Contractile Effects of Serotonin (5-HT) in the Rat Cauda Epididymis: Expression and Functional Characterization of 5-HT Receptors

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ABSTRACT

Serotonin [5-hydroxytryptamine (5-HT)] exerts multiple central and peripheral functions. High concentrations of 5-HT have been found in the epididymis, a ductal organ that plays pivotal roles in sperm transport and maturation. The contraction of the epididymal smooth muscle is essential for sperm transport and emission during ejaculation. The contributions of the epididymal 5-HT system to these events are poorly understood. Here, we assessed the contractile function of 5-HT in the rat cauda epididymis (CE), pharmacologically targeting the receptor(s) and the reuptake mechanism involved in this system. Segments of CE duct from adult Wistar rats were set up in an organ bath system for isometric tension recordings, and concentration-response curves to 5-HT and norepinephrine were obtained. 5-HT elicited concentration-dependent contractions of the CE duct ($pEC_{50} = 6.5 \pm 0.1$) that were potentiated

with high potency by the norepinephrine transporter (NET) inhibitor desipramine and with low potency by the highly selective serotonin transporter inhibitor paroxetine, indicating that the NET is the major mediator of 5-HT reuptake in vitro. CE contractions to 5-HT were antagonized by the α_1 -adrenoceptor (α_1 -AR) antagonist prazosin ($pA_2 \cong 8.9$), 5-HT_{2A/2C} antagonists ketanserin ($pA_2 \cong 9.4$) and fluoxetine ($pA_2 \cong 7.4$), and 5-HT_{1A} ligands WAY 100635 ($pA_2 \cong 8.9$) and buspirone ($pA_2 \cong 7.3$). Reverse transcriptase polymerase chain reaction analysis demonstrated that 5-HT_{1A} and 5-HT_{2A} transcripts are highly abundant in the cauda epididymis, whereas 5-HT_{2C} transcript was not found. Altogether, our results reveal that contractions of the CE duct to 5-HT encompasses at least activation of α_1 -ARs and 5-HT_{1A} and 5-HT_{2A} receptors, providing new insights into the roles of 5-HT on the epididymal function.

Introduction

Serotonin [5-hydroxytryptamine (5-HT)] is a largely distributed biogenic monoamine exerting several effects in both neuronal and non-neuronal tissues. In the central nervous system 5-HT controls behavioral processes and other functions such as emesis, body temperature, ejaculation, and circadian rhythms (Berger et al., 2009). Outside the central nervous system 5-HT is involved in a number of functions, such as vasoconstriction, nociception, intestinal motility and secretion, platelet aggregation, uterine smooth muscle contraction, among others (Hoyer et al., 1994; Berger et al., 2009). This myriad of actions of 5-HT are mediated by interactions with 14 different 5-HT receptors, which are classified into seven families (5-HT_{1–7}). All 5-HT receptors are metabotropic seven transmembrane domain receptors (or G protein-coupled

receptors) (Alexander et al., 2017a), with the exception of 5-HT₃, which is an ionotropic (cation-permeable) receptor (Alexander et al., 2017b). The variety of 5-HT receptor subtypes reflects the extraordinary biologic relevance of 5-HT as a neurotransmitter, as well as a paracrine and autocrine signaling molecule.

Concerning the metabotropic 5-HT receptors, the 5-HT₁ and 5-HT₂ classes are comprised of five (5-HT_{1A}, 1B, 1D, 1E, 1F) and three (5-HT_{2A}, 2B, 2C) subtypes, respectively (Hannon and Hoyer, 2008; Alexander et al., 2017a). In agreement with the widespread distribution of 5-HT receptors, the 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} subtypes have been described in male reproductive tissues. 5-HT is known to regulate testicular blood flow and testosterone secretion (Kinson et al., 1973), and to contract the smooth muscle of male reproductive organs, such as the seminal vesicle (Kim and Paick, 2004), vas deferens (Hay and Wadsworth, 1982; Campos et al., 1999; Pedroso et al., 2017), and prostate (Steidle et al., 1989), through activation of the 5-HT₁ and/or 5-HT₂ receptor subtypes. Interestingly, high concentrations of 5-HT have been found in the epididymis, a ductal organ that plays a crucial role in sperm transport, concentration, maturation, and storage until ejaculation (Korman and Penttilä, 1968; Anderson et al., 1979). Jiménez-Trejo et al. (2007) revealed that the rat caput

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ABBREVIATIONS: AR, adrenoceptor; CE, cauda epididymis; CR, concentration ratio; CRC, concentration-response curve; E_{max} , maximal response; 5-HT, serotonin (5-hydroxytryptamine); NET, norepinephrine transporter; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor.

epididymis possesses a local serotonergic system that includes the expression of 5-HT biosynthetic enzymes, 5-HT receptors (5-HT_{1A}, 5-HT_{2A}, etc.), and serotonin transporters by epididymal epithelial and non-epithelial cells. Notwithstanding, the contribution of 5-HT to epididymal physiology is still poorly understood.

It has been reported that 5-HT stimulates anion secretion via 5-HT_{1B} and 5-HT_{2B} receptors in rat cauda epididymal cell primary cultures (Leung et al., 1999), suggesting that 5-HT plays a role in the regulation of epididymal luminal content and sperm quiescence during storage in the epididymis. Furthermore, administration of exogenous 5-HT in rats resulted in fluid accumulation in the epididymis (Singh et al., 1987). In men, high blood 5-HT levels were associated with poor sperm count and motility (Gonzales et al., 1992), and treatment with the antidepressant paroxetine, a selective serotonin reuptake inhibitor (SSRI), caused ejaculatory difficulties and increased sperm DNA fragmentation (Tanrikut et al., 2010). Despite these negative outcomes, SSRIs, such as dapoxetine, can be used in the pharmacotherapy of premature ejaculation (Giuliano and Clément, 2005; Rowland et al., 2010; Abu El-Hamd and Abdelhamed, 2018). It is thought that the effects of SSRIs on the regulation of ejaculatory function are mediated by increasing 5-HT signaling in the central nervous system. Nevertheless, peripheral mechanisms in the male reproductive organs could also be involved in these clinical outcomes (Jannini et al., 2015).

Epididymal epithelial cells are surrounded by a smooth muscle layer, in which the thickness increases from the proximal (caput) to distal (cauda) epididymis. In the cauda epididymis (CE), the thicker smooth muscle layer is densely innervated by postganglionic sympathetic nerve fibers that release norepinephrine (Ricker, 1998; Silva et al., 2010). These sympathetic nerve fibers play a crucial role in the contraction of the CE smooth muscle, which is paramount for sperm transport during the emission phase of ejaculation and hence for reproduction (Ricker et al., 1997; Pacini et al., 2018). Likewise, the presence of a dense network of serotonergic nerve fibers has been detected in the CE (Leung et al., 1999), although the role of 5-HT in the contraction of the epididymal smooth muscle remains elusive.

Taking this into account and considering: 1) the ability of 5-HT to contract smooth muscle in reproductive organs, 2) the existence of a local serotonergic system in the epididymis, and 3) the reproductive outcomes of SSRI drugs, we hypothesized that 5-HT contracts CE smooth muscle. Thus, we systematically assessed the contractions induced by 5-HT in the rat CE smooth muscle *in vitro*, and investigated the roles of 5-HT receptor subtypes and reuptake systems in the pharmacological events mediated by 5-HT in this tissue.

Materials and Methods

Animals and Drugs. Adult male Wistar rats (90–150 days old) were maintained under controlled conditions (12-hour/12-hour light/dark cycle, 25 ± 2°C, and 40%–70% humidity) with food and water *ad libitum*. All animal procedures were approved by the Ethics Committee for the Use of Experimental Animals from the Institute of Biosciences of Botucatu, São Paulo State University (Process No. 749/15) and were in agreement with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and Animal in Research: Reporting *In Vivo* Experiments guidelines (Kilkenny et al.,

2010). The drugs used in the experiments were obtained from the following sources: L-(–)-norepinephrine bitartrate salt monohydrate, serotonin HCl, ketanserin tartrate, WAY 100635, buspirone, and corticosterone were purchased from Sigma; fluoxetine HCl was purchased from Fagron; paroxetine was purchased from Tocris; and desipramine HCl, prazosin HCl, yohimbine HCl, and (±)-propranolol HCl were purchased from Research Biochemicals Inc.

In Vitro Contraction Studies. Contraction experiments were conducted following the procedure detailed in Pacini et al. (2018). Rats were killed by decapitation and the epididymis was carefully excised. The CE was uncoiled, cleaned of adherent tissues, and freed from intraluminal content by flushing 1 ml of nutrient solution through a blunt-ended 30 gauge needle. For digital recording of isometric contractions, segments of the distal CE (~1.0 cm in length), corresponding to region 19 according to Jelinsky et al. (2007), were mounted in organ baths under 1.0 g of resting tension in a modified Tyrode's solution (138 mM NaCl, 5.7 mM KCl, 1.8 mM CaCl₂, 0.36 mM NaH₂PO₄, 15 mM NaHCO₃, and 5.5 mM dextrose) prepared in glass-distilled deionized water, maintained at 30°C and pH 7.4, and continuously bubbled with 95% O₂/5% CO₂. After a 30-minute stabilization period, CE segments were challenged with 80 mM KCl until reproducible contractions were obtained. Then, cumulative concentration-response curves (CRCs) to norepinephrine or 5-HT were obtained and taken as control curves. All curves were conducted in the presence of a cocktail of inhibitors containing corticosterone (10 μM), yohimbine (0.1 μM), and propranolol (0.1 μM) to block the monoamine uptake not mediated by the norepinephrine transporter (NET), serotonin transporter (SERT), and α₂- and β-adrenoceptors (ARs), respectively.

To investigate the role of the NET and/or SERT, CRCs to 5-HT and norepinephrine were built up in the presence of fluoxetine, paroxetine (selective SERT inhibitors), and desipramine (selective NET inhibitor). The involvement of α₁-ARs was investigated using prazosin (α₁-AR antagonist) and that of 5-HT receptors was investigated using ketanserin (5-HT_{2A/2C} antagonist), WAY 100635 (5-HT_{1A} antagonist), buspirone (5-HT_{1A} partial agonist), or fluoxetine (SERT inhibitor with high affinity for 5-HT_{2A/2C}). All inhibitors/antagonists were previously incubated for 45 minutes to allow equilibration, and then a new CRC to either norepinephrine or 5-HT was obtained. Contractions were expressed as percentage of maximal contraction of the control curves. The potencies of norepinephrine and 5-HT in the absence and presence of antagonists are presented as pEC₅₀ values, i.e., the negative logarithm of the concentration of 5-HT or norepinephrine producing 50% of its maximal response (*E*_{max}). The effects of each concentration of norepinephrine or 5-HT were measured as the peak of the respective induced contraction of agonist.

Schild Analysis. Antagonist potencies and affinities were evaluated by Schild analysis (Arunlakshana and Schild, 1959). The parallel rightward displacements induced by each antagonist concentration on 5HT or norepinephrine CRCs were quantified and used to calculate the concentration ratio (CR), which is the ratio between equieffective norepinephrine or 5-HT concentrations in the presence and absence of antagonist. The CR was plotted as log (CR – 1) versus the respective antagonist concentration in a Schild plot and analyzed by linear regression. The slopes of linear regressions were calculated, and when not different from unity the intercept in abscissa (pA₂) was taken as the antagonist dissociation constant (pK_B). However, as in most assays, since the slopes in the Schild plots were much lower than 1.0, the pA₂ values were taken as estimates of antagonist potencies calculated from the formula pA₂ = log (CR – 1) – log [B], where [B] is the lowest effective antagonist concentration.

Reverse Transcriptase Polymerase Chain Reaction. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to detect each specific gene target. Total RNA from epididymal regions (initial segment, caput, corpus, and cauda), testis, and brain was extracted using TRIzol Reagent (ThermoFisher) according to the manufacturer's instruction, followed by DNase treatment (DNase I Amplification Grade; Invitrogen) and first strand cDNA synthesis

(Thermoscript RT-PCR kit; Invitrogen). Oligo(dT)-primed cDNA was synthesized from total RNA (2 μ g) for 1 hour at 55°C in a reaction volume of 20 μ l. The resulting cDNA (2 μ l) was amplified by polymerase chain reaction (PCR) (SimpliAmp; Applied Biosystems) in a final volume of 20 μ l containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 1 U *Thermus aquaticus* DNA polymerase, and 0.4 μ M of each sense and antisense primer used to amplify specific nucleotide sequences present in *Htr1a*, *Htr1b*, *Htr2a*, *Htr2b*, *Htr2c*, and *Ppia* transcripts. Primer sequences, GenBank accession numbers, corresponding base sites, sizes of the PCR products, and PCR conditions are given in Table 1. Primers for *Htr1a*, *Htr1b*, *Htr2b*, *Htr2c*, and *Ppia* were designed using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and spanned at least one intron to ensure that PCR products were from cDNA and not genomic DNA. Primers for *Htr2a* were designed as described by Reist et al. (2003). Routinely, no-template negative control PCR reactions were performed to assess genomic DNA contamination in the template RNA. Positive control was employed to check that the PCR conditions used could successfully amplify the target sequences. DNA samples (10 μ l) were loaded onto agarose gels (1.5%, w/v) containing 10 μ g/ml Sybr Safe DNA Gel Stain (Invitrogen) and the results were imaged using the G:BOX XR5 (Syngene) imaging system.

Statistical Analysis. Curve fitting was performed by nonlinear regression using the three-parameter logistic equation from GraphPad Prism version 6 (GraphPad Software, San Diego, CA), and the $E_{\max}$, pEC₅₀, and pA₂ values were calculated accordingly. All values are shown as mean $\pm$ S.E.M. of n independent experiments. Differences between mean values were tested for statistical significance ($P < 0.05$) using ANOVA, followed by Dunnett's test for multiple comparisons.

Results

5-HT Contracts the Rat Cauda Epididymis Smooth Muscle. 5-HT elicited concentration-dependent contractions, presenting a pEC₅₀ value of 6.45 ± 0.14 and a maximal response that corresponded to $\sim 45\%$ of the norepinephrine $E_{\max}$ value (Fig. 1A). As shown in the typical recordings presented in Fig. 1, C and D, the contractions induced by 5-HT were less sustained than those produced by norepinephrine.

Uptake of 5-HT in the Rat Cauda Epididymis Is Mainly Driven by the NET Rather Than the SERT. To investigate the participation of the 5-HT reuptake system, CRCs to 5-HT were performed in the absence and presence of different concentrations of SERT inhibitors (Fig. 2) and the

influence of the reuptake was estimated in terms of agonist CRCs measured at the EC₅₀ values (Table 2).

At low concentrations (1–10 nM), both selective SERT inhibitors paroxetine and fluoxetine were ineffective in potentiating 5-HT-induced contractions in the CE duct (Fig. 2, A and E; Table 2). These low concentrations of both paroxetine and fluoxetine did not affect CRCs to norepinephrine (Fig. 2, B and F). However, at higher concentrations, 100–1000 nM of paroxetine increased the potency of 5-HT and norepinephrine by up to ~ 5 - and ~ 30 -fold, respectively (Fig. 2, C and D; Table 2). Conversely, 30–100 nM of fluoxetine reduced the potencies of 5-HT by up to ~ 3 -fold in the CE duct (Fig. 2G; Table 2); at such high concentrations, fluoxetine did not affect the potencies of norepinephrine in the CE duct (Fig. 2H; Table 2).

These data indicated that 5-HT is not taken up by the SERT in the CE duct, and that the NET could play such a role, as suggested by the effects of high concentrations of paroxetine on both 5-HT- and norepinephrine-induced contractions of the CE duct. We evaluated whether the NET could participate in the removal of 5-HT in the CE duct using the selective NET inhibitor desipramine. At concentrations of 10 and 30 nM, desipramine potentiated the effects of 5-HT in the CE duct by up to ~ 4 -fold (Fig. 2I; Table 2), whereas 10 nM of desipramine potentiated the effects of norepinephrine by ~ 10 -fold (Fig. 2J; Table 2).

α_1 -Adrenoceptors and 5-HT_{1A} and 5-HT_{2A/2C} Receptors Are Involved in the 5-HT-Induced Contractions of the Rat Cauda Epididymis. To investigate the receptors involved in the contractions induced by 5-HT, CRCs were built in the absence and presence of selective antagonists. Since 5-HT has been shown to activate adrenoceptors (Innes, 1962; Purdy et al., 1987; Shaw et al., 2000), and considering that these receptors are abundantly expressed in the CE (Ventura and Pennefather, 1991; Queiróz et al., 2002; White et al., 2013; Pacini et al., 2018), we determined the effects of the selective α_1 -AR antagonist prazosin on 5-HT-induced contractions in the CE duct. In fact, low concentrations (3–100 nM) of prazosin antagonized the contractions induced by 5-HT with a potency measured as a pA₂ value of 8.89, which well correlated to the potency obtained against norepinephrine (pA₂ = 8.92) (Fig. 3; Table 3), indicating that α_1 -ARs are involved in the contractions induced by 5-HT. However, the slope in the Schild plot

TABLE 1
Primers and conditions for RT-PCR

Gene	Primer Sequence	Amplicon Size	Accession Number	Initial Denaturation	35 Cycles			Final Elongation
					Denaturation	Annealing	Elongation	
		bp		°C/min	°C/s	°C/s	°C/s	°C/min
<i>Htr1a</i>	Forward: 5'-GTCACCTGCGACCTGTTTAT-3'	286	NM_012585.1	95/2	95/60	58/60	72/90	72/3
	Reverse: 5'-CGAAAGTGGAGTAGATGGTGT-3'	286	NM_012585.1	95/2	95/60	58/60	72/90	72/3
<i>Htr1b</i>	Forward: 5'-CACTGATGCGGTGGACTATT-3'	217	NM_022225.1	95/2	95/60	58/60	72/75	72/3
	Reverse: 5'-GAGCAGGGTGGGTAAATAGAA-3'	217	NM_022225.1	95/2	95/60	58/60	72/75	72/3
<i>Htr2a</i>	Forward: 5'-AGCTGCAGAATGCCACCACTAT-3'	322	NM_017254.1	95/2	95/60	60/60	72/75	72/3
	Reverse: 5'-GGTATTGGCATGGATATACCTAC-3'	322	NM_017254.1	95/2	95/60	60/60	72/75	72/3
<i>Htr2b</i>	Forward: 5'-CTGTGCTCCTGCCTGTTTATT-3'	224	NM_017250.1	95/2	95/60	58/60	72/75	72/3
	Reverse: 5'-TTGACCACATCAGCCTCTATTC-3'	224	NM_017250.1	95/2	95/60	58/60	72/75	72/3
<i>Htr2c</i>	Forward: 5'-GCTAGCGGGTTGTCAACTAT-3'	322	NM_012765.3	95/2	95/60	60/60	72/75	72/3
	Reverse: 5'-CGACGATTGAAAGTGCTGGC-3'	322	NM_012765.3	95/2	95/60	60/60	72/75	72/3
<i>Ppia</i>	Forward: 5'-AGCACTGGGGAGAAAGGATT-3'	174	NM_017101.1	95/2	95/60	60/45	72/60	72/3
	Reverse: 5'-GATGCCAGGACCTGTATGCT-3'	174	NM_017101.1	95/2	95/60	60/45	72/60	72/3

bp, base pair.

Fig. 1. Contractile effects of 5-HT and norepinephrine in the rat CE duct. (A) Mean concentration-response curves in the presence of cocktail of inhibitors for contractions induced by norepinephrine (○) and 5-HT (●), as measured by the peak of the phasic effect. Values represent mean $\pm$ S.E.M. of 11 and seven independent experiments to norepinephrine and 5-HT, respectively. (B) Representative recording of baseline before CRCs. (C and D) Representative recordings of contractions of the CE duct in vitro to norepinephrine and 5-HT, respectively.

for prazosin against 5-HT was much lower than theoretical unity (Fig. 3, A and C; Table 3), while the antagonism presented against norepinephrine was consistent with a competitive antagonism (Fig. 3, B and C; Table 3). Therefore, the effects of subtype-selective 5-HT receptor antagonists were evaluated in the presence of 100 nM prazosin to prevent activation of α_1 -ARs by 5-HT.

Increasing concentrations of the 5-HT_{2A}/5-HT_{2C} receptor antagonist ketanserin (1–30 nM) produced rightward shifts on the CRCs to 5-HT, although insurmountable antagonism was observed with concentrations from 3 to 30 nM, which reduced the $E_{\max}$ value for 5-HT (Fig. 4A). To further investigate the putative serotonergic receptors involved in 5-HT-induced contraction in the CE duct, the contraction evoked by 5-HT was evaluated in the presence of increasing concentrations of WAY 100635 (5-HT_{1A} antagonist) and buspirone (5-HT_{1A} partial agonist). Both acted as noncompetitive antagonists (slope less than unity), providing estimated pA_2 values of 8.90 ± 0.04 and 7.34 ± 0.14 , respectively (Fig. 4, B and C; Table 3). At concentrations in which fluoxetine displays affinity at 5-HT_{2A} and 5-HT_{2C} receptors (Bonhaus et al., 1997; Ni and Miledi, 1997; Owens et al., 1997; Rothman et al., 2000), 5-HT-induced contractions of the CE duct were right shifted in a surmountable manner with an estimated pA_2 value of 7.44 ± 0.16 (Fig. 4D; Table 3).

***Htr1a*, *Htr1b*, *Htr2a*, and *Htr2b*, but Not the *Htr2c* Transcript, Are Expressed in the Rat Epididymis.**

Based on the pharmacological data, we performed conventional RT-PCR assays to investigate the expression of *Htr1a*, *Htr1b*, *Htr2a*, *Htr2b*, and *Htr2c* transcripts along the rat epididymis (initial segment, caput, corpus, and cauda regions). The detection of these transcripts either in the brain or testis was used as positive controls (Fig. 5). Both the *Htr1a*

and *Htr2b* transcripts were detected in the corpus and CE, while the *Htr2a* transcript appeared in the CE only (Fig. 5). Conversely, the *Htr1b* transcript was found to be ubiquitously expressed throughout the epididymis, with higher abundance in the CE (Fig. 5). The expression of the *Htr2c* transcript was not detected in any region of the epididymis (Fig. 5).

Discussion

We revealed that 5-HT is a contractile agent in the rat CE. The 5-HT_{1A} and 5-HT_{2A} receptors are involved in the 5-HT-mediated contractions of the rat CE smooth muscle, suggesting that this serotonergic transmission might contribute to the coordinated contraction of the CE duct during the transport of spermatozoa within the epididymis. This epididymal event is crucial for the production of a fertile ejaculate since its disruption may affect fertility (Ricker et al., 1997; Solomon et al., 1997; Kempinas et al., 1998). Indeed, the blockade of epididymal and vas deferens contractions during the emission phase of ejaculation has been proposed as a pharmacological strategy for male contraception (Sanbe et al., 2007; White et al., 2013). Our results underscore that the serotonergic system in the epididymis should also be considered when targeting epididymal/vas deferens smooth muscle as a potential target for male contraception.

When used at concentrations within their selectivity windows, some of the most selective SERT inhibitors including paroxetine (0.1–10 nM) and fluoxetine (1–10 nM) were unable to increase the potency of 5-HT in the CE, indicating that the SERT is not involved in the removal of 5-HT. However, paroxetine at concentrations higher than 100 nM caused a 5-fold increase in the potency of 5-HT, which likely results from paroxetine's selectivity loss, and hence NET inhibition

Fig. 2. Differential effects of SERT and NET inhibitors on the 5-HT- and norepinephrine-induced contraction in the rat CE duct. Concentration-response curves to 5-HT are shown on the left and norepinephrine are shown on the right, in the presence of different concentrations of the selective SERT inhibitors paroxetine (A–D) and fluoxetine (E–H) and in the presence of the NET inhibitor desipramine (I and J). Each symbol represents the mean and the vertical bars, when larger than the symbols, represent the S.E.M. of independent experiments performed with CE ducts from four rats.

(Bolden-Watson and Richelson, 1993; Owens et al., 1997; Tatsumi et al., 1997). In fact, at such high concentrations paroxetine also potentiated the effect of norepinephrine, although to a greater extent than for 5-HT; this observation

is supported by the higher affinity of the NET for norepinephrine than for 5-HT (Andersen, 1989; Tatsumi et al., 1997; Rothman et al., 2001). The selective NET inhibitor desipramine also potentiated the effects of both norepinephrine and

TABLE 2
 pEC₅₀ values for 5-HT and norepinephrine in the absence and presence of monoamine reuptake inhibitors with the respective concentration ratio
 The values of control pEC₅₀ corresponds to the mean of independent experiments carried out in the absence (concentration = 0) of reuptake inhibitor. Data are expressed as mean ± S.E.M. of three to six experiments. The right displacement on the CRC to 5-HT or norepinephrine is denoted by the minus (–) signal before the CR value, which represents the ratio between agonist EC₅₀ in the absence and presence of reuptake inhibitor.

Inhibitor	Concentration	5-HT		Norepinephrine	
		pEC ₅₀	CR	pEC ₅₀	CR
	<i>nM</i>				
Paroxetine	0	6.31 ± 0.11		5.83 ± 0.10	
	0.1	6.39 ± 0.16	1.0	5.94 ± 0.10	1.3
	0.3	6.36 ± 0.17	1.0	5.97 ± 0.10	1.4
	1	6.21 ± 0.16	1.0	6.05 ± 0.13	1.6
	3	6.18 ± 0.14	1.0	6.05 ± 0.11	1.6
	10	6.41 ± 0.06	1.0	6.10 ± 0.11	1.8
	30	6.32 ± 0.13	1.0	6.19 ± 0.25	2.3
	100	6.74 ± 0.08	2.7	6.61 ± 0.10	6.0
	300	7.03 ± 0.11	5.2	7.05 ± 0.11	16.6
Fluoxetine	1000	7.03 ± 0.08	5.2	7.35 ± 0.15	33.1
	0	6.62 ± 0.13		5.90 ± 0.10	
	1	6.48 ± 0.13	−1.3	5.66 ± 0.08	−1.7
	3	6.42 ± 0.18	−1.6	5.80 ± 0.12	−1.2
	10	6.48 ± 0.20	−1.4	5.71 ± 0.08	−1.5
	30	6.33 ± 0.20	−1.9	6.12 ± 0.14	1.6
	100	6.12 ± 0.23	−3.1	6.23 ± 0.07	2.1
	300	ND		6.33 ± 0.10	2.7
	1000	ND		6.21 ± 0.14	2.0
Desipramine	0	6.26 ± 0.15		5.70 ± 0.12	
	10	6.52 ± 0.19	1.8	6.72 ± 0.17	10.5
	30	6.87 ± 0.19	4.1	ND	

ND, Not determined.

5-HT, further supporting the role of the NET in the removal of these two agonists in the CE. Although the presence of the SERT has been reported in epithelial, endothelial, and mast cells of the rat caput epididymis by immunohistochemistry (Jiménez-Trejo et al., 2007), we are not aware of studies describing its presence in epididymal smooth muscle. This may indicate the existence of region- and cell-specific mechanisms for 5-HT removal in the epididymis. It is worthwhile to establish the mechanisms of 5-HT removal in the epididymis in vivo considering both adverse and therapeutic effects of SSRIs on sperm parameters as well as on ejaculatory function (Giuliano and Clément, 2005; Kendirci et al., 2007; Rowland et al., 2010; Tanrikut et al., 2010; Abu El-Hamd and Abdelhamed, 2018).

It is known that 5-HT contracts vascular smooth muscles in vitro through α_1 -AR activation (Innes, 1962; Purdy et al.,

1987; Shaw et al., 2000). Our results showed that part of the 5-HT-mediated contraction of the CE smooth muscle is due to activation of α_1 -ARs. Indeed, the selective α_1 -AR antagonist prazosin, which exhibits low affinity for serotonergic receptors (Norman et al., 1985; Cossery et al., 1987; Lyon et al., 1987; Pauwels et al., 1993), antagonized 5-HT-induced contraction, yielding an estimated potency of $pA_2 = \sim 8.9$ (Table 3) that correlated with the value obtained for prazosin against norepinephrine as well with previous reports of pK_B values at α_1 -ARs (Pupo, 1998; Lima et al., 2005). Moreover, the Schild plot for prazosin against 5-HT yielded a regression line with a slope that was less than theoretical unity, indicating that 5-HT activates other receptor populations in addition to α_1 -ARs.

Considering that the presence of 5-HT G protein-coupled receptors (at least 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2B}) has already been demonstrated in the rat epididymis by histochemical and pharmacological approaches (Leung et al., 1999; Jiménez-Trejo et al., 2007), it is reasonable to hypothesize that the 5-HT metabotropic receptor(s) may be involved in the contractions of the CE smooth muscle to 5-HT. To explore this, the effects of selective 5-HT receptor antagonists on 5-HT-induced contractions were determined in the presence of desipramine to block the removal of 5-HT by the NET and prazosin to antagonize α_1 -ARs.

The potencies found for ketanserin (~ 9.4) and fluoxetine (7.4) in antagonizing the contractions of the CE to 5-HT are consistent with the participation of 5-HT_{2A}/5-HT_{2C}. This is due to the limited selectivity of ketanserin (Roth et al., 1992; Boess and Martin, 1994; Glennon et al., 2002; Knight et al., 2004) and fluoxetine (Sánchez and Hyttel, 1999; Rothman et al., 2000; Knight et al., 2004) for 5-HT_{2A} over 5-HT_{2C} receptors, and vice versa, thereby showing a weak ability to discriminate between 5-HT_{2A} and 5-HT_{2C} receptors. However, whereas mRNA encoding 5-HT_{2A} receptors was readily detected in the CE, transcripts for 5-HT_{2C} were not. Thus, it is likely that 5-HT_{2A} receptors have an important role in the contractions induced by 5-HT in the rat CE. It is worth noting that ketanserin presented insurmountable behavior against 5-HT in the CE. This may result from hemi-equilibrium, where the slow dissociation of ketanserin from the receptor within the short time frame required for the contraction leads to reduction in the observed 5-HT maximal response (Kenakin et al., 2006). Interestingly, ketanserin also presented insurmountable antagonism in the contractions induced by 5-HT in the rat uterus, another tissue whose contractions are fast and transient (Ichida et al., 1983).

Fig. 3. Effect of prazosin on the 5-HT- and norepinephrine-induced contractions in the rat CE duct. Concentration-response curves for 5-HT (A) and norepinephrine (B). The Schild plot for these antagonisms is presented in (C). Each symbol represents the mean and the vertical line, when greater than the symbol, represents the S.E.M. of independent experiments performed with CE ducts from three to six rats.

Fig. 4. Effect of 5-HT receptor antagonists on the 5-HT-induced contractions in the rat CE duct. Concentration-response curves to 5-HT in the presence of increasing concentrations of ketanserin (A), WAY 100635 (B), buspirone (C), and fluoxetine (D). All curves were performed in the presence of 100 nM prazosin, which was included in the inhibitor cocktail. The Schild plot for these antagonisms is presented in (E). Each symbol represents the mean and the vertical line, when greater than the symbol, represents the S.E.M. of independent experiments performed with CE ducts from three to four rats. * Denotes the mean depressed maximal response.

In addition to the participation of 5-HT_{2A} receptors, we also found evidence for the involvement of 5-HT_{1A} in the contractions induced by 5-HT in the CE. The contractions induced by 5-HT were antagonized with high potency by the selective 5-HT_{1A} ligands WAY-100635 ($pA_2 = \sim 8.9$) and buspirone ($pA_2 = \sim 7.3$); however, the Schild slopes were much less than unity, further supporting the participation of multiple 5-HT receptors. Indeed, our RT-PCR experiments demonstrated that 5-HT_{1A} and

5-HT_{1B} transcripts are abundantly present in the rat CE. However, additional studies are warranted to investigate the role of 5-HT_{1B} in the contraction of rat CE by 5-HT.

A dense network of interstitial serotonergic fibers has been identified in close proximity to the smooth muscle layer of the distal segment of the CE (Leung et al., 1999). This pattern of innervation is similar to the sympathetic innervation, which is known to play a major role in the contraction of epididymal

TABLE 3
Estimated pA_2 values of selective antagonists against contractile responses elicited by 5-HT in isolated rat CE duct
Mean $\pm$ S.E.M. When the Schild plot slopes were different from unity, the pA_2 values were calculated using the lowest positive values of log (CR - 1); n denotes number of CE preparations.

Antagonist	Agonist	<i>n</i>	<i>pA</i> ₂	Schild Slope (95% Confidence Interval)
Prazosin	Norepinephrine	4	8.92 $\pm$ 0.06	1.10 $\pm$ 0.10 (0.88–1.33)
Prazosin	5-HT	4	8.89 $\pm$ 0.20	0.58 $\pm$ 0.09 (0.37–0.79)
Ketanserin	5-HT	6	9.36 $\pm$ 0.17	— ^a
Fluoxetine	5-HT	6	7.41 $\pm$ 0.16	0.44 $\pm$ 0.14 (0.13–0.75)
WAY 100635	5-HT	4	8.90 $\pm$ 0.04	0.62 $\pm$ 0.13 (0.26–0.97)
Buspirone	5-HT	4	7.34 $\pm$ 0.14	0.20 $\pm$ 0.13 (–0.07 to 0.47)

^aThis could not be examined because the antagonism was insurmountable.

Fig. 5. Expression of 5-HT receptors in the rat epididymis. Representative inverted image of agarose gels showing the detection of *Htr1a*, *1b*, *2a*, *2b*, and *2c* mRNAs by RT-PCR as indicated (right arrow). MW indicates a 100 base pair (bp) standard DNA ladder. *Ppia* mRNA was used as the internal control. Legend: (+) positive control (brain for *Htr1a*, *1b*, *2a*, *2c*, and *Ppia* or testis for *Htr2b*); (–) negative control (H_2O). Results are representative of experiments performed in duplicate with tissues from one rat.

smooth muscle, transport of spermatozoa, and fertility (Kempinas et al., 1998; Ricker, 1998). In agreement with these observations, our results showed that the abundance of all 5-HT receptor transcripts analyzed was higher in the cauda epididymis when compared with proximal regions of this organ. It is recognized that proximal and distal epididymal regions display different morphologic and functional properties (Hinton and Robaire, 2015). Thus, further studies are warranted to understand the roles of 5-HT as a factor regulating the smooth muscle contractions also in the proximal regions of the epididymis.

It has been proposed that 5-HT regulates anion secretion from the CE epithelium via 5-HT_{1B} and 5-HT_{2B} receptors (Leung et al., 1999) and our data provide new insights into the potential roles of 5-HT as a signaling molecule regulating sperm transport and storage in the epididymis. Our study further contributes to the understanding of the peripheral effects of serotonergic drugs in the male reproductive tract.

Authorship Contributions

Participated in research design: Mueller, Kiguti, Silva, Pupo.

Conducted experiments: Mueller, Kiguti.

Contributed new reagents or analytic tools: Silva, Pupo.

Performed data analysis: Mueller, Kiguti, Silva, Pupo.

Wrote or contributed to the writing of the manuscript: Mueller, Kiguti, Silva, Pupo.

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