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Contractile effects of serotonin in the rat cauda epididymis: expression and functional characterization of 5-HT receptors

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Abstract

Serotonin (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±0.1) that were potentiated with high potency by the NET inhibitor desipramine and with low potency by the highly-selective SERT inhibitor paroxetine indicating that NET is the major mediator of 5-HT reuptake in vitro. CE contractions to 5-HT were antagonized by the α_1-AR antagonist prazosin (pA_2≅8.9), 5-$HT_{2A/2C}$ antagonists ketanserin (pA_2≅9.4) and fluoxetine (pA_2≅7.4) and 5-HT_{1A} ligands WAY 100635 (pA_2≅8.9) and buspirone (pA_2≅7.3). RT-PCR 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, 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 like 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 (7-TM or GPCRs) (Alexander et al., 2017a), with the exception of 5-HT_3, which is an ionotropic (cation-permeable) receptor (Alexander et al., 2017b). The variety of 5-HT receptor subtypes reflects the extraordinary biological relevance of 5-HT as a neurotransmitter, as well as a paracrine and autocrine signaling molecule.

Concerning the metabotropic 5-HT receptors, the 5-HT_1 and 5-HT_2 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, 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 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 5-HT_1 and/or 5-HT_2 receptor subtypes. Interestingly, high concentrations of 5-HT has been found in the epididymis, a ductal organ that plays a crucial role on sperm transport, concentration, maturation and storage until ejaculation (Kormano and Penttilä, 1968; Anderson et al., 1979). Jimenez-Trejo et al. (2006) revealed that the rat caput 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 transporter (SERT) by epididymal epithelial and non-epithelial cells. Notwithstanding, the contribution of 5-HT to epididymal physiology is still poorly understood.

It is reported that 5-HT stimulated 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; El-Hamd and Abdelhamed, 2018). It is thought that the effects of SSRI 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, whose thickness increases from proximal (caput) to distal (cauda) epididymis. In the cauda epididymis (CE), the thicker smooth muscle layer is densely innervated by post-ganglionic 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.

Taken this into account and considering: i) the ability of 5-HT to contract smooth muscle in reproductive organs, ii) the existence of a local serotoninergic system in the epididymis, and iii) 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 (12h / 12h light / dark cycle, 25 ± 2 ºC and 40 – 70% humidity) with food and water *ad libitum*. All experimental procedures were approved by the local Ethics Committee for the Use of Experimental Animals (process number 749/15) and are in agreement with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and with the ARRIVE guidelines (Kilkenny et al., 2010). Drugs used in experiments were purchased from the following sources: L-(−)-norepinephrine bitartrate salt monohydrate, serotonin HCl, ketanserin tartrate, WAY 100635, buspirone and corticosterone from Sigma; fluoxetine HCl from Fagron; paroxetine from Tocris; desipramine HCl, prazosin HCl, yohimbine HCl, (±)–propranolol HCl from Research Biochemicals Inc., RBI.

In vitro contraction studies

Contraction experiments were conducted according to 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 30G needle. For digital recording of isometric contractions, ~1.0 cm length segments of the distal CE (corresponding to region 19 according to Jelinsky et al. (2007)) were mounted in organ baths under 1.0 gram of resting tension in a modified Tyrode's solution (in mM: 138 NaCl; 5.7 KCl; 1.8 $CaCl_2$; 0.36 NaH_2PO_4; 15 $NaHCO_3$, 5.5 dextrose) prepared in glass-distilled deionized water, maintained at 30ºC, pH 7.4, and continuously bubbled with 95% O_2 / 5% CO_2. After a 30-min stabilization period, CE segments were challenged with 80 mM KCl until reproducible contractions were obtained. Then, cumulative concentration-response curves (CRC) 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 NET and SERT, α_2- and β-adrenoceptors, respectively.

To investigate the role of norepinephrine (NET) and/or serotonin (SERT) transporters, 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 α_1-ARs was investigated using prazosin (α_1-AR antagonist) and of 5-HT receptors 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 pEC_{50}, i.e. the negative logarithm of the concentration of 5-HT or norepinephrine producing 50% of its maximal response (E_{max}). Effects of each concentration of norepinephrine or 5-HT were measured as the peak of the respective induced contraction of agonist.

Schild analysis

Antagonists 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 concentration-ratios (CR), that 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

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different from unity the intercept in abscissa (pA_2) was taken as the antagonist dissociation constant (pK_B). However, as in most assays the slope in the Schild plots were much lower than 1.0, pA_2 values were taken as estimates of antagonist potencies calculated from the formulae: pA_2= log (CR-1) – log [B], where [B] is the lowest effective antagonist concentration.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

RT-PCR was performed to detect each specific gene target. Total RNA from epididymal regions (initial segment, caput, corpus and cauda), testis and brain were extracted using TRIzol® Reagent (Thermofisher) according to 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 cDNAs were synthesized from total RNA (2 μg) for 1 h at 55 °C, in a reaction volume of 20 μl. The resulting cDNAs (2 μl) were amplified by PCR (SimpliAmp, Applied Biosystems) in a final volume of 20 μl containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 units Taq DNA polymerase, and 0.4 μM of each sense and antisense primers 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 shown in Table 1. Primers for *Htr1a*, *Htr1b*, *Htr2b*, *Htr2c* and *Ppia* were designed using 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 μl) were loaded onto agarose gels (1.5%, w/v) containing 10 μg/ml Sybr Safe DNA

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Gel Stain (Invitrogen) and results were imaged using the G:BOX XR5® (Syngene) imaging system.

Statistical Analysis

Curve fitting was performed by nonlinear regression using the 3 parameter logistic equation from GraphPad Prism (version 6), and maximal response (E_{max}), pEC_{50} and pA_2 values were calculated accordingly. All values are shown as means ± standard error of mean (SEM) of *n* independent experiments. Differences between mean values were tested for statistical significance ($P < 0.05$) using ANOVA followed by Dunnet's for multiple comparisons.

Results

5-HT contracts the rat cauda epididymis smooth muscle

5-HT elicited concentration-dependent contractions, presenting a pEC_{50} of 6.45 ± 0.14 and a maximal response that corresponded to ~45% of norepinephrine E_{max} (Figure 1A). As shown in the typical recordings in Figure 1C and 1D, 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 NET rather than SERT

To investigate the participation of 5-HT reuptake system, CRCs to 5-HT were performed in the absence and presence of different concentrations of SERT inhibitors (Figure 2) and the influence of the reuptake was estimated in terms of agonist concentration-ratios measured at the EC_{50}s (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 (Figure 2A and 2E, Table 2). These low concentrations of both paroxetine and fluoxetine did not affect CRCs to norepinephrine (Figure 2B and 2F). At higher concentrations, however, paroxetine 100-1000 nM increased the potency of 5-HT and norepinephrine by up to ~5-fold and ~30-fold, repectively (Figure 2C, 2D and Table 2).. Conversely, fluoxetine 30-100 nM reduced the potencies of 5-HT by up to ~3-fold in the CE duct (Figure 2G and Table 2). At such high concentrations, fluoxetine did not affect the potencies of norepinephrine in the CE duct (Figure 2H and Table 2).

These data indicated that 5-HT is not taken up by SERT in the CE duct, and that 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 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

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the effects of 5-HT in the CE duct by up ~4-fold (Figure 2I and Table 2), whereas desipramine 10 nM potentiated the effects of NE by ~10-fold (Figure 2J and Table 2).

α_1-adrenoceptors, 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 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 pA_2 of 8.89, which well correlated to that potency obtained against norepinephrine (pA_2 = 8.92) (Figure 3A-C; Table 3), indicating that α_1-ARs are involved in the contractions induced by 5-HT. However, the slope in the Schild plot for prazosin against 5-HT was much lower than theoretical unity (Figure 3A and 3C; Table 3), while the antagonism presented against norepinephrine was consistent with a competitive antagonism (Figure 3B-C; Table 3). Therefore, the effects of subtype selective 5-HT receptors 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 the concentrations from 3 to 30 nM, which reduced the E_{max} for 5-HT (Figure 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$_{1A}$

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antagonist) and buspirone (5-HT_{1A} partial agonist). Both acted as noncompetitive antagonists (slope less than unity), providing an estimated pA_2 of 8.90 ± 0.04 and 7.34 ± 0.14, respectively (Figure 4B, 4C and 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 of 7.44 ± 0.16 (Figure 4D and Table 3).

***Htr1a, Htr1b, Htr2a, Htr2b*, but not *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 in the testis was used as positive controls (Figure 5). Both *Htr1a* and *Htr2b* transcripts were detected in the corpus and CE, while *Htr2a* transcript appeared in the CE only (Figure 5). Conversely *Htr1b* transcript was found to be ubiquitously expressed throughout the epididymis, with higher abundance in the CE (Figure 5). The expression of *Htr2c* transcript was not detected in any region of the epididymis (Figure 5).

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Discussion

We revealed that 5-HT is a contractile agent in the rat CE. 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 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 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 (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 at a greater extent than for 5-HT; this observation is supported by the higher affinity of 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 5-HT, further supporting the role of NET in the removal of these two agonists in the CE. Although the presence of SERT has been reported in epithelial, endothelial and mast cells of the rat caput epididymis by immunohistochemistry (Jimenez-Trejo et al., 2006), 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; 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_2~8.9, Table 3) that correlated with the value obtained for prazosin against norepinephrine as well with previous reports of pK_B at α_1-ARs (Pupo, 1998; Lima et al., 2005). Moreover, the Schild plot for prazosin against 5-HT yielded a regression line with slope less than theoretical unity, pointing that 5-HT activates other receptor population in addition to α_1-ARs.

Considering that the presence of 5-HT GPCRs (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; Jimenez-Trejo et al., 2006), it is reasonable to hypothesize that 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 NET and of 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 little 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. It may result from a hemi-equilibrium where the slow dissociation of ketanserin from the receptor within the short time frame required for the contraction lead to a 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).

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_2~8.9) and buspirone (pA_2~7.3), but 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. Additional studies, however, 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 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 to proximal regions of

this organ. It is recognized that proximal and distal epididymal regions display different morphological 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, and Pupo.

Conducted the experiments: Mueller, Kiguti.

Contributed new reagents or analytic tools: Silva and Pupo

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

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

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Footnotes

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Legends for Figures and Tables

Figure 1 – Contractile effects of 5-HT and norepinephrine in the rat CE duct. (A) Mean concentration-response curves (CRCs) 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 ± S.E.M of 11 and 7 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.

Figure 2 – Differential effects of SERT and NET inhibitor(s) 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 on the right, in the presence of different concentrations of the selective SERT inhibitors paroxetine (A, B, C, D) and fluoxetine (E, F, G, H) and in the presence of the NET inhibitor desipramine (I, J). Each symbol represents the mean, and the vertical bars, when larger than the symbols, the SEM of independent experiments performed with CE duct from 4 rats.

Figure 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, the SEM of independent experiments performed with CE duct from 3 to 6 rats.

Figure 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 was 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, the SEM of independent experiments performed with CE duct from 3 to 4 rats. *means depressed maximal response.

Figure 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 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.

Table 2 – The values of control pEC_{50}, aligned to the left, corresponds to the mean of independent experiments carried out in the absence (concentration = 0) of reuptake inhibitor.
Data are expressed as mean ± SEM of 3-6 experiments.
CR = ratio between agonist EC_{50} in the absence and presence of reuptake inhibitor.
The right displacement on CRC to 5-HT or norepinephrine is denoted by the minus (-) signal before the CR value.

Table 3 – Mean ± S.E.M., n = number of CE preparations. When Schild plot slopes were different from unity, pA_2 were calculated using the lowest positive values of log (CR – 1).
---[a] It could not be examined because the antagonism was insurmountable.

TABLES

Table 1 – Primers and conditions for RT-PCR

Gene		Primer sequence (5' – 3')	Amplicon size (bp)	Accession number	Initial denaturation (ºC/min)	Denaturation (ºC/s)	Annealing (ºC/s)	Elongation (ºC/s)	Final Elongation (ºC/min)
						35 cycles			
Htr1a	F:	GTCACCTGCGACCTGTTTAT	286	NM_012585.1	95 ºC/2min	95 ºC/60 s	58 °C/60 s	72 ºC/90 s	72 ºC/3min
	R:	CGAAAGTGGAGTAGATGGTGT							
Htr1b	F:	CACTGATGCGGTGGACTATT	217	NM_022225.1	95 ºC/2min	95 ºC/60 s	58 °C/60 s	72 ºC/75 s	72 ºC/3min
	R:	GAGCAGGGTGGGTAAATAGAA							
Htr2a	F:	AGCTGCAGAATGCCACCAACTAT	322	NM_017254.1	95 ºC/2min	95 ºC/60 s	60 °C/60 s	72 ºC/75 s	72 ºC/3min
	R:	GGTATTGGCATGGATATACCTAC							
Htr2b	F:	CTGTGTCCTGCCTGGTTATT	224	NM_017250.1	95 ºC/2min	95 ºC/60 s	58 °C/60 s	72 ºC/75 s	72 ºC/3min
	R:	TTGACCACATCAGCCTCTATTC							
Htr2c	F:	GCTAGCGGGTTGTCAACTAT	322	NM_012765.3	95 ºC/2min	95 ºC/60 s	60 °C/60 s	72 ºC/75 s	72 ºC/3min
	R:	CGACGATTGAAAGTGCTGGC							
Ppia	F:	AGCACTGGGGAGAAAGGATT	174	NM_017101.1	95 ºC/2min	95 ºC/60 s	60 °C/45 s	72 ºC/60 s	72 ºC/3min
	R:	GATGCCAGGACCTGTATGCT							

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Table 2 – pEC_{50} values for 5-HT and norepinephrine in absence and presence of monoamine reuptake inhibitors with the respective concentration-ratio

Inhibitor	Concentration (nM)	5-HT		Norepinephrine	
		pEC_{50}	CR	pEC_{50}	CR
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
	1000	7.03 ± 0.08	5.2	7.35 ± 0.15	33.1
Fluoxetine	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	--		6.33 ± 0.10	2.7
	1000	--		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	--	

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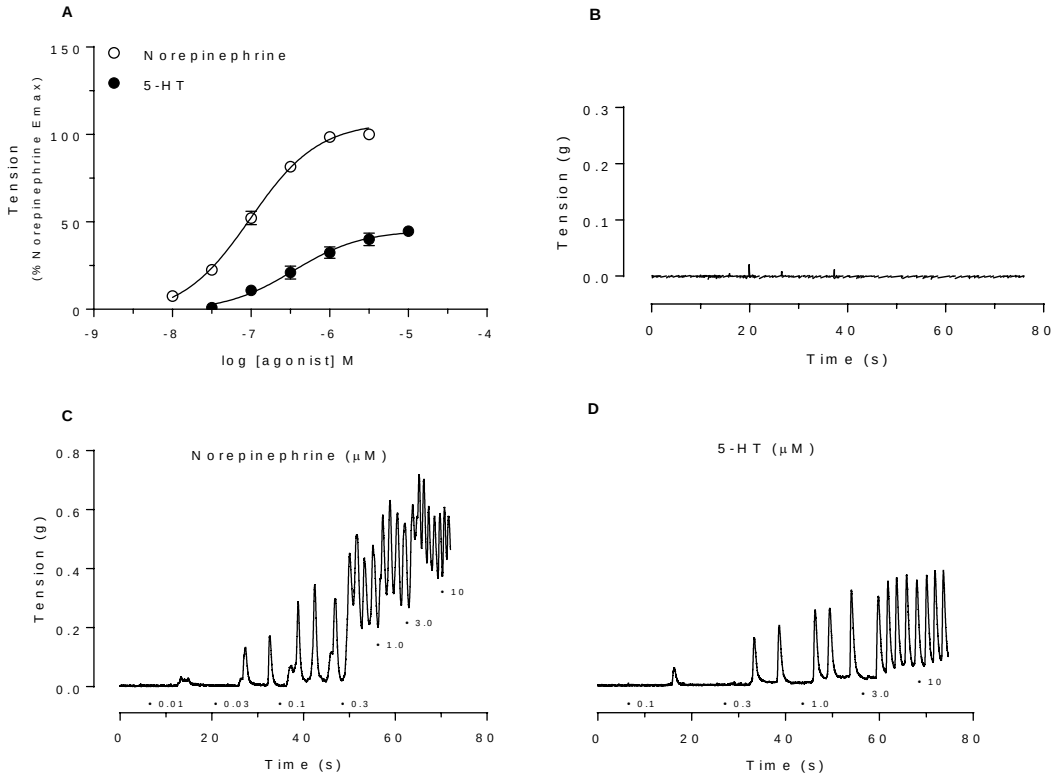
Table 3 – Estimated pA_2 of selective antagonists against contractile responses elicited by 5-HT in isolated rat CE duct

Antagonist	**Agonist**	**n**	**pA_2**	**Schild Slope** (95% confidence intervals)
Prazosin	Norepinephrine	4	8.92 ± 0.06	1.10± 0.10 (0.88 – 1.33)
Prazosin	5-HT	4	8.89 ± 0.20	0.58 ± 0.09 (0.37 – 0.79)
Ketanserin	5-HT	6	9.36 ± 0,17	----[a]
Fluoxetine	5-HT	6	7.41 ± 0.16	0.44 ± 0.14 (0.13 – 0.75)
WAY 100635	5-HT	4	8.90 ± 0.04	0.62 ± 0.13 (0.26 – 0.97)
Buspirone	5-HT	4	7.34 ± 0.14	0.20 ± 0.13 (-0.07 – 0.47)

Mueller et al. - FIGURE 1



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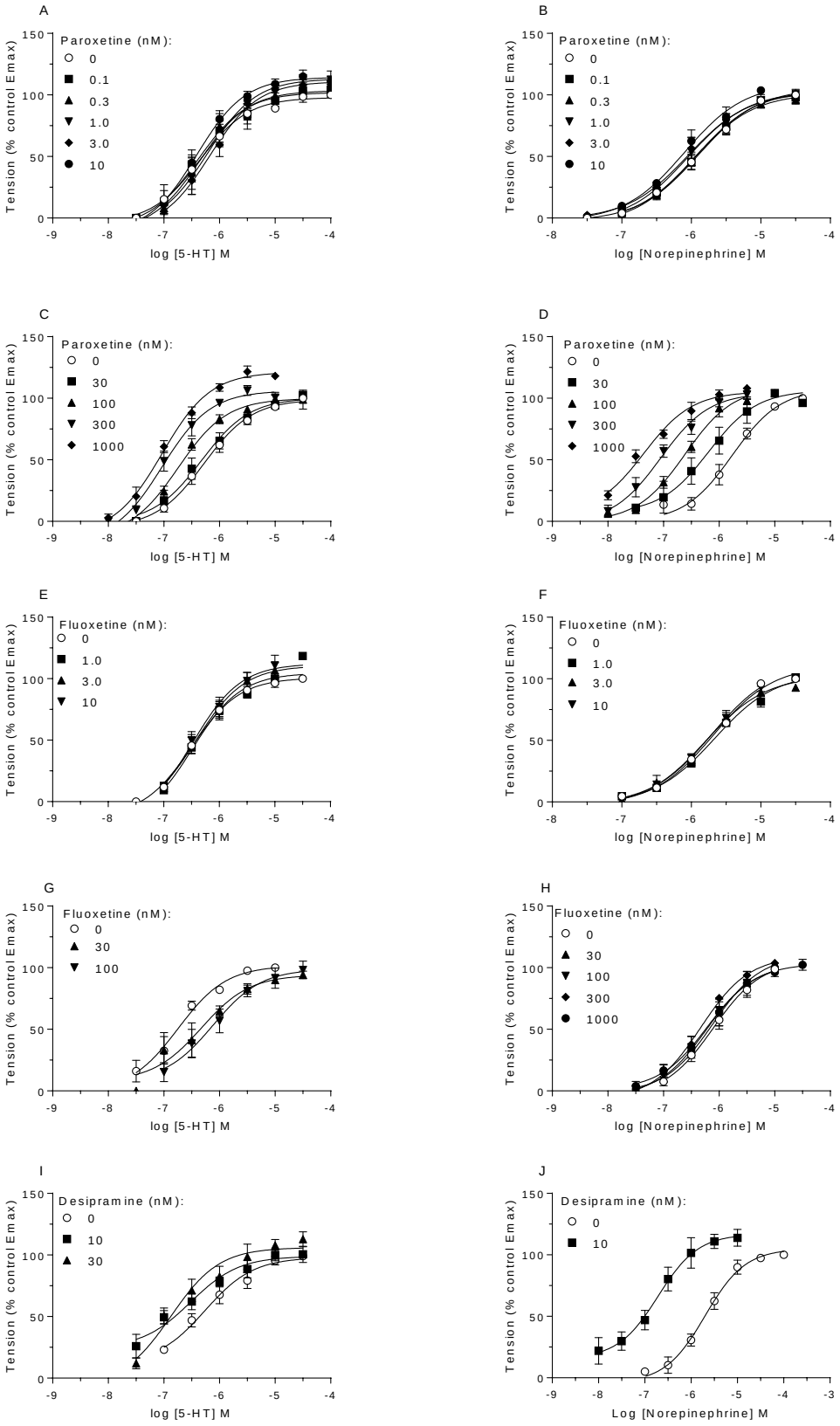
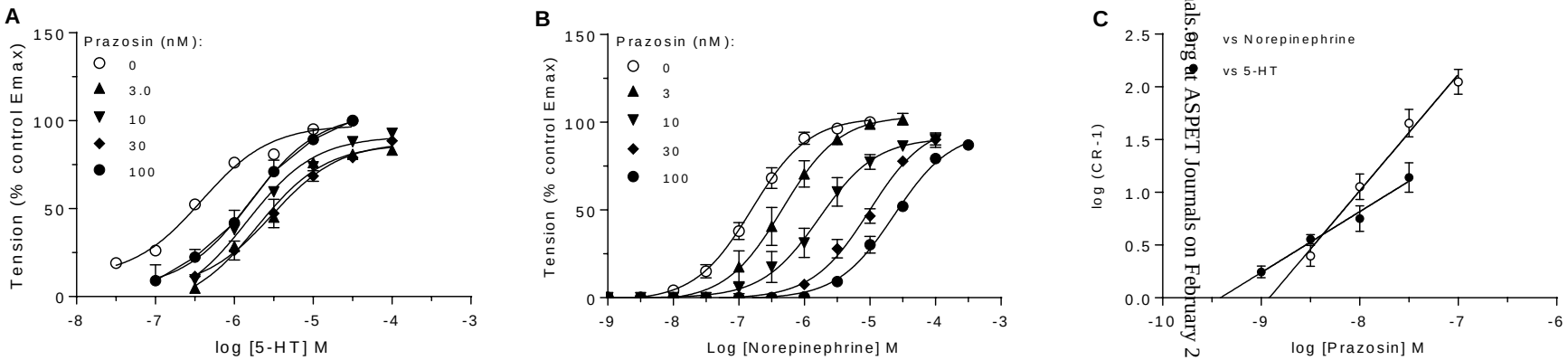
Mueller et al. - FIGURE 2



Mueller et al. - FIGURE 3



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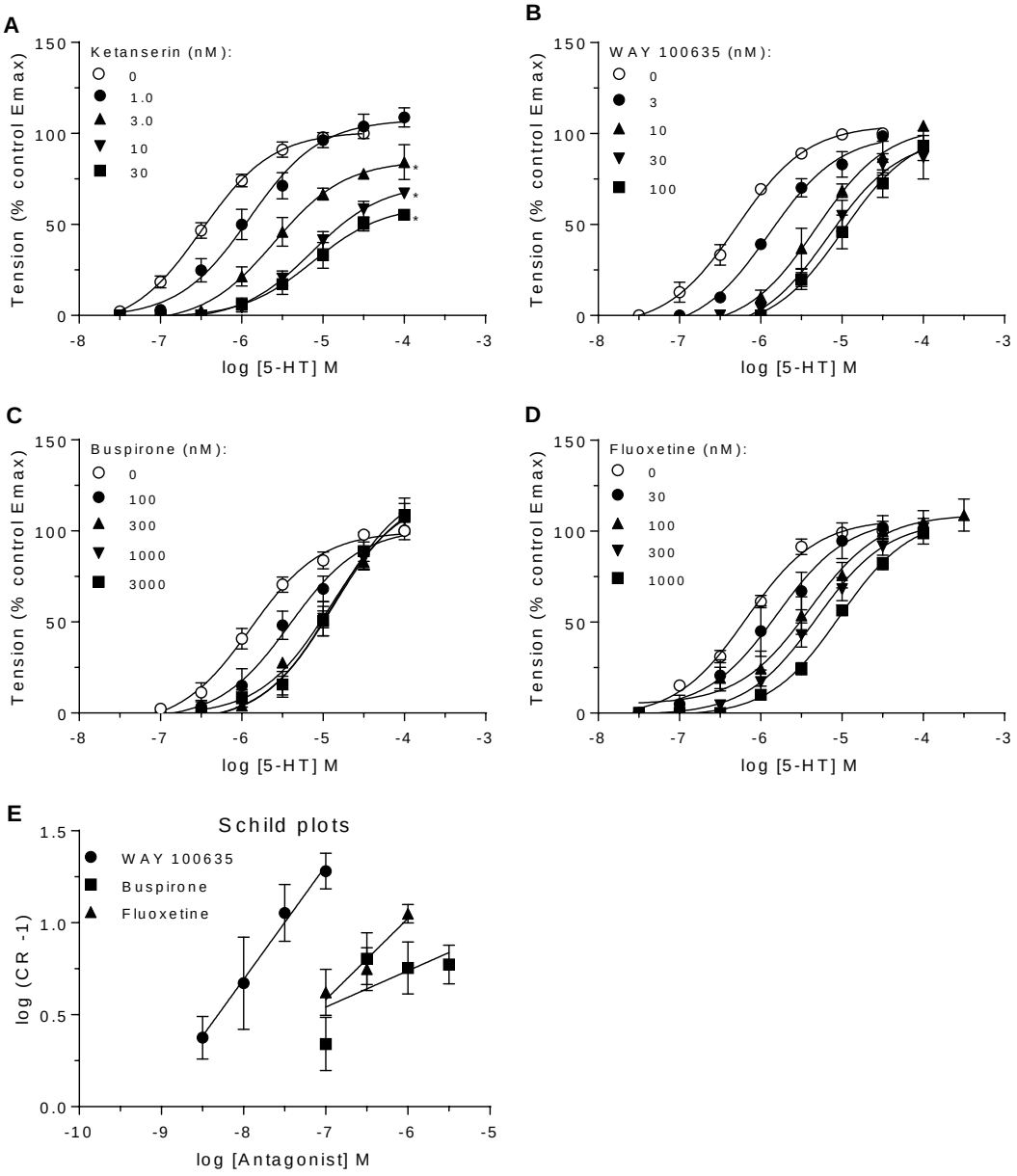
Mueller et al. - FIGURE 4



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Mueller et al. - FIGURE 5