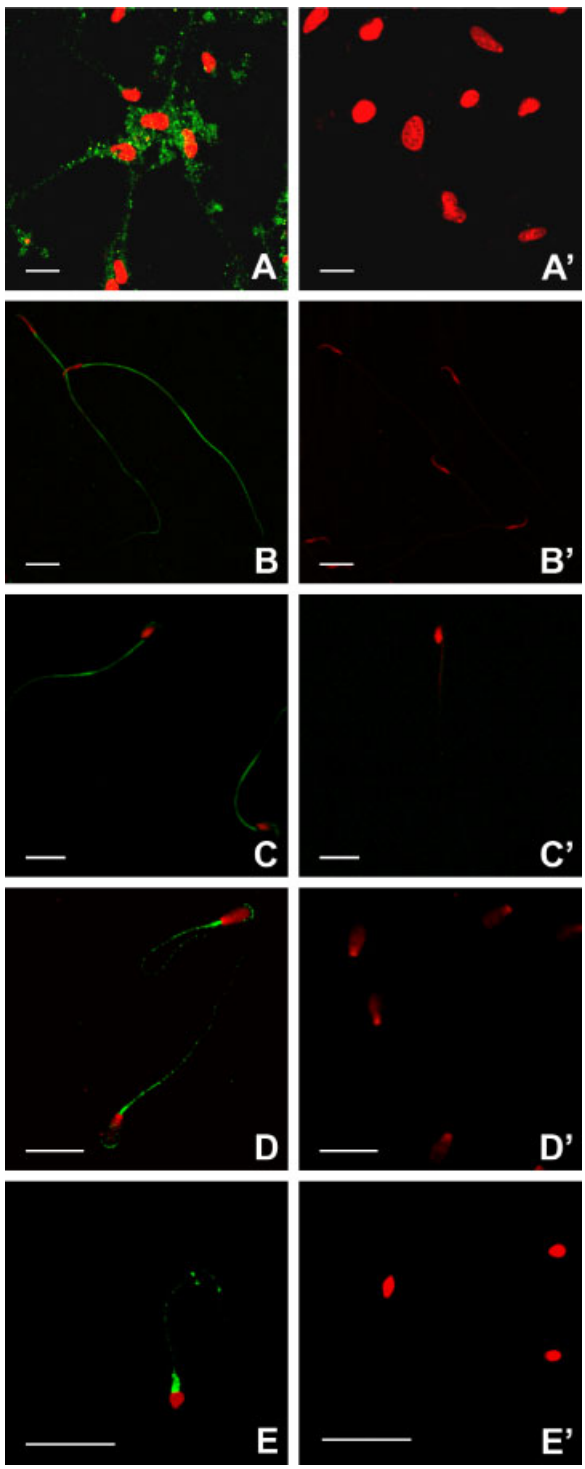


Fig. 2. Localization of D2 receptor in rat testis, spermatocytes, and spermatids. Testis sections from adult rats (**A** and **B**) spermatocytes (**C** and **D**) and spermatids (**E** and **F**) were incubated with D2 receptor antibody followed by incubation with a secondary antibody conjugated to horseradish peroxidase. Nuclei were counterstained with hematoxylin. Confocal laser scanning fluorescence microscopy analysis of spermatocytes (p) and spermatids (st) are shown in **G** and **H**. The sections and isolated male germ cells that were incubated with the pre-adsorbed antibody did not show positive reaction (**B**, **D**, **F**, and **H**). Scale bars, 10 μm. These are representative images from six independent determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and couple through pertussis toxin-sensitive G proteins ($G_{i/o}$). The peripheral location of dopamine receptors and the significant physiological effects of dopaminergic agents on renal, adrenal, cardiovascular function, and corpus cavernosum have been of great interest [Aperia et al., 1987; Siragy et al., 1989, 1990, 1992; Van Woerkens et al., 1991; Kujacic et al., 1995].

In the present study we have identified the peripheral dopamine receptors D2 in all germ cells (pre- and post-meiotic phase) of the tubule (Fig. 2). In later stages during spermatogenesis, D2 receptor localizes at the membrane and intracellularly in spermatids and spermatozoa. Using molecular biology techniques we identified both isoforms of the D2 receptor [the long form (D2L) and the short form (D2S)] in RNA

samples. Immunoblotting analysis with a polyclonal antibody detected two bands with an apparent molecular mass of 47–50 kDa and bands with a higher molecular mass in brain and testis membrane proteins (Fig. 1B), similar to that observed by some authors in other cell systems [Farooqui et al., 1992; Zawarynski et al., 1998; Lee et al., 2000; Scarselli et al., 2001]. This heterogeneity can be explained by homodimerization [Zawarynski et al., 1998; Guo et al., 2003; Lee et al., 2003] and heterooligomerization of the D2 receptor [Rocheville et al., 2000; Canals et al., 2003: Kearn et al., 2005]. Immunoprecipitation detection analysis with a monoclonal antibody only detected a predominant broad band of an apparent molecular mass of 50 kDa that can correspond to both isoforms (Fig. 4). Future studies using a specific antibody (anti-D2L isoform) and optimizing the electrophoresis resolution condition will help to know which D2 receptor isoform is predominant in the testis and sperm.



Spermatogenesis is a complex process of cellular multiplication and differentiation, and its regulation is only partially understood [Parvinen, 1982; Kierszenbaum, 1994]. In addition to the role of pituitary hormones (mainly FSH and LH), a multitude of cell–cell interactions are also involved in this regulation process, which involves soluble and membrane bound factors produced by both somatic and germ cells of the seminiferous tubule [Skinner, 1991; Mauduit and Benahmed, 1996]. The presence of dopamine receptors in rat testis germ cells may suggest unknown interactions between the nervous and reproductive systems [Hyun et al., 2002]. This opens many new possibilities for future research, such as studying the activation of D2 receptor by dopamine inside the seminiferous tubules. Until now, there is no conclusive evidence to support catecholaminergic phenotype of Sertoli cells. To solve this problem dopamine should be transported through Sertoli cells by dopamine transporter (DAT) or the vesicular monoamine transporter (VMAT). If dopamine and D2 receptor participate in the proliferation and/or differentiation of the male germ cells or if they only participate in sperm function after release

Fig. 3. Immunolocalization of D2 receptors in rat, mouse, bull, and human spermatozoa. For immunofluorescence, the rat (**B** and **B′**), mouse (**C** and **C′**) bull (**D** and **D′**), and human (**E** and **E′**) spermatozoa were spread onto coated slides and reacted with D2 receptor antibody and visualized using an anti-rabbit IgG-Alexa 488. Rat neuronal primary cultures were used as positive controls (**A** and **A′**). Cells incubated with the pre-absorbed antibody did not show positive reaction (**B**, **D**, **F**, **H**, and **J**). Nuclei were counterstained with propidium iodide, and confocal images were acquired. Scale bars, 20 μm. These are representative images from five independent determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

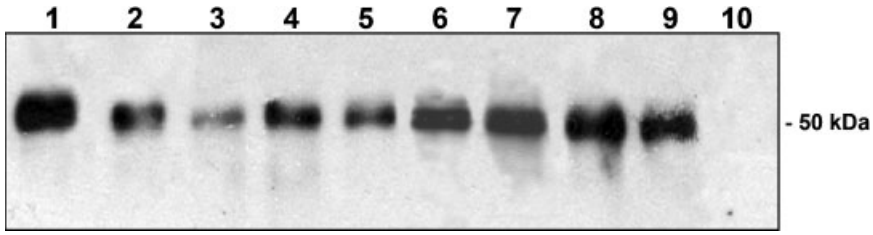


Fig. 4. Immunoprecipitation of D2 receptors in rat, mouse, human, and bull. Total proteins isolated from rat brain, testis, spermatocytes, spermatids, and spermatozoa (**lanes 1–5**, respectively), mouse brain and sperm (**lanes 6**, **7**, respectively), human (**lane 8**), and bull (**lane 9**) spermatozoa were immunoprecipited with rabbit polyclonal anti-D2R antibodies and resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, transferred to PVDF membranes, and probed with mouse monoclonal anti-D2R antibodies, followed by incubation with a secondary antibody coupled to peroxidase. Pre-absorbed polyclonal antibody incubated with the peptides for the D2 receptor and adult rat testis total protein were used as negative controls (**lane 10**). ECL sizes on the right are kDa and indicate the migration of molecular mass standards.

into the female genital tract, will be the basis for future studies of dopamine signaling in these cells.

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