RESEARCH

mPRs represent a novel target for PRL inhibition in experimental prolactinomas

María Andrea Camilletti¹, Alejandra Abeledo-Machado¹, Pablo A Perez², Erika Y Faraoni¹, Fernanda De Fino³, Susana B Rulli¹, Jimena Ferraris⁴, Daniel Pisera⁴, Silvina Gutierrez², Peter Thomas⁵ and Graciela Díaz-Torga¹

¹Instituto de Biología y Medicina Experimental (IBYME), CONICET, Buenos Aires, Argentina

²Centro de Microscopia Electrónica, Instituto de Investigaciones en Ciencias de la Salud (INICSA-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

³Instituto de Investigaciones Farmacológicas, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

⁴Instituto de Investigaciones Biomédicas (INBIOMED), Facultad de Medicina, UBA-CONICET, Buenos Aires, Argentina

⁵Marine Science Institute, University of Texas at Austin, Port Aransas, Texas, USA

Correspondence should be addressed to G Díaz-Torga: gdiaz@ibyme.conicet.gov.ar

Abstract

Membrane progesterone receptors are known to mediate rapid nongenomic progesterone effects in different cell types. Recent evidence revealed that mPR α is highly expressed in the rat pituitary, being primarily localized in lactotrophs, acting as an intermediary of P4-inhibitory actions on prolactin secretion. The role of mPRs in prolactinoma development remains unclear. We hypothesize that mPR agonists represent a novel tool for hyperprolactinemia treatment. To this end, pituitary expression of mPRs was studied in three animal models of prolactinoma. Expression of mPRs and nuclear receptor was significantly decreased in tumoral pituitaries compared to normal ones. However, the relative proportion of mPR α and mPR β was highly increased in prolactinomas. Interestingly, the selective mPR agonist (Org OD 02-0) significantly inhibited PRL release in both normal and tumoral pituitary explants, displaying a more pronounced effect in tumoral tissues. As P4 also regulates PRL secretion indirectly, by acting on dopaminergic neurons, we studied mPR involvement in this effect. We found that the hypothalamus has a high expression of mPRs. Interestingly, both P4 and OrgOD 02-0 increased dopamine release in hypothalamus explants. Moreover, in an *in vivo* treatment, that allows both, pituitary and hypothalamus actions, the mPR agonist strongly reduced the hyperprolactinemia in transgenic females carrying prolactinoma. Finally, we also found an interesting gender difference: males express higher levels of pituitary mPR α/β , a sex that does not develop prolactinoma in these mice models. Taken together, these findings suggest mPRs activation could represent a novel tool for hyperprolactinemic patients, especially those that present resistance to dopaminergic drugs.

Key Words

- ▶ prolactinomas
- ▶ progesterone
- ▶ lactotrophs
- ▶ membrane progesterone receptors

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Introduction

Pituitary tumors are benign adenomas, monoclonal in nature (Melmed 2011), where classic oncogene mutations, metastases and progress to malignancy are rarely observed (Ewing *et al.* 2007, Melmed 2015). Benign pituitary tumors account for approximately 15–25% of intracranial tumors,

and among them, prolactinomas are the most frequently found (40%) (Ciccarelli *et al.* 2005).

These prolactin-secreting adenomas represent an important cause of sexual dysfunction due to the hyperprolactinemia. In addition, these patients may

suffer from other symptoms such as severe headaches and visual disturbances related to the tumor mass effect (Colao *et al.* 2003, Gillam *et al.* 2006). Usually, treatment with dopamine agonists, such as cabergoline, carries a high probability of disease control. However, between 15 and 20% of patients do not normalize their prolactin levels in response to dopamine agonists (Vroonen *et al.* 2012), are resistant to classical pharmacological treatments, their tumors tend to be large and invasive and require extirpation. Therefore, alternative therapies are desired for these patients.

The effect of progesterone (P4) on pituitary tumorigenesis remains poorly understood with reports on both stimulatory and inhibitory effects in different animal models. Piroli *et al.* described that P4 treatment significantly reduces pituitary weight and serum PRL in female rats chronically exposed to diethylstilbestrol (DES rats), by acting directly on the hypothalamus and increasing tyrosine hydroxylase (TH) mRNA, the main enzyme in the synthesis of dopamine (Piroli *et al.* 1996). In support of this finding, it was shown that P4 antagonizes the permissive action of estradiol on tumor necrosis factor- α -induced apoptosis in anterior pituitary cells of ovariectomized (OVX) rats (Candolfi *et al.* 2005), and there is also evidence that the synthetic progestin levonorgestrel reduces the number of proliferating lactotrophs in estradiol-treated rats by diminishing pituitary galanin expression (Piroli *et al.* 2001).

On the other hand, a role for progesterone as a contributor of prolactinoma development was also suggested. Transgenic female mice models with elevated LH or overexpressing the human chorionic gonadotrophin β subunit (hCG β +) develop large prolactinomas despite normal estrogen levels. As both models present persistently high serum P4 levels, it was proposed that elevated P4 levels could be involved in tumor growth (Ahtiainen *et al.* 2010). In addition, other groups showed that, in estradiol primed-animals, progesterone treatment increased PRL release (Caligaris *et al.* 1974, Libertun *et al.* 1979, Cho *et al.* 1993).

In view of the aforementioned results, we postulate that some of the discrepancies found in the literature could be explained by two major facts: (i) the employment of different animal models with substantially different hormonal milieu and (ii) the progesterone receptor type involved in P4 actions.

Regarding the latter, during the last fifteen years many efforts have been made to identify receptors mediating rapid, nongenomic progesterone actions. In 2003,

Dr Thomas and his group first discovered and characterized a novel group of membrane progestin receptors (mPRs) structurally unrelated to nuclear receptors in the fish ovaries (Zhu *et al.* 2003a). These receptors were later identified in mammals, including humans, on the surface of different cell types including gametes, myometrial and vascular muscle cells, cancer cells, ovarian follicle cells, neuronal cells and glial cells, where they elicit rapid biological responses to progestins by mechanisms that do not involve gene transcription (Thomas *et al.* 2007, Dressing *et al.* 2012, Zuloaga *et al.* 2012, Meffre *et al.* 2013, Pang *et al.* 2013, 2015, Castelnovo *et al.* 2019).

The mPRs belong to the progesterone and adipoQ receptor family (PAQR) and have been classified into five subtypes (mPR α , β , γ , δ and ϵ). Among them, mPR α is the most extensively characterized in vertebrates. It was described as an intermediary of many rapid nongenomic progesterone actions including: the regulation of meiosis in oocytes, sperm hypermotility, gonadotropin-releasing hormone (GnRH) secretion, immune function and upregulation of nitric oxide synthesis in human vascular endothelial cells, among others (Karteris *et al.* 2006, Ashley *et al.* 2009, Meffre *et al.* 2013, Pang *et al.* 2015).

Recent evidence revealed that mPRs are also expressed in the rat pituitary gland. We have recently described that the mPR α subtype is the most abundant mPR within the pituitary gland, expressed mainly in lactotrophs, acting in the P4-inhibitory actions on PRL secretion. Moreover, this effect was found to be mediated, at least partially, by activating transforming growth factor β 1 (TGFB1), a known inhibitor of lactotroph function (Camilletti *et al.* 2018). Interestingly, TGFB1 has been proposed recently as a possible target in the treatment of dopamine-agonist-resistant prolactinomas (Recouvreux *et al.* 2016, Faraoni *et al.* 2017).

Since mPR α mediates the P4-inhibitory effect on lactotrophs, we propose that mPR activation may represent an alternative tool for the treatment of pathological hyperprolactinemia.

To this end (1) pituitary expression of mPRs was studied in three experimental models of prolactinomas: Drd2 KO female mice (mice lacking functional dopamine receptor type 2) (Kelly *et al.* 1997), hCG β mice (Rulli *et al.* 2003) and DES rats (Piroli *et al.* 1996) in comparison to their wild-type (WT)/control counterparts and (2) the effect of a selective mPR agonist (Org OD 02-0) (Kelder *et al.* 2010, Pang *et al.* 2013) on PRL secretion and TGFB1 activation in tumoral pituitaries was studied using the Drd2 KO female mice model.

Materials and methods

Drugs

Diethylstilbestrol (DES), a synthetic estrogen: (E)-3,4-bis (4-hydroxyphenyl)-3-hexene, was purchased from Steraloids (Newport, RI, USA); 10-ethenyl-19-norprogesterone Org OD-02-0, a selective mPR agonist, was obtained from Organon (Oss, The Netherlands). Progesterone was purchased from Sigma.

Animals

Rodents were maintained at $22 \pm 2^\circ\text{C}$ and 12 h light–dark cycle, lights on 07:00–19:00 h and provided with food and water *ad libitum*. All the animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Biology and Experimental Medicine (IByME), in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th Ed. 2011, National Academies Press, Washington, DC, USA) and with the European Communities Council Directive of November 2010 (2010/63/UE).

Animals were killed by decapitation. Trunk blood was collected, and serum samples were separated by centrifugation and stored at -20°C for biochemical analyses. Anterior pituitaries and hypothalami were immediately removed and stored in TRIzol reagent (Invitrogen) for posterior RNA isolation and RT-qPCR procedure or in supplement medium for *ex vivo* assays. For HPLC assay, hypothalami were stored at -70°C until use.

Animal models of prolactinoma

Transgenic D2 dopamine receptor-deficient mice (Drd2 KO mice)

The official strain designation is B6; 129S2-Drd2^{tm1low} (JAX stock #003190). In Drd2-knockout mice (KO) both isoforms of dopamine receptor (D2L and D2S) are nonfunctional. Due to the lack of dopaminergic inhibitory control females, but not males, develop lactotroph hyperplasia (Kelly *et al.* 1997). Animals were used at 8 months old, at which time pituitaries from Drd2 KO females were hyperplastic. WT littermates were used as controls.

Transgenic mouse model overexpressing the hCG β subunit (hCG β + mice)

These mice overexpress the hCG β subunit under the control of human ubiquitin C promoter (hCG β +) and

belong to FVB/N background. hCG β + males are fertile, with normal spermatogenesis but reduced serum FSH levels and testis size (Rulli *et al.* 2003). On the other hand, females present precocious puberty, enhanced ovarian steroidogenesis, infertility and develop prolactinomas at 6 months, and mammary tumors at 9–10 months (Rulli *et al.* 2002).

Six-month-old mice of both sexes were used. At 6 months of age pituitaries from hCG β + females were hyperplastic. These mice were genotyped as previously described (Rulli *et al.* 2002). In all cases WT littermates were used as controls.

Estrogen-treated rats

The estrogen-treated rat is a well-known model of pituitary lactotroph hyperplasia and hyperprolactinemia (Heaney *et al.* 1999, 2002). Briefly, adult female Sprague–Dawley (SD) rats (3-month old, 250 ± 30 g) were implanted subcutaneously with a 20 mg pellet of DES. The procedure was performed under anesthesia (50 mg/kg ketamine, 10 mg/kg xylazine, i.p.). Animals were killed by decapitation after 4 weeks of DES treatment. Female SD rats in diestrus were used as controls. Cycling rats were monitored daily, by vaginal smears, and killed in diestrus. In both groups, anterior pituitary glands were removed immediately after decapitation.

Reverse transcription-qPCR (RT-qPCR)

Extraction of total RNA of anterior pituitaries/hypothalami from the experimental groups was performed using TRIzol Reagent (Ambion, Life Technologies) following the manufacturer's protocol. The concentration of the isolated RNA was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) and the purity and integrity of RNA was considered acceptable when the ratio between 260/280 nm absorbance was above 1.8. For cDNA synthesis, reverse transcription reaction was performed using 1 μg RNA, Random primers (Tecnolab S.A., Buenos Aires, Argentina) and the Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Promega). The reaction was performed in a thermocycler (70°C for 5 min, 37°C for 60 min and 95°C for 5 min). Then, a working cDNA solution (6 ng/mL) was prepared with RNase-free water and added (1:2) to a master mix containing EVA green qPCR mix (Solis BioDyne, Estonia) and specific forward and reverse primers (Table 1). Primers were designed using Primer-BLAST, selected considering those with less probability of dimer formation by

Table 1 Specific forward and reverse primers for each gene.

Gene	Accession no.	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
Mouse <i>CypB</i>	NM_011149.2	GACCCTCCGTGGCCAACGAT	ACGACTCGTCCTACAGATTCATCTC
Mouse <i>mPRα</i>	NM_027995.2	TCTGACCACAGTTTTCCTGAC	GCTGGACAGAAGGTGGTTGAA
Mouse <i>mPRβ</i>	NM_028829.4	TACAAAGGTTGTCTAACTGAGCCC	CCACTCATTGACAGGGTGCT
Mouse <i>mPRγ</i>	NM_028748.2	TTGGTTACCGACACCCACAG	ACCTCCACACAAAGAACCAGAA
Mouse <i>mPRδ</i>	NM_001310597.1	ACGCGGCAACAGGTCA	GCCTTCTCCCAAAACACCC
Mouse <i>mPRϵ</i>	NM_198414.2	GGAGTGCTTCATCCTGTCCG	TTGAGCGTCTCATTGGTGCGG
Mouse <i>nPR</i>	NM_008829.2	GGTCCTTGGAGGTCGTAAGT	CTGGCTCTCGTTAGGAAGGC
Rat <i>CypB</i>	NM_022536	GACCCTCCGTGGCCAACGAT	GTCACCTCGTCCTACAGGTTCTCTC
Rat <i>mPRα</i>	NM_001034081	CCGCATCATAGTGTCTCCCC	TGTCCCTGCCCAAAGATGTG
Rat <i>mPRβ</i>	NM_001014099	TACAAAGGTTGTCTACTGAACCC	AGAATCTTGGGCAGACGACG
Rat <i>mPRγ</i>	NM_001014092	GTGCCACAGGTGTTCCATGA	ATGCGTCCAGATGTTGAGGG
Rat <i>mPRδ</i>	NM_001191077	CCACTTGGTACTTCTGTGGC	CGAGGCGACATGGAGCTAAA
Rat <i>mPRϵ</i>	NM_001271152	CAGTGTAGCCTGTGCAAGA	AAGTAGCGGCGGTAGAAGTG
Rat <i>nPR</i>	NM_022847	GACAACACAAAGCCCCGACAC	CGGAAACCTGGCAGAGACTT
Rat <i>TH</i>	NM_012740.3	TCCAGTACAAGCACGGTGAA	GGCATGGGTAGCATAGAGGC

OligoAnalyzer 3.1 and synthesized by Tecnolab S.A. Quantitative real-time PCR analysis was performed using a Bio-Rad real-time PCR detection system setting the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 1 min and then analyzed with a CFX Manager Software (Bio-Rad laboratories). All cDNA samples were assayed in duplicate for each gene and melt curve analyses were performed to ensure specificity of amplification. qPCR efficiency of each pair of primers was previously tested using serially diluted samples and established by means of calibration curves. Amplification efficiency was determined from the slope of the log-linear portion of the calibration curve. Specifically, PCR efficiency was calculated as $10^{1/\text{slope}-1}$, when the logarithm of the initial template concentration was plotted on the x axis and Ct was plotted on the y axis. All primers showed similar efficiencies, approximately 95–100%. A reference gene, *Cyclophilin B* (*Cyp B*), was used as an internal control to normalize differences in the amount of starting template between samples, as previously described (Faraoni *et al.* 2017). Target gene expression was quantified using the comparative Ct method ($\Delta\Delta\text{Ct}$). To analyze the relative mRNA abundance among all the receptor types, we compared $2^{-\Delta\text{Ct}}$ ($2^{-(\text{Ct PR}-\text{Ct Cyp B})}$) values of each receptor.

Ex vivo assays

Drd2 KO and WT mice pituitary explants incubated with selective mPR agonist Org OD 02-0

Female Drd2KO and WT littermates (8-month-old) were killed and anterior pituitaries were collected in 300 μL of DMEM supplemented with 15 v/v horse serum (Internegocios, Argentina), 2.5 v/v fetal bovine

serum (Natocor, Argentina) and gentamicin (20 $\mu\text{g}/\text{mL}$, Sigma-Aldrich). Immediately, anterior pituitaries were cut into small fragments, washed and incubated with supplemented media during 2 h at 37°C in a CO_2 incubator. Then, tissues were washed with fresh supplemented medium and stimulated for 1 h with 100 nM Org OD 02-0, the selective mPR agonist, in serum-free DMEM or with serum-free DMEM alone (vehicle) at 37°C. At the end of the treatment period, both medium and pituitaries were collected and stored at -20°C until measurement of PRL and TGF β 1.

Female rat hypothalamus explants incubated with progesterone and Org OD 02-0

Female rats in diestrus (confirmed by microscopic examination of vaginal smears) were killed and whole hypothalami were excised considering the optic chiasma as the anterior limit, the hypothalamic fissures as laterals and a plane passing in the limit with the mammillary bodies (excluding them), as the posterior limit. The hypothalami were collected in 500 μL of DMEM supplemented with horse serum, fetal bovine serum and gentamicin and then incubated for 2 h. Then, tissues were washed and treated for 30 min with 100 nM progesterone (P4); 100 nM Org OD 02-0 or in serum-free medium alone (vehicle). Dopamine (DA) levels were measured by HPLC in tissue homogenates and in the media.

In vivo experiments

1. Female rats (3-month-old) in diestrus were injected subcutaneously with P4 at a dose of 6.5 mg/kg, sc or castor oil (vehicle, control group) and then, animals were killed by decapitation after 30 min, 2 h or 24 h.

The hypothalami were carefully excised (as described for *ex vivo* assays) and collected in TRIzol reagent for RT-qPCR studies.

- Female Drd2 KO mice (8-month-old) were anesthetized (ketamine 50 mg/kg + xylazine 10 mg/kg, i.p.) to avoid PRL rise due to stress during manipulation and then animals were injected with Org OD 02-0 (15 mg/kg, s.c.) or castor oil (vehicle). Blood samples were obtained from the tail vein at 0, 15 and 30 min and PRL was measured by RIA.

Measurement of active TGFB1 levels by ELISA

The content of active TGFB1 of the pituitary fragments (pg/mg protein) incubated *ex vivo* was determined using a specific TGFB1 DuoSet ELISA Kit (DY1679-05, R&D Systems) following the manufacturer's instructions.

Measurement of PRL levels by radioimmunoassay (RIA)

PRL levels were measured by RIA using a kit provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) National Hormone and Pituitary Program (NHPP) (Dr AF Parlow, NHPP, Torrance, CA, USA). Results were expressed in terms of mouse or rat prolactin standard RP3. Intra- and inter-assay coefficients of variation were 7.1 and 12.3%, respectively.

Determination of DA concentration in hypothalamic tissue by HPLC

The content of dopamine (DA) levels of the hypothalamic fragments incubated *ex vivo* (pmol/mg tissue) and of the collected medium were detected by HPLC using a Varian 5000 liquid-chromatograph coupled to an electrochemical detector (BAS LC-4C). Hypothalami were weighed and homogenized before being de-proteinized in 0.2 M perchloric acid (1/20). The homogenates obtained were centrifuged, and 50 μ L of supernatants were injected onto a 12.5 cm $\times$ 4 mm Nova-Pak C18 (Waters) reverse-phase column. The mobile phase contained $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$ 0.076 M, EDTA 0.99 mM, PICB8 5.24 mL/L and 6% methanol. The electrode potential was fixed at 0.7 V. The Peak Simple Chromatography Data System (Model 302 Six Channel USB) was used to measure the peak heights, using standard curves in the same software. DA concentration was related to tissue wet weight or to μ L of media.

Confocal laser scanning microscopy and electron microscopy immuno-labeling were performed as previously described in Perez *et al.* (2015, 2018) respectively. See details in Supplementary materials and methods (see section on [supplementary data](#) given at the end of this article).

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. $P < 0.05$ was considered significant. Results of mPRs mRNA expression in tumoral vs normal pituitaries were analyzed by Student's *t* test in the three animal models of prolactinomas. Results of mPRs expression by RT-qPCR in Drd2 KO/WT females and males were compared using two-way ANOVA, since the effects of two factors (sex and genotype) were evaluated. Similarly, the effect of Org OD 02-0 in female Drd2 KO and WT mice pituitaries was analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. The effect of P4 or Org OD 02 *ex vivo* on pituitary/hypothalami explants and the effect of P4 *in vivo* at different times were evaluated by one-way ANOVA followed by Dunnett's *post hoc* test. Data set was transformed when required and analyzed using Graphpad Prism 6.0 software (GraphPad Software).

Results

mPRs expression is reduced in female Drd2 KO mice pituitaries

The pituitary mRNA expression of mPR α , β , γ , δ and ϵ was studied in a well-known model of prolactinoma (Kelly *et al.* 1997), the transgenic D2 dopamine receptor-deficient mice (Drd2 KO mice) in comparison to their WT counterparts. In this model, both isoforms of dopamine receptor (long and short) are nonfunctional. Interestingly, despite the lack of dopaminergic inhibition, only females, but not males, develop lactotroph hyperplasia and hyperprolactinemia (Kelly *et al.* 1997, Recouvreux *et al.* 2013) (Fig. 1A and B). Our results showed that mPRs expressions, except for mPR γ , were found to be significantly decreased in tumoral pituitaries from Drd2 KO (prolactinoma) compared to their WT counterparts (Fig. 1C).

We recently reported that mPRs represent about a 15% of the total progesterone receptors in the rat pituitary, with mPR α and β being the most abundant among all mPR subtypes.

Table 2 Values of each gene calculated as $2^{-\Delta Ct}$ (Ct gene–Ct Cyclophilin B).

Gene	$2^{-\Delta Ct} \pm S.E.M.$	
	F WT	F Drd2KO
<i>mPRα</i>	0.192 $\pm$ 0.052	0.080 $\pm$ 0.011
<i>mPRβ</i>	0.413 $\pm$ 0.155	0.103 $\pm$ 0.021
<i>mPRγ</i>	0.023 $\pm$ 0.014	0.001 $\pm$ 0.000
<i>mPRδ</i>	0.020 $\pm$ 0.009	0.003 $\pm$ 0.001
<i>mPRϵ</i>	0.018 $\pm$ 0.008	0.005 $\pm$ 0.001
<i>nPR</i>	0.875 $\pm$ 0.274	0.113 $\pm$ 0.024

The values for each gene obtained in female mice pituitaries (averages) expressed as $2^{-\Delta Ct}$ ($2^{-(Ct \text{ gene} - Ct \text{ Cyclophilin B})}$, relative expression) are shown in Table 2. Using these data, and as previously described in rat pituitaries (Camilletti *et al.* 2018), we performed a qualitative analysis of the relative proportion of each gene comparatively in both genotypes. Pie charts (Fig. 2A and B) are shown to better visualize the relative gene proportions in both genotypes. Figure 2A shows that *mPRs* represent nearly 45% of total progesterone receptors in the female WT pituitaries, and this proportion is even higher in KO (tumoral) pituitaries. Among all the *mPRs* (Fig. 2B), *mPR α* and *mPR β* were the most abundant in both genotypes.

Although *mPR α* expression was reduced in tumoral pituitaries (KO) when compared to WT ones (Fig. 1), its relative proportion was increased in tumoral pituitaries at the expense of a pronounced decrease in the *nPR*.

mPR agonist Org OD 02-0 decreases PRL secretion in normal and tumoral pituitaries

Due to the high proportion of *mPR α* and β found in mice pituitaries, we next evaluated the direct effect of a selective mPR agonist (Org OD 02-0) (Pang *et al.* 2013) on anterior pituitaries explants. Pituitaries from female WT and KO mice (8 months old) were incubated *ex vivo* in medium with Org OD 02-0 or vehicle for 30 min. At the end of the treatment period, PRL levels were measured in the secreted media by RIA. As expected, secreted PRL levels were higher in Drd2 KO than in WT tissue (Fig. 3A). Org OD 02-0 treatment significantly inhibited PRL release in both, normal and tumoral pituitaries, but the inhibition of PRL release was found greater in tumoral pituitaries than the observed in WT pituitaries (Fig. 3B), probably due to a higher *mPRs* relative proportion among all pituitary *PRs* in this genotype.

We recently described that *mPR α* -mediated inhibitory effect on PRL secretion involves an increase in local TGF β 1, a known inhibitor of PRL secretion (Camilletti *et al.* 2018).

Therefore, after treatments, active TGF β 1 levels were measured by ELISA in the pituitary homogenates. As Fig. 3C and D show, active TGF β 1 levels were significantly increased after Org OD 02-0 treatment (vs vehicle) only in Drd2 KO pituitaries.

Gender differences in pituitary mPRs expression

It is well known that in the Drd2 KO mice model, females, but not males develop prolactinomas at 8 months despite the lack of dopaminergic inhibition in both sexes (Kelly *et al.* 1997, Recouvreux *et al.* 2013). Continuing our assessment of mPR involvement in prolactinoma development, we next explored the sex and genotype differences in pituitary *mPRs* expression in our experimental model. Figure 4 shows that the mRNA expressions of *mPR α* (4A), *mPR β* (4B), *mPR γ* (4C) and *mPR ϵ* (4E) are higher in male pituitaries. *mPR δ* expression, however, was found to be lower in males compared to female pituitaries (Fig. 4D).

Regarding genotype differences, and similarly to what is shown in female pituitaries (Fig. 1), the expression of *mPRs* was found to be reduced also in male Drd2KO pituitaries compared to WT (Fig. 4A, B, C, D and E).

Figure 5

Study of mPRs mRNA expression in the transgenic D2 dopamine receptor-deficient mice (Drd2KO) animal model. (A) Pituitary weight of 8-month-old mice ($N = 20$ – 25 /group). Results were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test, **** $P < 0.0001$ vs. WT females, * $P < 0.05$ males vs WT females. (B) Serum prolactin levels measured by RIA ($N = 20$ – 25 /group). Significant differences among genotypes were observed only in females. Two-way ANOVA followed by Bonferroni's test, **** $P < 0.0001$ vs WT females. (C) Expression of mPRs mRNA in pituitaries from Drd2 KO and WT female mice was measured by RT-qPCR. Results of each gene were relativized to WT values and analyzed by Student's *t* test, * $P < 0.05$, *** $P < 0.001$, ($N = 5$).

Figure 2

Pie charts representing *mPRs* and *nPR* relative proportion in Drd2 KO and WT female pituitaries. Table 2: Values of each gene expressed as $2^{-\Delta Ct}$ ($2^{-(Ct \text{ gene} - Ct \text{ Cyclophilin B})}$), $N = 5$. (A) Qualitative representation of the putative abundance of *nPR* and *mPRs* in pituitaries from female WT and Drd2KO mice by Real-Time qPCR. (B) Qualitative representation of the putative abundance of *mPRs* in female WT and Drd2KO pituitaries by Real-Time qPCR, based on data shown in Table 2.

Based on $2^{-\Delta Ct}$ results showed in Table 3, a qualitative and comparative analysis of the relative *PRs* proportions is presented as pie charts (Fig. 5). An increased relative proportion of *mPR α* and *mPR β* in male pituitaries is observed, at the expense of a lower expression of *nPR*, compared to females.

Pituitary *mPR* expression in other animal models of prolactinoma

As previously assayed in Drd2 KO mice (Fig. 1), the pituitary expression of *mPR α* , β , γ , δ and ϵ was studied in other two animal models of prolactinoma: hCG β + mice (Rulli *et al.* 2002, Ahtiainen *et al.* 2010) and DES-rats, in comparison to their WT/control counterparts by RT-qPCR. As it is shown in Fig. 6, the occurrence of prolactinoma in female hCG β + mice and DES-rats can be observed by the increased pituitary weight (Fig. 6A and D) with concomitant hyperprolactinemia (Fig. 6B and E).

Figure 3

Effect of Org OD 02-0 on PRL secretion and TGF β 1 activation in Drd2 KO and WT female (F) pituitary explants. (A and B) PRL release was measured by RIA in the secreted media of Drd2KO and WT pituitaries incubated *ex vivo* with *mPR α / β* agonist, Org OD 02-0 (100 nM) or vehicle (control). Data set was analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. (A) * $P = 0.0476$ Drd2 KO vs. WT; * $P < 0.05$ WT Org OD 02-0 vs. WT control; * $P < 0.05$ Drd2 KO Org OD 02-0 vs. Drd2 KO control. (B) ** $P < 0.01$ WT Org OD 02-0 vs. WT control; *** $P < 0.0001$ Drd2 KO Org OD 02-0 vs. Drd2 KO control. ($N = 7$). (C and D) Active TGF β 1 levels were measured by ELISA in pituitary homogenates of female Drd2 KO and WT pituitaries incubated with Org OD 02-0 (100 nM) or vehicle (control). Data set was analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. ** $P < 0.01$ Drd2 KO Org OD 02-0 vs. Drd2 KO control. ($N = 7$).

Figure 6C shows that *mPRs* expressions, except for *mPR γ* , are significantly decreased in tumoral pituitaries from hCG β + female mice (prolactinoma) compared with their respective WT counterparts, whereas in the rat model, the expression of all *mPRs* isoforms was found reduced in tumoral pituitaries from DES-treated rats in comparison to control pituitaries (females in diestrus), (Fig. 6F).

In addition, *mPR α* protein expression in lactotroph population (PRL+) was confirmed in the hCG β + mice model, in both, normal (WT) and tumoral (hCG β +) pituitaries, by immunostaining analysis using confocal laser scanning microscopy as well as immunogold electron microscopy (Fig. 7), as previously demonstrated in rats (Camilletti *et al.* 2018)

Progesterone action in the hypothalamus mediated by *mPRs*

In addition to the direct action on the lactotroph population, progesterone also regulates prolactin secretion by acting on dopaminergic neurons in the hypothalamus through its *nPR*. This effect has been well studied in the

Table 3 Values of each gene calculated as $2^{-\Delta\text{CT}}$ (Ct gene–Ct Cyclophilin B).

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<i>mPRα</i>	0.192 $\pm$ 0.052	0.080 $\pm$ 0.011	0.207 $\pm$ 0.008	0.113 $\pm$ 0.015
<i>mPRβ</i>	0.413 $\pm$ 0.155	0.103 $\pm$ 0.021	0.407 $\pm$ 0.013	0.197 $\pm$ 0.033
<i>mPRγ</i>	0.023 $\pm$ 0.014	0.001 $\pm$ 0.000	0.002 $\pm$ 0.000	0.001 $\pm$ 0.000
<i>mPRδ</i>	0.020 $\pm$ 0.009	0.003 $\pm$ 0.001	0.015 $\pm$ 0.001	0.007 $\pm$ 0.001
<i>mPRϵ</i>	0.018 $\pm$ 0.008	0.005 $\pm$ 0.001	0.024 $\pm$ 0.001	0.038 $\pm$ 0.007
<i>nPR</i>	0.875 $\pm$ 0.274	0.113 $\pm$ 0.024	0.207 $\pm$ 0.018	0.146 $\pm$ 0.016

rat model (Arbogast & Voogt 1993, 1994). However, the contribution of mPRs in this effect is unknown.

Therefore, we assayed in three female rats the expression of all progesterone receptor types by RT-qPCR in two different hypothalamic areas separately, in order to determine whether there were differences in the expression of these genes: the anterior hypothalamus (including the pre-optic area), and the medial

basal-posterior hypothalamus (including the median eminence), as previously described by Catalano *et al.* (2010). Since no differences were found among these areas (data not shown), the following experiments were performed in whole hypothalami.

According to data shown in Table 4, a qualitative analysis of the relative abundance of each mRNA shows that mPRs represent about 90% of the total progesterone receptors in the rat hypothalamus (Fig. 8A). This percentage is very high, compared to nPR expression (about a 10%) and to mPRs expression in the rat pituitary. The analysis derived from ΔCT s results suggests that mPR α and mPR β have the highest expression among the mPRs in the hypothalamus.

We next examined the progesterone effect on TH expression within the hypothalamus in our rat model. Progesterone was administered *in vivo* in female rats in diestrus. Animals were killed at different times: 30 min, 2 h or 24 h, and then TH mRNA expression was analyzed by RT-qPCR. A P4-stimulatory action on hypothalamic TH was observed at 30 min and 2 h but normalized after 24 h of treatment (Fig. 8B).

Based on these results we next evaluated the direct effect of progesterone and Org OD 02-0 on dopamine release, measured by HPLC, in rat hypothalamus explants incubated *ex vivo* (Faraoni *et al.* 2017). After 30 min of treatment, both progesterone as well as Org OD 02-0 increased dopamine (DA) release (Fig. 8C); however, no differences were found in the hypothalamic content of DA after treatments (Fig. 8D).

Finally, since mPRs were found highly expressed in the rat hypothalamus, we next explored the hypothalamic mRNA expression of nPR and mPRs, in the Drd2 KO mice model, comparatively among sexes and genotypes. Table 5 shows the $2^{-\Delta\text{CT}}$ values for each gene. No significant differences were found among groups, then, only one pie chart is shown in Fig. 9 representing the qualitative analysis of the relative proportion among PRs in female WT hypothalamus. As observed in rat hypothalamus (Fig. 8A and Table 4), mPRs also represent a very high percentage of total PRs in mice tissue.

Figure 4

Sex differences in mPRs mRNA expression in the anterior pituitary from Drd2 mice model. Results of mPR mRNA expression (RT-qPCR) between Drd2 KO/WT females and males were compared using two-way ANOVA followed by Bonferroni's *post hoc* test. (A) *** $P < 0.0001$ F Drd2 KO vs. F WT; *** $P < 0.0001$ M Drd2 KO vs. M WT; *** $P < 0.01$ F WT vs. M WT; *** $P < 0.0001$ F Drd2 KO vs. M Drd2 KO. Interaction $P = 0.011$ ($N = 5$). (B) *** $P < 0.0001$ F Drd2 KO vs. F WT; *** $P < 0.0001$ M Drd2 KO vs. M WT; *** $P < 0.01$ F WT vs. M WT; *** $P < 0.0001$ F Drd2 KO vs. M Drd2 KO ($N = 5$). Interaction $P = 0.010$. (C) ** $P < 0.001$ M Drd2 KO vs. M WT; *** $P < 0.01$ F WT vs. M WT. Interaction $P = 0.015$ ($N = 5$). (D) *** $P < 0.0001$ F Drd2 KO vs. F WT; *** $P < 0.0001$ M Drd2 KO vs. M WT; ** $P < 0.001$ F WT vs. M WT; *** $P < 0.001$ F Drd2 KO vs. M Drd2 KO ($N = 5$). (E) *** $P < 0.0001$ F Drd2 KO vs. F WT; *** $P < 0.0001$ M Drd2 KO vs. M WT; *** $P < 0.01$ F WT vs. M WT; *** $P < 0.0001$ F Drd2 KO vs. M Drd2 KO. Interaction $P < 0.001$, ($N = 5$).

Figure 5

Gender differences among *mPRs* and *nPR* relative proportion in WT mice pituitaries. Table 3: Values of each gene expressed as $2^{-\Delta\text{Ct}}$ ($2^{-(\text{Ct gene}-\text{Ct Cyclophilin B})}$), $N = 5$. Pie charts: Qualitative representation of the putative abundance of *nPR* and *mPRs* in pituitaries from female and male WT mice by real-time qPCR based on data shown in Table 3.

Effect of Org OD 02-0 *in vivo* on PRL serum levels in female *Drd2* KO mice

As Org OD 02-0 was probed to reduce the secretion of PRL in the pituitary *ex vivo* but also at the hypothalamus level by promoting DA release, we finally explored whether *mPRs* activation was capable of inhibiting PRL levels in prolactinomas *in vivo*. To this end, *Drd2* KO female mice (8 months old) were anesthetized to avoid PRL rise due to stress during manipulation and then animals were injected with Org OD 02-0 (15 mg/kg, s.c.) or castor oil (Vehicle). Blood samples were obtained from the tail vein at 0, 15 and 30 min and PRL was measured by RIA. One single dose of Org OD 02-0 significantly diminished serum PRL

in just 15 min (Fig. 10) and maintained those levels up to 30 min, demonstrating a rapid *in vivo* mechanism to reduce high PRL levels in this animal model. Although further studies are needed (e.g. longer term data), our results suggest that *mPR* agonists may represent novel candidates for the treatment of hyperprolactinemia, especially in dopaminergic agonists-resistant prolactinomas.

Discussion

In the present work, we described how the pituitary expression of *mPRs* is altered in different animal models of prolactinomas. Besides the lower *mPRs* expression, in comparison with normal pituitaries, their relative proportion was found to be increased, with *mPRs* representing more than 60% of total progesterone receptors in prolactinomas. This fact implies alterations in progesterone effects in tumoral pituitaries.

The regulation of lactotroph function by progesterone has always been controversial. Most of the early studies were carried out in the rat model. Progesterone action on prolactin secretion was thought for a long time, to be indirect, acting on dopaminergic neurons, as *nPR* expression in the rat pituitary is restricted to gonadotrophs (Fox *et al.* 1990). However, in contrast to rat pituitaries, mice express *nPRs* also in the lactotroph population (Turgeon *et al.* 2001), complicating the study of the final action of progesterone on prolactin secretion, with the possibility of direct (pituitary) and/or indirect (hypothalamus) effects. Moreover, during

Figure 6

Pituitary mRNA expression of *mPRs* in other animal models of prolactinoma. (A) Pituitary weight of 6-month-old female transgenic mice ($N = 13/\text{group}$). Results were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test, $***P = 0.0027$ vs. WT females. (B) Serum prolactin levels measured by RIA ($N = 9/\text{group}$). $****P < 0.0001$ vs. WT females. (C) *mPRs* mRNA expression in *hCGβ+* and WT female mice measured by RT-qPCR. Results were analyzed by Student's *t* test: $****P < 0.001$ vs. WT females, ($N = 6$). (D) Pituitary weight increased 3-fold after DES treatment compared to untreated rats in diestrus (CTRL). Student's *t* test: $****P < 0.0001$ vs. CTRL rats, ($N = 16$). (E) Serum prolactin levels were measured by RIA. Student's *t* test: $****P < 0.0001$ vs. CTRL rats, ($N = 16$). (F) *mPRs* mRNA expression in DES-treated female rats vs female rats in diestrus. Results were analyzed by Student's *t* test: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. CTRL rats, ($N = 6$).

Figure 7

mPR α expression in lactotroph cells of WT and hCG β + female mice. (A) Identification of mPR α (arrowhead, red) and prolactin (*, green) by double indirect immunofluorescence. Nuclei were immunostained with 4',6-diamidino-2-phenylindole (blue). White arrows show lactotrophs expressing mPR α . Scale bar = 20 μ m. (B) Immuno electron labeling for mPR α and prolactin. Lactotroph cells with gold particles of 15 nm indicating the presence of mPR α in plasmatic membrane (arrows) with secretory granules (g) immunolabelled for PRL (5 nm gold particles). N, nucleus; pm, plasmatic membrane; RER, rough endoplasmic reticulum. Scale bar = 0.2 μ m.

the last 15 years, the scene became even more complex with the discovery of membrane progesterone receptors (Zhu *et al.* 2003b).

We have recently described that mPRs are expressed in normal rat pituitaries, and moreover, activation of mPR α inhibits PRL secretion (Camilletti *et al.* 2018), demonstrating a P4-direct effect on rat lactotroph function.

Our current results show a decreased mRNA expression of both, nPR and mPRs in three different animal models of prolactinoma when compared to normal tissue. However, in these pathological situations the relative proportion of mPRs/nPR is increased. In fact, mPR α and β represent more than 60% of the total progesterone receptors in female Drd2 KO (tumoral) pituitary. Interestingly, their activation (*ex vivo* and *in vivo*) induced a clear inhibitory action on prolactin secretion. The effect on pituitary explants *ex vivo* is independent of dopamine action and mediated, in part, by an increase in the local TGF β 1

Figure 8

Progesterone effect in the rat hypothalamus. (A) Pie chart showing relative abundance of mPRs and PR transcripts in the rat hypothalamus, measured by RT-qPCR based on data shown in Table 4. (B) Effect of progesterone (P4, 6.5 mg/kg) *in vivo* on hypothalamic TH mRNA expression in rat, measured by RT-qPCR. Results were analyzed by one-way ANOVA followed by Dunnet's test. *** P = 0.0004 (N = 6). (C and D) Effect of Org OD 02-0 and progesterone (P4) on dopamine (DA) levels in hypothalamus explants. (C) DA release was measured by HPLC in collected medium. Data set was analyzed by one-way ANOVA followed by Dunnet's test. **** P < 0.0001, (N = 5). (D) DA content in hypothalamus homogenates measured by HPLC. One-way ANOVA followed by Dunnet's test, ns (N = 5).

Table 4 Values of each gene calculated as $2^{-\Delta CT}$ (Ct gene–Ct Cyclophilin B).

Gene	$2^{-\Delta CT} \pm S.E.M.$
mPR α	1.270 $\pm$ 0.097
mPR β	1.875 $\pm$ 0.124
mPR γ	0.021 $\pm$ 0.006
mPR δ	0.736 $\pm$ 0.103
mPR ϵ	0.424 $\pm$ 0.055
nPR	0.498 $\pm$ 0.045

Table 5 Values of each gene in whole hypothalami of female and male Drd2 KO and WT mice calculated as $2^{-\Delta CT}$ (Ct gene–Ct Cyclophilin B).

Gene	$2^{-\Delta CT} \pm S.E.M.$			
	F WT	F Drd2KO	M WT	M Drd2KO
<i>mPRα</i>	0.529 $\pm$ 0.038	0.482 $\pm$ 0.034	0.482 $\pm$ 0.027	0.498 $\pm$ 0.025
<i>mPRβ</i>	1.186 $\pm$ 0.183	1.176 $\pm$ 0.080	1.309 $\pm$ 0.109	1.096 $\pm$ 0.047
<i>mPRγ</i>	0.009 $\pm$ 0.001	0.007 $\pm$ 0.001	0.015 $\pm$ 0.004	0.008 $\pm$ 0.000
<i>mPRδ</i>	0.227 $\pm$ 0.047	0.221 $\pm$ 0.021	0.201 $\pm$ 0.027	0.217 $\pm$ 0.009
<i>mPRϵ</i>	0.047 $\pm$ 0.005	0.047 $\pm$ 0.004	0.050 $\pm$ 0.003	0.037 $\pm$ 0.002
<i>nPR</i>	0.259 $\pm$ 0.042	0.233 $\pm$ 0.029	0.336 $\pm$ 0.043	0.340 $\pm$ 0.016

(a known inhibitor of lactotroph function), as previously described in rat pituitaries (Camilletti *et al.* 2018). This finding provides a new tool for the pharmacological treatment of hyperprolactinemia.

Regarding the hypothalamus, we observed high *mPR* expression in both the mice and rat models, without gender or genotype differences. This result is in accordance with previous findings in rat and human tissues where a high expression of mPRs was observed by RT-qPCR and confirmed by Western blot (Zuloaga *et al.* 2012, Pang *et al.* 2013). Moreover, it was shown that mPRs represent the most abundant progesterone receptors, not only in hypothalamus, but also in other human brain regions (Pang *et al.* 2013).

As previously mentioned, progesterone regulation of PRL secretion also involves an indirect effect in the hypothalamus, by acting on the neurons of the arcuate nucleus where it regulates mRNA levels of TH, a key enzyme in dopamine synthesis. However, this action is also controversial in the bibliography. Although inhibition of TH by progesterone treatment was

described, facilitating PRL release (Arbogast & Voogt 1993, 1994), progesterone induction of TH expression was also observed counteracting chronic estradiol treatments and decreasing serum PRL levels (Piroli *et al.* 1996). This discrepancy could be due to the type of treatment, the hormonal status, but also to the progesterone receptor type involved. We here demonstrated that the specific activation of the most abundant progesterone receptors in the hypothalamus (*mPR α* and β) leads to a rapid dopamine release in hypothalamus explants, effect that, *in vivo* would relay in an inhibition of prolactin secretion.

Then, taking together both, the Org OD 02-0 effect on the lactotroph population, and on the hypothalamus, we postulate that specific *mPR α/β* activation would represent a novel tool to reduce prolactin secretion in pathological situations of hyperprolactinemia. In fact, in the present work, we demonstrated that a short-term *in vivo* treatment with Org OD 02-0 was able to significantly reduce the hyperprolactinemia in Drd2 KO female mice.

Another interesting finding in the present results is related to the gender differences. Sexual differences in prolactinoma, incidence, size and behavior were described, not only in humans, but also in animal models of prolactinoma. In humans, during the fertile period (between 20-50 years), the prevalence is higher in women (Colao *et al.* 2003, Gillam *et al.* 2006). It is clear that the symptoms caused by hyperprolactinemia in women (menstrual cycle disorders) lead to an earlier diagnosis; in contrast, the polymorphic clinical manifestations in men cause a delay in the diagnosis (Colao *et al.* 2003). However, this is not the only explanation for the sex differences found in tumor size and aggressiveness, suggesting a gender-specific behavior for human prolactinomas (Gillam *et al.* 2004, 2006).

Interestingly, sexual differences in prolactinoma incidence and behavior have also been observed in several animal models of prolactinoma. In the Drd2-knockout mouse, despite the lack of dopaminergic inhibition, only females develop hyperprolactinemia and lactotroph hyperplasia at 8 months of age (Kelly *et al.* 1997, Asa *et al.* 1999, Diaz-Torga *et al.* 2002), while Drd2-deficient males

Figure 9

Pie chart showing relative abundance of mPRs and nPR transcripts in the female mice hypothalamus, measured by RT-qPCR based on data shown in Table 5. *N* = 4.

Figure 10

Serum PRL levels after Org OD 02-0 treatment *in vivo* in female Drd2KO mice. PRL levels were measured by RIA at different times. To normalize the values, data was plotted as a % of PRL secretion over time (respect to time zero) and then analyzed by ANOVA of repeated measures. * $P < 0.033$, ($N = 5$).

develop smaller but more aggressive lactotroph adenomas at 17–20 months of age (Asa *et al.* 1999). Similar findings were observed in other animal models of prolactinomas (reviewed in Recouvreur *et al.* 2018): the hCG β + mice (Rulli *et al.* 2002, Faraoni *et al.* 2017), the transgenic mice overexpressing galanin in lactotrophs (Cai *et al.* 1999), the mouse models with overexpression of NGF (Borrelli *et al.* 1992), overexpression of FGFR4 (Ezzat *et al.* 2017), overexpression of TGF α directed to lactotrophs (McAndrew *et al.* 1995) and the PRL receptor-knockout mice (Lines *et al.* 2016). In all these models, the hyperprolactinemia and the prolactinoma development are observed only in females or are more pronounced in females.

The participation of ovarian steroids in these gender differences cannot be excluded. In fact, Drd2 KO and hCG β + female mice do not develop prolactinomas when ovariectomized in prepuberal ages, even with estradiol replacement (Hentges & Low 2002, Ahtiainen *et al.* 2010) suggesting the participation of other ovarian factors in the gender differences of prolactinoma behavior.

The present results showed higher expression of pituitary mPR α/β in male pituitaries compared to females, representing nearly 80% of the total progesterone receptors. It is known that, in addition to its synthesis in ovaries and placenta in females, progesterone is also synthesized by adrenal glands and by the nervous system in both males and females. Then, as mPR α/β activation leads to inhibition of prolactin secretion, the higher mPR expression found in male pituitaries could be protecting this sex from prolactinoma development.

Collectively, our present findings clearly demonstrate that mPR α/β activation inhibits prolactin secretion acting directly on lactotroph population, and indirectly increasing dopamine secretion on hypothalamus.

Considering that about 20% of patients with prolactinomas are resistant to current treatments with dopaminergic drugs, and up today there are no alternative treatments, our present results position the Org OD 02-0, as a putative pharmacological tool for prolactinoma treatment.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0409>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M A C, D P, P T and G D T conception and design of research; M A C, P A P, J F, A A M, F D F, E Y F and S G performed experiments; M A C and G D T analyzed data; M A C, A A M, S G, P T and G D T interpret results of experiments; M A C and P A P prepared figures; M A C and G D T drafted manuscript; M A C, A A M, E Y F, P A P, S G, P T and G D T edited and revised manuscript; M A C and G D T approved final version of manuscript.

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