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Dopamine-$_3$ receptor modulates intraocular pressure: Implications for glaucoma

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ABSTRACT

The aim of the present study was to investigate the role of D_3 receptor on intraocular pressure regulation using WT and KO $D_3R^{-/-}$ mice. Both mice were used with normal eye pressure or steroid-induced ocular hypertension. As measured by tonometry, the topical application of 7-OH-DPAT, a dopamine D_3-preferring receptor agonist, significantly decreased, in a dose-dependent manner, the intraocular pressure in WT mice both in an ocular normotensive group and an ocular hypertensive group. Pretreatment with U-99194A, a D_3 receptor antagonist, reverted 7-OH-DPAT induced ocular hypotension in WT mice. No change of intraocular pressure was observed after topical application of 7-OH-DPAT in KO $D_3R^{-/-}$ mice. PCR analysis demonstrated the presence of all dopamine receptor genes in eye tissues obtained from WT mice, and the lack of D_3R mRNAs in KO mice. The present study identified the D_3R subtype as the most important receptor of the dopaminergic system to modulate intraocular pressure with relevant implications for glaucoma that represents one of the most crippling optic neuropathies.

1. Introduction

Glaucoma is a progressive optic neuropathy and is the leading cause of blindness in industrialized countries. Elevated eye pressure is the main risk factor for glaucoma, and intraocular pressure (IOP) is caused by an impaired outflow of aqueous humor resulting from abnormalities within the drainage system of the anterior chamber angle (primary open-angle glaucoma, POAG) or impaired access of the aqueous humor to the drainage system (angle-closure glaucoma, ACG). In Western countries, POAG is much more common than angle-closure glaucoma. Epidemiological studies have shown that the risk of glaucoma increases by 12% with every 1 mmHg increase in IOP [1]. The molecular events responsible for glaucoma are currently poorly understood, complicating the design of therapies based on the underlying disease mechanisms. We know that several systems are implicated in the IOP regulation such as adrenergic, cholinergic, serotonergic, purinergic, and dopaminergic. The latter is still unclear and represents one of the most intriguing systems in view of the value of potential hypotensive effects in pathological conditions such as glaucomatous optic neuropathy. Dopamine is the predominant neurotransmitter in the retina. The role of dopamine in the eye has been studied for the last 30 years, particularly in the retina where it has been highlighted that dopamine plays a key role during retina development. The biogenic amine dopamine has been identified in the aqueous humor [2,3] and various dopamine receptor subtypes are present in ocular tissues [4]. Dopamine exerts its action through five subtypes of receptors, classified into two superfamilies that differ in their binding profiles: D_1-like receptors (D_1 and D_5) are principally coupled to stimulatory Gα-proteins and enhance the activity of adenylyl cyclase (AC), whereas D_2-like receptors (D_2, D_3, and D_4) are primarily coupled to inhibitory Gα-proteins and suppress the activity of AC. D_2 and D_3 receptors display a high degree of sequence similarity, and they share a predicted binding site for dopamine and synthetic ligands at the interface of transmembrane helices [5]. D_2 and D_3 receptors also show similar patterns of signal transduction, though under certain conditions the latter couple less broadly and robustly to intracellular messengers like AC [6,7]. Classical D_2 receptor agonists such as bromocriptine, lergotrile, lisuride, pergolide, cianergoline and cabergoline have been shown to elicit ocular hypotension in a number of species [8,9,11–17]. Two dopaminergic agonists, bromocriptine and SDZ-GLC-756, administered topically in the human eye were shown to lower IOP [16,18,19], while D_1 receptor agonists, ibopamine and fenoldopam, increased IOP in glaucomatous and ocular normotensive patients apparently stimulating aqueous humor production [20,21].

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We believe that the dopaminergic system has an important role in the regulation of IOP, and that the D_3 receptor is the key binding site in IOP-lowering effects. Because the sequence similarity between D_2 and D_3 receptors and the lack of selectivity of the above mentioned IOP-lowering dopaminergic agonists, it has not been possible, so far, to figure out the real contribution of these two receptors on IOP regulation. Therefore, the aim of the present study was to investigate the precise role of D_3 receptor on IOP modulation using KO $D_3R^{-/-}$ mice and D_3 selective receptor ligands.

2. Materials and methods

2.1. Animals

All experiments were carried out on $D_3R^{-/-}$ and WT littermates (male mice 8–12 weeks old). The animals were fed on standard laboratory food and were allowed free access to water in an air conditioned room with a 12-h light/12-h dark cycle. All the experimental procedures were performed during the light cycle. $D_3R^{-/-}$ mice used in these experiments were 5th–8th generation of congenic C57BL/6J mice, and generated by a backcrossing strategy. These $D_3R^{-/-}$ mice were created by Accili et al. [22]. The genotypes of the dopamine D_3 receptor mutant and WT mice were identified by a PCR method with two pairs of primers flanking either exon 3 of the wild-type dopamine receptor D_3 or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene [22]. All the animals were treated according to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research, and the Directive 2010/63/EU of the European Parliament and of the Council.

2.2. IOP measurement

Similar to the procedure previously described by Ding et al. [23] we trained mice for awake IOP measurements. IOP (mmHg) was measured before and after the drug treatment using a Tono-Pen XL tonometer (Mentor Corp., Norwell, MA, USA) calibrated according to the manufacturer's instructions [24]. Ten microliters of 0.4% oxybuprocaine hydrochloride were applied to the cornea to minimize any discomfort to the animal. IOP was measured both in the treated (OD) and the contralateral (OS) eye. Three baseline readings were taken 60, 30 and 0 min before the drug administration. IOP determination was made 30, 60, 120 and 240 min after the topical application of the drug in the right eye. IOPs of animals were measured between 10 am and 12.30 pm. All measurements, under the same environmental conditions, were made by the same operator blind to treatment.

2.3. Drugs and treatment

The following compounds were used: 7-OH-DPAT·HBr (7-hydroxy-2-dipropylaminotetralin hydrobromide); U99194A maleate were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were procured from standard commercial suppliers unless otherwise noted. Pharmacological treatments were performed after a baseline IOP measurement, defined as three baseline readings. The dopamine D_3 agonist 7-OH-DPAT was diluted in sterile phosphate buffered saline (PBS) and unilaterally instilled in the right eye at 0.01, 0.1, 1 and 5% (w/v) in a volume of 10 μl. The D_3 antagonist U99194A maleate was diluted in sterile PBS and instilled (1% (w/v), 10 μl) 30 min before the application of 7-OH-DPAT. In each experimental group, animals received either drugs or the appropriate vehicle. The animals were randomly assigned to treatment groups (n = 6–7) and were used only once.

2.4. Animal model of steroid-induced ocular hypertension

As previously described by Whitlock et al. [25], Alzet micro-osmotic pumps (Model 1004, DURECT Corp., Cupertino, CA, USA) were filled with PBS containing dexamethasone (Sigma–Aldrich, St. Louis, MO, USA) or PBS alone (sham). Dexamethasone (DEX) was formulated at a concentration of 34.5 mg/ml (w/v). The flow rate for the micro-osmotic pumps was 0.11 μl per hour, which delivers 0.09 mg of DEX per day. Animals were anesthetized with Zoletil 100 (tiletaminehydrochloride + zolazepan hydrochloride, Virbac, Milan, Italy) during implantation of osmotic mini-pumps delivering DEX or PBS-filled pumps (controls). Loss of consciousness was determined by a toe pinch. Surgical instruments were sterilized before use. The surgical area was shaved to remove excess fur and sanitized with 70% isopropyl alcohol and iodine. A small incision was made midline at the base of the scapula. Using a sterile hemostat, a small pocket was made subcutaneously along the side of the animal and pumps were inserted with the flow moderator pointed posterior away from the surgical site. The tissue was pulled together and blotted dried. Tissue bond adhesive (3 M, St. Paul, MN, USA) was placed on the surgical site and allowed to dry. Mice were then single housed and placed on a heating pad to recover. Baseline IOP values were obtained between 8 and 11 am one day prior to the pump implantation surgery. Weekly IOP measurements were made on several cohorts in the study to determine the onset of DEX-induced elevation in IOP. The final IOP is obtained at comparable times during the fourth week following surgery. For each animal, the IOP is presented as the average of 3 measurements taken from the right eye.

2.5. Analysis of mRNA expression by RT-PCR

Iris-ciliary body (ICB) tissue samples were pooled and total RNA extracts were isolated by 1 ml TRIzol reagent (Invitrogen, Milan, Italy) and 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. Pellet was washed with 75% ethanol and air dried. Single stranded cDNAs were synthesized incubating total RNA (5 μg) with SuperScript III RNase H-reverse transcriptase (200 U/μl) (Invitrogen, Milan, Italy); Oligo-(dT)20 primer (100 nM) (Invitrogen, Milan, Italy); 1 mM dNTP mix (Invitrogen, Milan, Italy), dithiothreitol (DTT, 0.1 M), recombinant RNase-inhibitor (40 U/μl) at 42 °C for 1 h in a final volume of 20 μl. Reaction was terminated by incubation of samples at 70 °C for 10 min. Aliquots of cDNA were amplified using specific primers for D_1R, D_2R, D_3R, D_4R, D_5R and S18 ribosomial subunit. Oligonucleotide sequences are listed in Table 1. Each PCR reaction contained 0.4 μM specific primers, 200 μM dNTPs, 1.25 U AmpliTaq Gold DNA polymerase and GeneAmp buffer containing 2.5 mM $MgCl^{2+}$ (Applied Biosystem, San Francisco, CA, USA). PCR was performed using the following three cycle programs: (1) denaturation of cDNA (1 cycle: 95 °C for 12 min); (2) amplification (40 cycles: 95 °C for 30 s, 59 °C for 30 s, 72 °C for 45 s); (3) final extension (1 cycle: 72 °C for 7 min). Amplification products were separated by electrophoresis in a 1.8% agarose gel in 0.045 M Tris-borate/1 mM EDTA (TBE) buffer.

2.6. HPLC analysis

Mouse ICB levels of 7OH-DPAT were determined by high-performance liquid chromatography (HPLC). HPLC apparatus consisted of a Hewlett-Packard HP 1100 system interfaced with the HP Chemstation software and equipped with a binary pump G1312A, a UV–visible diode array (UV-DAD) detector G1315A, a thermostated column compartment G1316A and a Rheodyne sample injector with a 10 μl loop. A LiChroCART® 125-4 reverse-phase column packed with LiChrosfer® 60 RP-select B (125 mm × 4 mm i.d., 5 μm, Merck, Milan, Italy) with a LiChroCART® 4-4 precolumn packed with

Table 1
Primer sequences.

Gene	Forward	Reverse	bp length
Dopamine D_1 receptor Acc# NM_010076.1	GAGCAGGACATACGCCATTT	GCTTCTGGGCAATCCTGTAG	101
Dopamine D_2 receptor Acc# NM_010077.1	TGCCATTGTTCTTGGTGTGT	GTGAAGGCGCTGTAGAGGAC	111
Dopamine D_3 receptor Acc# NM_007877.1	GGGGTGACTGTCCTGGTCTA	AAGCCAGGTCTGATGCTGAT	110
Dopamine D_4 receptor Acc# NM_007878.2	CTGCAGACACCCACCAACTA	CCTGGACCTCGGAGTAGACA	100
Dopamine D_5 receptor Acc# NM_013503.1	GGCTATTTCCAGACCCTTCC	TGAGTTGGACCGGGATAAAG	116
Ribosomal protein S18 Acc# NM_011296.2	GAGGATGAGGTGGAACGTGT	GGACCTGGCTGTATTTTCCA	115

LiChrosfer® 60 RP-select B was used. The mobile phase consisted of 95% CH_3CN and 5% water containing 0.1% H_3PO_4 and 0.05% TEA. Prior to use, the mobile phase was filtered through a 0.2 μm nylon membrane filter. The UV-DAD detector was set at 282 nm that represents the optimum wavelength to detect 7-OH-DPAT. Chromatography was performed at 25 °C with a flow rate of 1 ml/min. The iris-ciliary body was weighed, added with 100 μl of a mixture of 30% CH_3CN and 70% water containing 0.1% H_3PO_4 and 0.05% TEA and homogenated. The homogenate was centrifuged at 10,000 × *g* for 5 min, then filtered and injected into the HPLC system. The detection limit was 100 ng/ml.

2.7. Statistical analysis

For statistical analysis the GraphPadInstat statistical package (version 5 GraphPad software, San Diego, CA, USA) was used. The data were analyzed by analysis of variance (ANOVA) followed, when significant, by an appropriate post hoc comparison test.

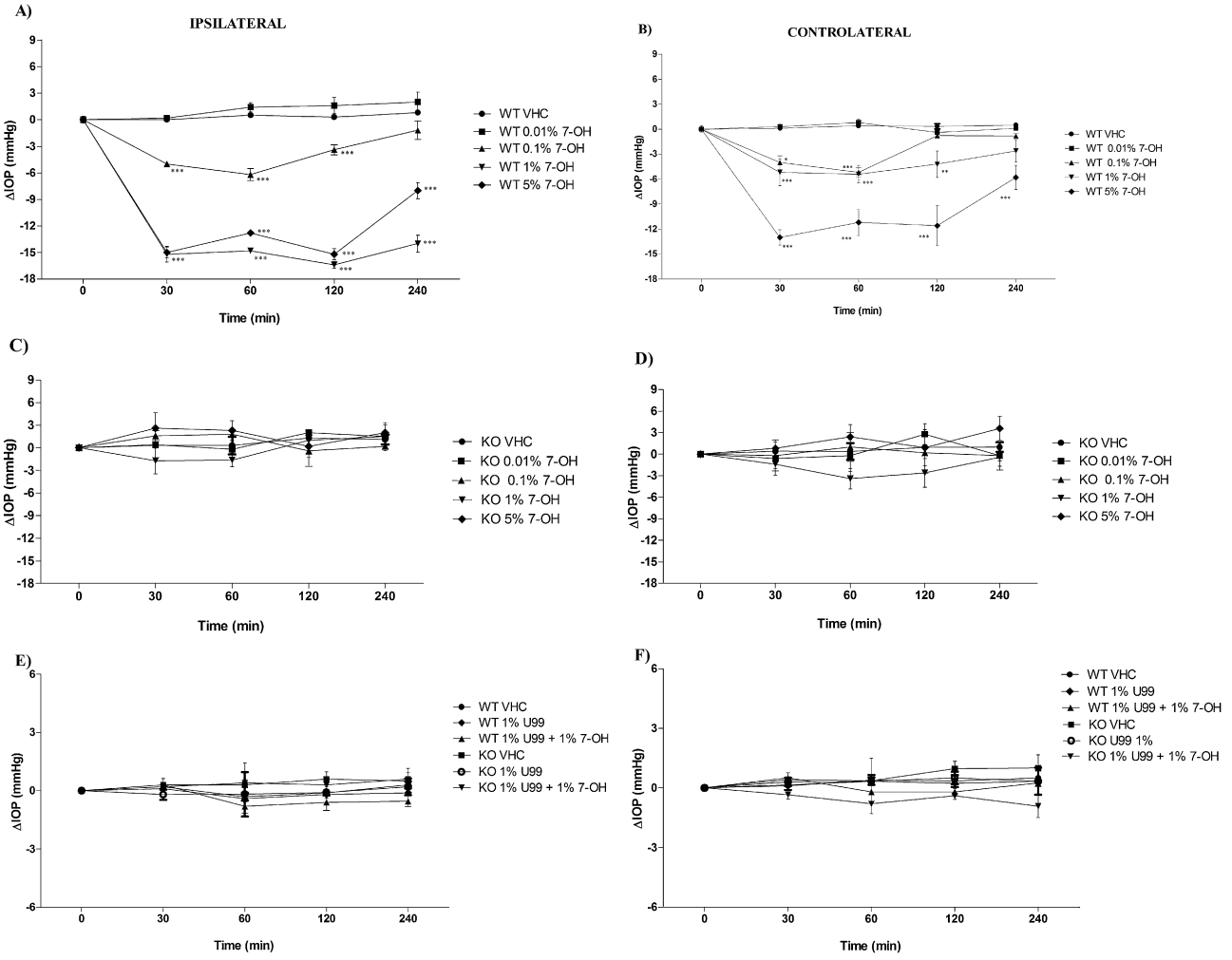

Fig. 1. IOP in ocular normotensive WT and KO $D_3^{-/-}$ mice. Unilateral topical application of the D_3R agonist 7-OH-DPAT (0.01, 0.1, 1 and 5% (w/v)) on IOP of normotensive ipsilateral (treated, Panels A and C) and controlateral (Panels B and D) eyes of WT and KO $D_3^{-/-}$ mice. Unilateral topical application of the D_3R antagonist U 99194A (1%, w/v) plus the D_3R agonist 7-OH-DPAT (1%, w/v) on IOP of normotensive ipsilateral (treated, Panel E) and controlateral (Panel F) eyes of WT and KO $D_3^{-/-}$ mice. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* vehicle (VHC) treated group.

Differences were considered statistically significant when p values ≤ 0.05.

3. Results

3.1. IOP in ocular normotensive WT and KO $D_3R^{-/-}$ mice

As shown in Fig. 1 (Panels A and B), unilateral topical application of 7-OH-DPAT caused dose-related ocular hypotension in ipsilateral (treated) and contralateral eyes of WT mice. The maximum IOP-lowering effect (15 mmHg and 12 mmHg in the ipsilateral and contralateral eyes, respectively), obtained with 1% and 5%, occurred at 30 min and persisted up to 4 h. At 0.01% the maximum IOP-lowering effect (6 mmHg in the ipsilateral eye and no change in the contralateral eye, respectively) occurred at 60 min and returned at baseline level at 4 h. The same D_3R agonist did not cause any effect in terms of IOP modification in D_3R KO mice when topically instilled at different concentrations (Fig. 1C and D), indirectly showing a clear involvement of this receptor subtype in the IOP regulation. To confirm the involvement of a D_3 receptor mechanism on ocular hypotension, experiments were performed using selective D_3 antagonist (U99194A). A bilateral topical pretreatment was carried out with 1% of U99194A followed (30 min) by a subsequent challenge with 7-OH-DPAT. As shown in Fig. 1 (Panels E and F), pretreatment with U99194A completely antagonized the IOP-lowering effect induced by 7-OH-DPAT in WT mice and no difference were observed in KO mice. U99194A, given alone, did not cause any change in terms of IOP (data not shown).

3.2. IOP in ocular hypertensive WT and KO $D_3R^{-/-}$ mice

In order to reduce the number of animals we picked the two doses between the lowest and highest that showed a significant effect on IOP in ocular normotensive WT mice. Topical administration of 1% 7-OH-DPAT in steroid-induced ocular hypertension WT mice caused a decrease of IOP in ipsilateral (14 mmHg) and contralateral (10 mmHg) eyes (Fig. 2A and B). On the contrary, topical application of 7-OH-DPAT in steroid-induced ocular hypertension KO $D_3R^{-/-}$ mice did not produce any effect on IOP at all doses tested (Fig. 2C and D). Further, experiments were performed using selective D_3 antagonist (U99194A). A bilateral topical pretreatment was carried out with 1% of U99194A followed (30 min) by a subsequent challenge with 7-OH-DPAT both in steroid-induced ocular hypertension WT and KO mice. As shown in Fig. 2 (Panels E and F),

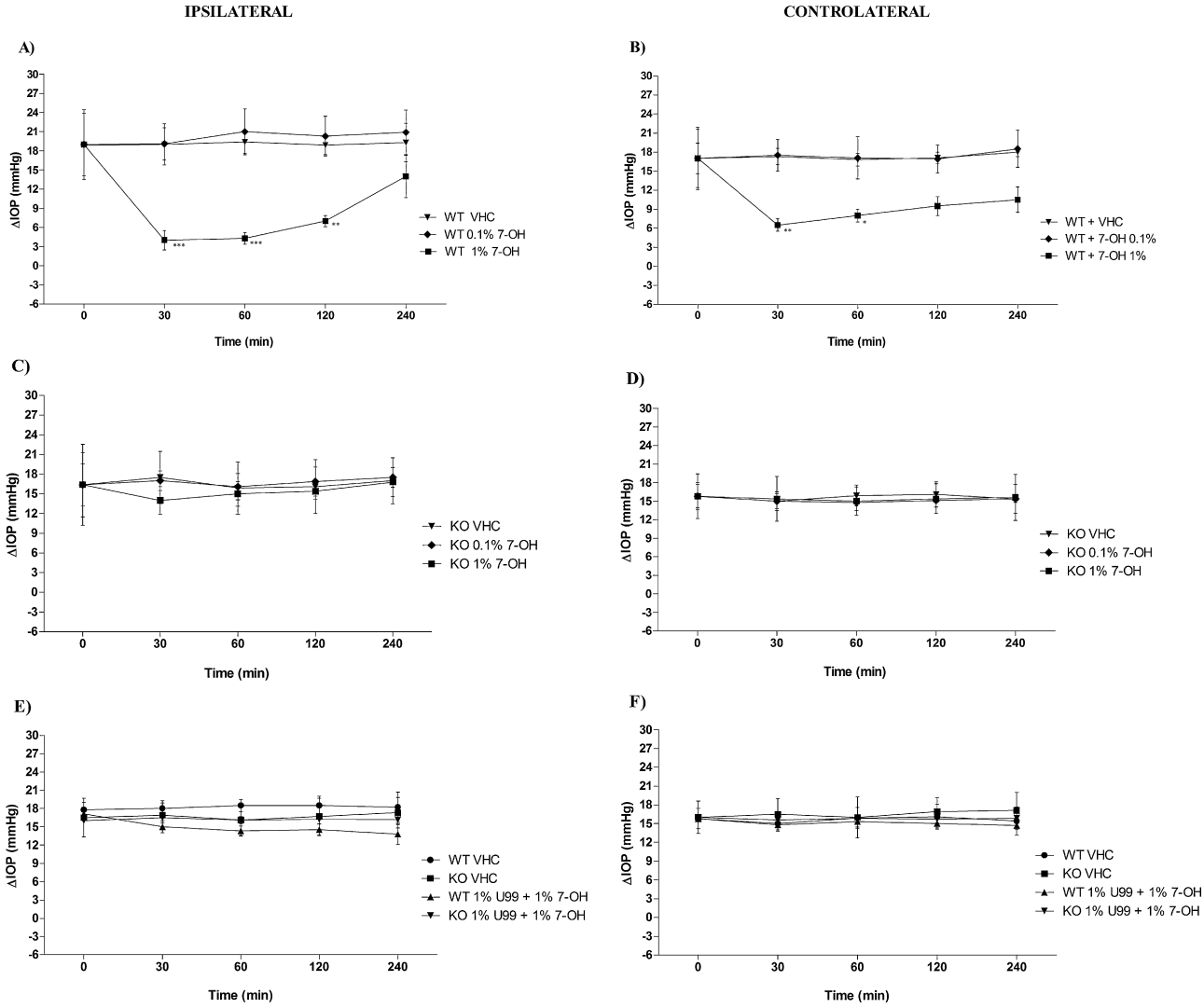

Fig. 2. IOP in ocular hypertensive WT and KO $D_3^{-/-}$ mice. Unilateral topical application of the D_3R agonist 7-OH-DPAT (0.1, 1% (w/v)) on IOP of hypertensive ipsilateral (treated, Panels A and C) and controlateral (Panels B and D) eyes of WT and KO $D_3^{-/-}$ mice. Unilateral topical application of the D_3R antagonist U99194A 1% plus the D_3R agonist 7-OH-DPAT 1% on IOP of hypertensive ipsilateral (treated, Panel E) and controlateral (Panel F) eyes of WT and KO $D_3^{-/-}$ mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* vehicle (VHC) treated group.

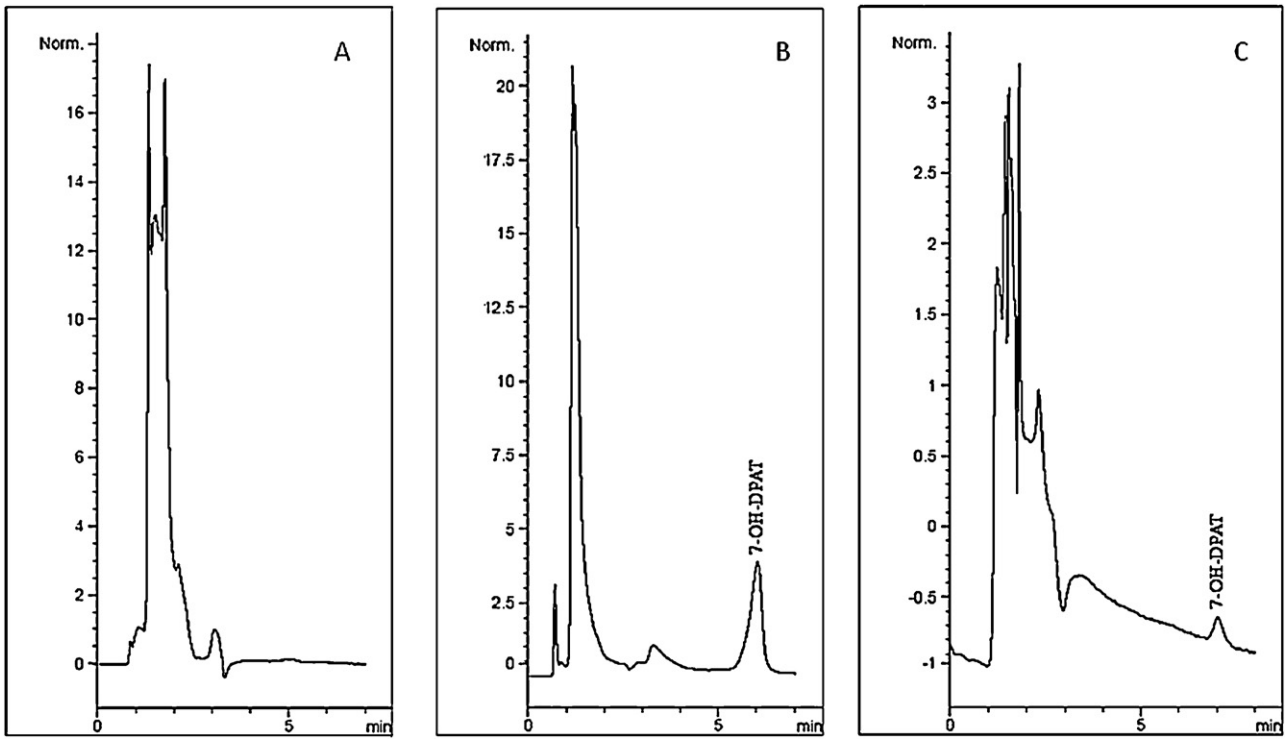

Fig. 3. HPLC analysis. Representative chromatograms of 7-OH-DPAT in mouse iris-ciliary body (ICB) detected by HPLC. Chromatogram obtained from ICB of untreated mouse (Panel A). Chromatogram from mouse ICB obtained 30 min after topical instillation (10 μl) of 0.1% 7-OH-DAPT (ipsilateral eye Panel B; contralateral eye Panel C). Y-axis: milli-absorbance units; x-axis: minutes.

pretreatment with U99194A completely antagonized the IOP-lowering effect induced by 7-OH-DPAT in WT mice and no difference were observed in KO mice. U99194A, given alone, did not cause any change in terms of IOP. This confirms, beyond any reasonable doubt, the involvement of a D_3 receptor mechanism in the ocular hypotension induced by 7-OH-DAPT. U99194A, given alone, did not cause any change in terms of IOP (data not shown).

3.3. HPLC analysis

It is noteworthy that 7-OH-DPAT crosses the corneal barrier and reaches the target tissue after topical instillation. Levels of 7-OH-DPAT (0.147 μg/mg) were detected, by HPLC analysis, in the iris-ciliary body 30 min after single topical administration of 0.1% eye drops. The representative chromatograms are presented in Fig. 3.

3.4. RT-PCR analysis

Identification of dopamine receptors transcripts by RT–PCR analyses were carried out in ICB tissue samples obtained from both WT and D_3R KO mice with the aim of evaluating whether ICB tissues express dopamine receptors mRNAs. Amplification products obtained using specific primer pairs (Table 1) demonstrated that all dopamine receptor genes were expressed in ICB obtained from WT mice (Fig. 4). On the contrary, D_3R mRNAs were not identified in KO mice. Primers for S18 ribosomal subunit were used as control in each PCR amplification and generated bands of the expected length. Forward and reverse primers were selected from the 5′ and 3′ region of each gene mRNA. The expected length of each PCR amplification product is indicated in the right column (Table 1).

4. Discussion

The present study demonstrated that D_3R has an important role to modulate the IOP, and that the topical instillation of a D_3R agonist is able to decrease the IOP in wild-type mice. It is noteworthy that unilateral topical application of a D_3R-preferential agonist (7-OH-DPAT) caused dose-related ocular hypotension in ipsilateral (treated) and contralateral eyes in these mice. This latter evidence is explained by the fact that the instilled agonist reaches the contralateral eye by the systemic blood stream as detected by HPLC analysis. We also demonstrated that 7-OH-DPAT crosses the corneal barrier and reaches the target tissue. Levels of 7-OH-DPAT were detected in the ipsilateral eye after single topical administration of 0.1% eye drops. The same D_3R agonist did not cause any effect in terms of IOP modification in KO mice when topically instilled, indirectly showing a clear involvement of this receptor in the IOP regulation. The PCR confirm that the KO mice lack only for the D_3R receptor and that the other dopamine receptors are present including the D_2R.

IOP remains the major risk factor for glaucoma and its lowering is of priority interest, this is also the reason why there has been considerable attention in the IOP responses to dopaminergic ligands [26]. However, dopamine's involvement in IOP regulation

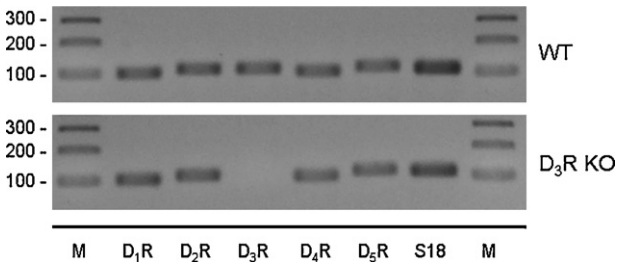

Fig. 4. RT-PCR analysis. Identification of dopamine receptors transcripts in iris-ciliary body tissue samples of WT and KO $D_3^{-/-}$ mice. Aliquots of cDNA were amplified using specific primers for D_1R, D_2R, D_3R, D_4R, D_5R and S18 ribosomial subunit. Amplification products demonstrated that all dopamine receptor genes were expressed in iris-ciliary bodies of WT mice.

and aqueous humor flow (production and outflow) are ambiguous. In 1976 Shannon et al. [27] reported that dopamine caused dose-dependent decreases in IOP in conscious rabbits after topical, intravitreous, and intravenous administration. These data were similar to the results generated by Reitsamer and Kiel [28] obtained during dopamine infusion. Also other authors [29] reported an ocular hypotensive response to intravenous dopamine infusion in anesthetized rabbits. In contrast to the hypotensive response to topical dopamine reported by the above-mentioned groups, Potter and Rowland [30] observed an increase in IOP in response to 2% topical dopamine in conscious rabbits. Similar results were observed by Hariton [31], they evaluated a range of topical doses in conscious rabbits and also found hypertensive responses at higher dopamine concentrations (0.05–1%), but hypotensive responses at lower concentrations (0.005–0.01%). It is noteworthy that most of the studies above mentioned were carried out in rabbits, while mice were used in the present study, therefore we cannot rule out the possibility of species differences in these pharmacological responses.

In order to understand the mechanism/s of these controversial findings we should focus the attention of the reader to the process of aqueous production. Three mechanisms are involved in the aqueous humor formation: diffusion, ultrafiltration, and active secretion. The first two mechanisms are passive and do not entail active cellular participation. There are few studies of dopamine's effects on aqueous production; however, the existing evidence suggests a stimulatory response. Green et al. [32] observed that, in isolated rabbit ciliary epithelium, dopamine increased passive permeability and active secretion, and that H^3-inulin dilution measurements of aqueous flow suggest that intracameral dopamine increases aqueous production in anesthetized rabbits [29]. Chiou and Chiou [33] also suggested that topical dopamine stimulates aqueous production based on its ability to accelerate the recovery of IOP from intravenous hypertonic saline in conscious rabbits. In contrast to these early studies with dopamine, subsequent work with selective ligands for dopaminergic receptor subtypes suggest that dopamine should have a more variable effect on aqueous production. It has been proposed [26] that aqueous production is stimulated by activation of D_1 and inhibited by activation of D_2. Recently, selective activation of D_2 and D_3 receptors was also shown to decrease aqueous production, most likely by postganglionic, prejunctional inhibition of norepinephrine release [34,10]. Reitsamer and Kiel [28] showed that aqueous flow responded to dopamine in a biphasic manner, increasing at the low infusion rate (dopamine 40 μg/min) and decreasing at the high infusion rate (dopamine 600 μg/min). It is noteworthy that dopamine at high doses binds to other receptors (e.g. α- and β-adrenergic receptors) in addition to dopamine receptors. Activation of α_2 receptor in addition to the D_2 stimulation causes inhibition of norepinephrine release, reducing aqueous production as consequence. This would be offset by activation of D_1, α_1, and β_2 receptors, complicating the mechanisms responsible for the decrease in aqueous flow at the high concentration of dopamine.

To complicate the above mentioned frame there are the data generated by Chu et al. [34] that demonstrated the involvement of a specific dopamine's subtype receptor (D_3) to decrease the IOP in rabbits. According to these findings, in the present study we showed that D_3 receptor has an important role in the modulation of IOP both in physiological and pathological conditions. Actually, the social impact of glaucoma is quite relevant considering that this optic neuropathy affects a large number of the population. Therefore, despite several classes of drugs are known to reduce IOP and five of them are used clinically (prostaglandin derivatives, beta blockers, carbonic anhydrase inhibitors, sympathomimetics and miotics), there continues to be a need for more potent agents with fewer undesirable effects. In their compelling work on dopaminergic system and IOP Reitsamer and Kiel [28] concluded that dopamine modulates ciliary blood flow and aqueous production in a dose-dependent manner with a significant decrease of IOP. However, these authors did not investigate the receptors responsible for these effects, and they encouraged further study to identify the receptor subtype. The present study represents the first work that generate a tangible contribute to understand this point, identifying the D_3R subtype as the most important receptor of the dopaminergic system to modulate IOP. Therefore, based on the data produced in this study, and thanks to the animal models and the pharmacological tools used, we concluded that the D_3 receptor has important implications for glaucoma that is the most crippling optic neuropathy.

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