Universidade do Minho
Escola de Medicina

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The Role of Serotonin in Regulation of Benign Prostatic Growth – Implications for Benign Prostatic Hyperplasia Etiology

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UMinho | 2021 Emanuel Carvalho-Dias **The Role of Serotonin in Regulation of Benign Prostatic Growth – Implications for Benign Prostatic Hyperplasia Etiology**

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Tese de Doutoramento
Doutoramento em Medicina

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Statement of integrity

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

O Papel da Serotonina na Regulação do Crescimento Benigno da Próstata – Implicações para a Etiologia da Hiperplasia Benigna da Próstata.

Resumo

O envelhecimento e a presença de testosterona causa quase inexoravelmente hiperplasia benigna da próstata (HBP) nos homens, no entanto a etiologia da HBP é largamente desconhecida. A serotonina (5-HT) é uma amina biogénica produzida pelas células neuroendócrinas da próstata e está presente em altas concentrações na próstata normal, mas a sua função na fisiologia prostática é desconhecida. A evidência acumulada tem demonstrado que as células neuroendócrinas e a 5-HT estão diminuidas ou ausentes no tecido com HBP comparativamente à próstata normal. Aqui, nós demonstramos que a 5-HT é um forte regulador negativo do crescimento prostático. *In vitro*, a 5-HT inibe na próstata de rato o processo de morfogénese por ramificação e esta função reguladora negativa é associada à diminuição do receptor de androgénio (AR). Este mecanismo inibirtório da 5-HT está também presente em células humanas de próstata normal e próstata com HBP, mas apenas em linhas celulares que expressam o AR e tratadas com testosterona. Uma vez que a produção periférica de 5-HT é especificamente regulada pela triptofano hidroxilase 1 (*Tph1*), nós usamos ratinhos knockout para a *Tph1* e demonstramos que estes ratinhos exibem uma próstata com maior massa comparativamente a ratinhos selvagem. Demonstrámos também aqui que o tratamento de ratinhos knockout para a *Tph1* com 5-HT restaura a massa prostática para os níveis de ratinhos selvagem. Finalmente, nós demonstramos neste trabalho que a suplementação oral com uma dieta rica em triptofano diminui a massa prostáticas em ratinhos. Uma vez que a serotonina está diminuida na HBP nós apresentamos aqui evidência que liga a deplecção de serotonina à etiologia da HBP através de modulação do recetor de androgénios. A via serotoninérgica prostática deverá ser explorada como um novo alvo terapêutico para a HBP.

Palavras-chave: hiperplasia benigna da próstata; receptor androgénios; serotonina.

The Role of Serotonin in Regulation of Benign Prostatic Growth – Implications for Benign Prostatic Hyperplasia Etiology.

Abstract

Aging and the presence of testosterone cause almost inexorably benign prostatic hyperplasia (BPH) in human males, however the etiology of BPH is largely unknown. Serotonin (5-HT) is a biogenic amine produced by neuroendocrine prostatic cells and present in high concentration in normal prostate but its function in prostate physiology is unknown. Evidence have demonstrated that neuroendocrine cells and 5-HT are decreased or absent in BPH tissue comparatively to normal prostate. Here, we show that 5-HT is a strong negative regulator of prostate growth. *In vitro*, 5-HT inhibits rat prostate branching morphogenesis and this negative regulatory function is associated with a down-regulation of androgen receptor (AR). This 5-HT inhibitory mechanism was also present in human cells of normal prostate and BPH, but only in cell lines expressing AR and treated with testosterone. Since peripheral 5-HT production is specifically regulated by tryptophan hydroxylase 1 (*Tph1*), we used *Tph1* knockout mice and showed that this mice have higher prostate mass comparatively to wild-type. We show here also that treatment of *Tph1* knockout mice with 5-HT restores prostate mass to levels of wild-type. Finally, we demonstrate in this work that oral supplementation with a tryptophan rich diet decreases de prostate mass in mice. As serotonin is decreased in BPH we present here evidence that links 5-HT depletion to BPH etiology through modulation of AR. Serotoninergic prostate pathway should be explored as a completely new therapeutic target for BPH.

Keywords: androgen receptor; benign prostatic hyperplasia; serotonin .

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Abbreviations

ABs – alfa adrenergic blockers

AR – Androgen Receptor

BPE - Benign Prostatic Enlargement

BPH – Benign Prostatic Hyperplasia

DHT- Dihydrotestosterone

EGF- Epidermal growth factor

ER – Estrogen receptor

FDA – Federal Drug Administration

FGF- Fybroblast growth factor

FGFR- Fybroblast growth factor receptor

GFR – Glomerular filtration rate

HBP – Hiperplasia Benigna da Próstata

hPrE - human prostatic epithelium

IIEF-5- Índice Internacional de Função Erétil-5

IPSS -International Prostate Symptom Score

LUTS – lower urinary tract symptoms

MSC- Mesenchymal stem cells

PALT- prostate-associated lymphoid tissue

PSA – prostate specific antigen

STUI – Sintomas do trato urinário inferior

TGF-beta- Transforming growth factor beta

T_H1- T-helper1

T_H2- T-helper23

TPH1 – Tryptophan hydroxylase type 1

TPZ – transition and peri-urethral zone

UGS- Urogenital sinus

UTI - urinary tract infection

5-HT- serotonin

5-HTR- serotonin receptors

5- ARI - 5α-reductase inhibitor

CHAPTER 1 | Introduction

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Introduction

1.1. The Prostate Gland

The first draft of the prostate gland on Earth was built probably around 251 and 201 million years ago during the Triassic of the Mesozoic Era. In that geologic period, the ancestral mammals develop for the first time a urethra (distinct from the cloaca of birds and reptiles) that was the basis for the formation of rudimentary male accessory sex glands (Price et al., 1961). As mammals' evolution continued the urethra accessory glands evolved in different anatomy and function but all the three classes of mammals: monotremes, marsupials and eutherian share a "prostate gland" (Rodger, 1975).

The historic evolution of the knowledge of prostate gland started a long time ago and evolved for centuries. Important for the understanding the history of prostate we need to go back to the 3th century B.C. In that century, the Greek Anatomist Herophilhus (the father of Anatomy), under the school of Hippocrates in Alexandria identified for the first time the seminal vesicles and used to describe them the term *parastatai adenoeides* - "glandular assistants", however, the anatomically description of the prostate gland was not made in that time (Marx et al., 2009). Only eighteen centuries after, in 1536, the Venetian physician Niccoló Massa in his work "Introductory book of Anatomy" made reference to a new anatomical structure that he described as "glandular fesh upon which rests the neck of the bladder" (Marx et al., 2009). However, only in 1538, Andreas Vesalius (the father of Modern Anatomy), recognized, described and drew the prostate gland in humans, in its observations of the male accessory glands and described this structure as *corpus glandulosum* (Goddard, 2019). In the year 1600, the French Anatomist, André du Laurens, first called, to the chestnut-sized structure under the bladder drew by Vesalius, as *prostatae* (Vesalius et al., 1950). The term *prostatae* used by André du Laurens resulted from two important errors. First Du Laurens, assumed that the structures described by Herophilus (*parastatai adenoiedes*) were in fact the prostate gland drew by Vesalius. Second, in most of the medieval books the *parastatai adenoiedes* were incorrectly translated to *prostatai adenoids.* However, in the next decades until our days the word *prostatae* introduced by Du Laurens was perpetuated for the description of a fibromuscular gland, with an inverted cone shape, having the base bellow the urinary bladder neck and the apex immediately proximal to external urethral sphincter.

Functionally prostate gland is exquisitely dependent from testes, and this dependence was probably first recognized by the Scottish surgeon John Hunter in 1786. Hunter described in his 1786 work " Observations on the glands situated between the rectum and the bladder, called vesiculae seminales"

that "the prostate and Cowper's glands and those of the urethra which in the perfect male are soft and bulky with a secretion salty to the taste, in the castrated animal are small, flabby, tough and ligamentous and have little secretion" (Hunter, 1840).

The prostate gland is a male reproductive accessory gland involved in fertility. Male fertility requires the cooperation of different organs of the male urogenital system, namely the testes, epididymis, prostate, seminal vesicles and bulbourethral glands (Verze et al., 2016). After ejaculation, a synchronized cascade of events occurs with the unique objective of optimize the capacity of spermatozoa to reach and fertilize an egg (Hayward et al., 2000). The human male ejaculate is one of the most complex body fluids. Almost 70% of the ejaculate is produced in seminal vesicles and the predominant proteins produced by these are seminogelin and fibronectin (Lilja et al., 1984; Lilja et al., 1989) and both are mainly involved in coagulation of semen post-ejaculation (Lilja et al., 1984; Lilja et al., 1987). The prostate gland produces approximately 25% of human ejaculate, and the major protein produced by prostate is a serine protease of the kaliikrein-type known most commonly as prostate-specific antigen (PSA). PSA is involved in liquefaction of semen and because of that one of the major physiologic functions of prostate gland is the liquefaction of the semen coagulum 5 to 20 minutes after its formation (Lilja, 1985). The process of liquefaction enables the sperm to be released and move to the Fallopian tube. Prostatic secretions are not absolutely required for human fertility, but without prostate the human fertility is significantly impaired (Verze et al., 2016).

The great attention that Medicine in general and Urology in particular give to the prostate gland is because this organ is a target of two very common diseases: Benign Prostatic Hyperplasia (most frequent disease in Human male) and Prostate Cancer (most frequent non-cutaneous cancer in Human male). This Thesis will focus on the etiology of one of the most frequent Human male disease: Benign Prostatic Hyperplasia.

1.2. The Prostate Gland Development and Growth

The human embryo has the potential to develop towards a male or female phenotype. In normal individuals, this is genetically determined and depends of four critical structures: the fetal gonad, the Wolff and Muller ducts and the Urogenital sinus (UGS). Under the influence of androgens produced by fetal testis the prostate gland will start its development from the UGS (Aaron et al., 2016).

Prostatic development has been studied in many mammalian species, and while species-specific details of prostatic development have been noted, the development process is remarkably similar in all species studied. The most detailed description of prostatic development has been reported from mouse and rat (Price 1936; Price 1963; Staack et al., 2003), while the knowledge of human prostatic development is especially incomplete. In general, prostate development is subdivided into the following sequential stages: Prostate induction, initial budding from UGS, bud elongation and branching, ductal canalization of solid epithelial chords and cellular Differentiation (Aaron et al., 2016).

The first event, prostate induction from the UGS, is poorly understood but is in general accepted that is direct or indirectly dependent from androgens (Marker et al., 2003). In fact, the production of testosterone in fetal male embryo starts at 9 weeks of gestation and precedes immediately in time the prostatic induction. Moreover, the induction of prostate is dependent of the paracrine interaction between UGS mesenchyme and UGS epithelium with the main role attributed to mesenchyme (Cunha et al., 1987; Donjacour and Cunha, 1988).

The next two phases, epithelial budding and branching morphogenesis, are characterized by the outgrowth of solid epithelial buds from the UGS epithelium. During this period, individual and bilateral sets of prostatic buds emerge from specific locations from the UGS epithelium, this buds elongate and invade the UGS mesenchyme and starts the prostate tissue outgrowth, through a process of branching morphogenesis (Timms et al., 1994; Sugimura et al., 1986; Donjacour and Cunha, 1988).

The Branching morphogenesis of the exocrine glands (prostate, salivary, mammary and pancreas) or organs like lung, kidney and pancreas is an extremely complex process but with numerous similarities in all these glands/organs. In humans, prostate branching morphogenesis occurs at the solid tips of elongating prostatic buds after 12 weeks of gestation (Cunha et al., 2018). This process is totally dependent of AR stimulation by androgens (Thomson, 2001). After testes start the production of testosterone at 8-10 weeks of gestation, this testosterone is converted to DHT that is a tenfold more potent then testosterone (Siiteri et al., 1974; Andersson et al., 1989; Wilson 2011). This conversion is mainly executed in UGS mesenchyme that express 5-α reductase. Over the next weeks, binding of DHT to AR stromal cells will secret several paracrine growth factors collectively known as Andromedins, mainly,

insulin-like growth factor, vascular endothelium growth factor and FGF 7 and 10 (Yan et al., 1992; Richard et al., 2002; Marker et al., 2003; Ohlson et al., 2007). These Andromedins diffuse to the epithelial compartment as well to stroma where they will bind to their receptors. In the stroma, Andromedins in conjugation with epithelial sonic hedgehog will stimulate myogenesis and in epithelium they will induce budding from the initially AR-negative UGS epithelium into the surrounding UGS mesenchyme (Lamm et al., 2002; Karhadkar et al., 2004; Peng et al., 2013).

Contrarily to the majority of organs that develop trough branching morphogenesis, at birth, the prostate is small with a limited number of undeveloped buds. Postnatally the prostate will complete its development through cellular differentiation (Sugimura et al., 1986). The prostatic buds proliferate mainly at the tips and undergo a process of canalization in a proximal to distal direction (from the urethra to the tips). At the same time, the epithelial cells differentiate in two major cell types: the luminal and basal cells (Hayward et al., 1996) and the UGS mesenchyme proliferates and originates two cell types: the fibroblasts and smooth muscle (Hayward et al., 1996). The mechanism of cellular differentiation is poorly understood but from studies in genetically engineered mice some candidates have emerged, most notably, the transcription factors Foxa1, Nkx3.1, Sox9 and the transcriptional regulator Hoxb13 (Timms, 2008).

The human prostate gland is small in childhood weighing around 2 g and this will be the size until puberty. At puberty, the prostate will growth significantly to a size of about 20 g (Schauer et al., 2011). This is directly related to the rise of plasmatic testosterone during puberty. As a general rule the most important stimulator of post-natal prostate growth are also the Androgens (Schauer et al., 2011). In prostate, the most biological active androgen is 5α-dihydrotestosterone (DHT) which is produced by the local reduction of testosterone (produced in the testes) by the enzyme Δ^4-3-ketoesteroid-5α-reductase (5α-reductase) (Wilson, 2011). In humans two forms of 5α-reductase exists, type 1 and type 2, but in the genital tract the active form is the type 2 which is localized both in epithelium and prostatic stroma. The reduction of testosterone to DHT originates a much more potent agonist of AR and the great stimulatory effects of androgens over the prostatic growth are executed mainly by DHT (Azzouni et al., 2012).

Interestingly, the prostatic weigh stabilizes after puberty and remains almost the same until the end of the third decade of life when the mean prostatic weight begins to rise slowly (Marker et al., 2003; Aaron et al., 2017). The growth kinetics of prostate gland is very curious because the human prostate is an extremely slow-growing organ. Even during puberty (the peak rate of prostatic growth) the gland as a doubling time of nearly 2.76 years (Berry et al., 1984).

We can resume human prostate growth in three waves (Schauer et al., 2011). The first wave takes place during fetal life, the second wave during puberty and the third wave begins somewhere at midlife and proceeds to senescence. Is consensual today that the first and second waves are caused by the rising in plasmatic testosterone. In fact, testosterone in male fetuses begin to increase at the 8th week of gestation and peak at 16th week at a concentration similar to adulthood life and slowly decrease to the low levels detected at birth (Rastrelli et al., 2019). Interestingly, the prostate branching morphogenesis follows this first testosterone surge (Kellokumpu-Lehtinen et al., 1980). Shortly after birth, testosterone increases again for a 6-month period, known as mini-Puberty, and then declines to undetectable levels during childhood (Cunha GR et al., 1992). Paralleling the declining of testosterone after mini-puberty, prostate volume decreases slightly Rastrelli et al., 2019) and its growth is completely quiescent until puberty where the great production of testosterone is associated with the second wave of prostatic growth and the prostate reaches the weight of 20 g (Schauer et al., 2011). In contrast to almost all human organs the prostate starts enlarging in the middle age and continues to enlarge in the elderly period (Rastrelli et al., 2019). However, this third wave of growth is unique because it affects only a specific region of the prostate and starts at the moment of the age-related decline in plasmatic testosterone.

1.3. The Prostate Gland Anatomy and Its Implications for Disease

In 1912, Lowsley made a revolution in the anatomy of the prostate gland. Lowsley identified five separate groups of prostatic ducts originating from the UGS and used for the first time the term “lobes” to describe them. These were designated the middle lobe, the two lateral lobes, the posterior lobe and the ventral lobe in relation with the site of origin from the UGS (Lowsley, 1912). The nomenclature proposed by Lowsley to describe prostate anatomy started a debate about the nomenclature used to describe anatomy of human prostate that continued for 70 years. Lowsley made his observation in human fetuses but in the adult prostate the lobes that he described are fused and cannot be separated by dissection, giving rise to many different views and controversies on the anatomic division of the human prostate (Franks, 1954; Hutch et al., 1970, McNeal, 1984; Tissel, 1984).

In 1984, John McNeal proposed a different division of the human prostate into three major areas that are histologically and anatomically distinct. These areas are the non-glandular fibromuscular anterior stroma, the central zone and the peripheral zone. The central zone was described as a wedge of glandular tissue at the base of the prostate and surrounding ejaculatory ducts. The peripheral gland made up the remainder of the gland, surrounding most of the central zone extending caudally to surround the distal part of the urethra (McNeal, 1984). Importantly, after this initial description McNeal identified an

additional fourth smaller glandular region surrounded the prostatic urethra that he called "Transition Zone" (McNeal, 1990; Selman, 2011).

The new anatomic classification proposed by McNeal is now the most accepted nomenclature used to describe the heterogeneity of prostate gland. The "Zonal Anatomy" of McNeal defines areas histologically and anatomically different but more important defines zones that are the origin of tremendous different diseases.

The peripheral Zone is composed by ducts originating from the postero-lateral urethral wall. This zone is composed by small acini emptying in long and narrow ducts surrounded by a loose stroma. Ducts and acini are lined with simple columnar epithelium. Clinically, this area is the main site of prostate carcinoma and prostatitis although not of Benign Prostatic Hyperplasia (BPH) (Aaron et al., 2017). The Central Zone is composed by ducts originating proximally closely following the ejaculatory duct. The acini and ducts are much larger and the stroma is much more compact than the peripheral zone. Clinically, the central zone has a low incidence of disease (Aaron et al., 2017). Lastly, the Transition Zone surrounds the urethra between the bladder and the veromontanum. This is a very small volume of the prostate, less than 5% in the normal young prostate, but is the principal and almost exclusive site of BPH origin (Aaron et al., 2017).

1.4. Benign Prostatic Hyperplasia

The prostate gland can be pathological affected mainly by three conditions: BPH, Prostate Cancer and Prostatitis. BPH is a nonmalignant growth of prostatic gland and represents the microscopic evidence of stromal and epithelial hyperplasia. This proliferative process occurs exclusively in the Transition Zone of the prostate gland. Frequently, but not always, microscopic BPH is associated with Benign Prostatic Enlargement (BPE). This "Macroscopic BPH" represents the enlargement of the prostate Transition Zone arising from the stromal and epithelial proliferation (Bushman, 2009).

Histologic BPH have an extraordinary Prevalence in Human male. Approximately 50% of men in the sixth decade and 90% of men by the ninth decade of life exhibited histologic evidence of BPH, however, histologic BPH was rarely observed in men under the age of 30 (Berry et al., 1984). Data from almost around all the world demonstrates that histologic prevalence of BPH is similar throughout the world (Roehrborn, 2005).

By other side, "Clinical BPH" represents all the possible clinical manifestations attributed to the prostate enlargement caused by BPH. These manifestations include, lower urinary tract symptoms (LUTS), bladder outlet obstruction, acute and chronic urinary retention, urinary tract infection (UTI),

bladder stones and hematuria (Lepor, 2005). Hopefully, most commonly, the prostate enlargement causes only LUTS that can be clinical divided in obstructive (weak stream, straining, hesitancy, intermittency, incomplete bladder emptying) and irritative (frequency, nocturia and urgency with or without urinary incontinence). Many urological and non-urological diseases, other than BPE, can cause all the possible clinical manifestations of BPH, including prostate cancer, prostatitis, urethral stenosis, bladder stones, bladder cancer, overactive bladder, interstitial cystitis, UTI, diabetes mellitus, Parkinson´s disease, Multiple Sclerosis among others (Gratzke et al., 2015). By this fact, Clinical BPH is a diagnosis of exclusion once these clinical entities have been ruled out. LUTS attributed to BPH will affect about 20% of men in the fourth decade of life and almost 50% of men in their 80s. This highlights the enormous burden of this disease for Humankind (Boyle et al. 2003).

The management of LUTS secondary to BPH has changed significantly over the last three decades. Until 1990s surgery and watchful waiting were the only therapeutic options for patients with clinical BPH but since those years, pharmacological management of clinical BPH has become the first-line of treatment.

In 1976, Caine et al, demonstrated for the first time in humans that α-adrenergic receptors caused prostatic smooth muscle contraction and that α-adrenergic blockers (ABs) could inhibit this contraction (Caine et al., 1976). In 1992, Lepor et al, published the first randomized placebo-controlled trial of the AB Terazosin for the treatment of BPH associated LUTS (Lepor et al., 1992). The beneficial effects of Terazosin in symptoms and peak urine flow rate led to the Federal Drug Administration (FDA) approval of ABs for the management of BPH in 1992. Over the years, others ABs were approved for treatment of BPH, namely, Doxazosin, Tamsulosin, Alfuzosin, and lastly Silodosin (Lepor et al., 2012).

Additionally, in the 1970s, several families in Dominican Republic were identified has having genotypically male children with ambiguous genitalia that raised as females until puberty and then became phenotypically male, although their prostates remained small and dysplastic for all life (Okeigwe and Kuohung, 2014; Kahokehr and Gilling, 2014). The study of these children demonstrated that a defect on 5α-reductase impaired the conversion of testosterone to DHT (Marks, 2004). In 1992, Gomerley et al published its results of a randomized controlled trial demonstrating the safety and efficacy of Finasteride for BPH treatment (Gormley et al., 1992) and in the same year Finasteride was the first inhibitor of 5α-reductase (5 ARI) approved by FDA for the treatment of BPH. Currently, there are two 5 ARIs, finasteride (inhibits only type 2) and dutasteride (inhibits both type 1 and 2) that can be used for patients with clinical BPH (Nickel et al., 2011).

Although both ABs and 5 ARIs can improve symptoms and improve peak flow urine rate, near 40% of patients have no response or progress despite therapy. Additionally, after initiation of medical therapy for BPH only 30% of patients continued with medical therapy at 1 year (Gul 2019). These, emphasizes the urgent need for new effective medications for prevention and treatment of Clinical BPH.

1.5. Etiology of Benign Prostatic Hyperplasia

One of the first philosophic attempts to establish the etiology of BPH comes in the book Lectures on the Diseases of the Urinary Organs by Sir Benjamin Collins Brodie in 1842. In its 5th Lecture, Brodie states that "When the hair becomes gray and scanty, when specks of earthy matter begin to be deposited in the tunics of artery and when a white zone is formed at the margin of the cornea, at this same period the prostate gland usually - I might say perhaps - invariably becomes increased in size". At this moment, it was for the first time recognized that BPH is a disease of the Aging Human male (Broddie, 1842). Later, several authors, most notably Charles Huggins is 1940s, proposed that BPH was certainly related to a stimulus acting for a very long period of time on prostate gland, and he presumed that the stimulus was of testicular origin since the disease does not occur in the absence of the gonad (Huggins, 1947).

From that moment, BPH was almost exclusively seen as a disease resulting from the action of testosterone in the aged prostate gland and the focus for the Etiology of BPH were the Androgens. However, a big question was completely unsolved: Why BPH appears during aging while testosterone is decreasing in human male? (Wu et al., 2008). From the 1970s until now several attempts have been made to clarify the almost universal development of BPH in humans, however, the exact etiology of BPH remains unsolved.

1.5.1. Embryonic Reawakening Theory

In 1970s, during his seminal work on the anatomy of the prostate gland, John McNeal observed a subset of periurethral glands independent of the central and peripheral zone of the prostate (McNeal, 1972). McNeal argued that periurethral glands should not be considered a part of the prostate, however, he claimed that they have exclusive predilection to developing BPH. McNeal observed that these glands were surrounded by a cylindrical urethral sphincter that he called "preprostatic sphincter" and he believed that both urethral stroma and the smooth muscle of the preprostatic sphincter were capable of interacting with the periurethral glands to produce BPH nodules. He concluded, in 1972, that BPH should be referred to as benign prostatic hyperplasia of the prostatic urethra (McNeal, 1972).

However, in 1978, McNeal work in this concept was completely changed. After examining prostates from 63 patients ranging in age from 15 to 80 years, that were sectioned in a new plane parallel to the coronal axis of the preprostatic sphincter, McNeal introduced a new area that he designated as "Transition Zone". McNeal describes this transition zone as a small wedge of tissue lateral to the distal end of the periurethral sphincter, different from the previous described periurethral glands which were enclosed inside the sphincter, and he stated that Transition Zone was the almost exclusive site of BPH origin (McNeal, 1978).

In 1978, McNeal proposed that BPH results from a "reawakening" of inductive potential in adult prostatic stroma resulting in formation of new epithelial branching morphogenesis specifically in the Transition and Periurethral zone (TPZ) (McNeal, 1978). One of the crucial points in McNeal theory was that adult prostatic epithelium of TPZ should retain an ability to respond to inductive signals from stroma with new ductal branching morphogenesis, recapitulating the embryonic mechanism of prostate development (McNeal, 1981; McNeal, 1984). In 1986, the seminal work of Cunha et al., showed for the first time the induction of new ductal growth in adult human prostatic epithelium (hPrE) in response to an embryonic prostatic inductor (Norman elal., 1986), and later, he demonstrated that rat UGS mesenchyme could be an inductor of this new ductal-acinar tissue, involving epithelial proliferation, ductal branching morphogenesis and functional cytodifferentiation in hPrE. (Hayward et al., 1998).

The embryonic reawakening theory proposed by McNeal and supported by the findings of Cunha et al., call the attention for the interactions between epithelium and stroma as the core for BPH etiology. Several accumulated evidences support that these interactions may be mediated by abnormal levels of several growth factors, mainly, FGF3, Epidermal growth factor (EGF), basicFGF and Transforming growth factor β (TGF-β) from either the epithelial or stroma compartment, resulting in the reawakening of embryonic cellular growth potential and leading to hyperplasia. (Hayward et al., 1998; Thomson et al., 2002; Peehl et al., 1998).

However, several questions in McNeal´s and Cunha hypothesis remains to be elucidated: first, why the adult stroma reawakens later in life (between the third and fourth decade of life), second why this occurs only in TPZ and third, what is the trigger for this reawakening. Some experiments have demonstrated that under hypoxic conditions human prostatic stromal cells secrete increased levels of FGF7 and TGF-β (Berger et al., 2003; Saito et al., 2014), suggesting that hypoxia could trigger the embryonic reawakening of the prostatic stroma. However, the two first questions about the Reawakening theory remain without any answer.

1.5.2. Stem Cell Theory

In 1980s, Isaacs and Coffey proposed the stem cell theory for BPH pathogenesis (Isaacs and Coffey, 1989). They suggest that an increase in the number of prostatic stem cells or a clonal expansion of resident stem cells into transient amplifying cells might be the initial trigger responsible for the development of BPH. The precise number and distribution of stem cells in the adult prostate is currently unclear. However, biomarkers characteristics of stem cells have been identified in cultures of stromal cells of human BPH (Lin et al., 2007). Recently, Brennen et al described the existence of Mesenchymal stem cells (MSC) with tri-lineage differentiation potential (adipogenesis, osteogenesis, and chondrogenesis) in prostate tissue from men undergoing open prostatectomy for symptomatic BPH, yet the number of this cells is extremely rare (0,1% of total cells) (Brennen et al., 2017). This MSCs are multipotent cells derived from bone marrow that can be mobilized to peripheral organs in response to an inflammatory stimulus (Brennen et al., 2013).

Based on this findings, Brennen and Isaacs proposed recently that inflammation in TPZ could induce the recruitment of stromal progenitors, such as mesenchymal stem cells (MSCs) from the bone marrow and these MSCs could be the inductor of new branching morphogenesis in the TPZ (Brennen and Isaacs, 2018). The stem cell theory defends that the stroma-epithelial interactions in the reawakening theory are triggered by the infiltration of MSCs in prostate in response to prostatic inflammation. However, the same major unsolved questions in McNeal theory namely: why the adult stroma reawakens almost universally in all human males later in life? and why this occurs only in TPZ? remain unsolved questions in this theory.

1.5.3. Endocrine Theory

Multiple hormonal theories for BPH etiology have emerged since Charles Huggins proposed that an androgenic stimulus coming from testes would be at least in part the cause of BPH. The essential role of Androgens in normal prostate development and growth its well documented and widely accepted (Rastrelli et al., 2019). The central role for Androgens in the development of BPH was concluded from basic and clinical observations that castration significantly improved LUTS associated with BPH (Huggins and Stevens, 1940; Huggins and Clark, 1940; Schroeder et al., 1986). Additionally, the observation that genetic deficiency of 5α-reductase causes abnormal prostatic development and prevents BPH highlighted the importance of DHT in prostate growth (Roehrborn, 2003).

Despite the consensual acceptance of androgens role in prostate development and growth, it is completely unclear why BPH develops at a stage of life (after third decade) when plasmatic levels of androgens are gradually decreasing. Multiple studies have examined the possible relationship between

the risk of BPH and circulating levels of testosterone and DHT (Roberts et al., 2004; Rohrmann et al., 2007; Kristal et al.,2008; Ansari et al., 2008) but most of them have demonstrated an inverse relationship between plasmatic testosterone and BPH. Additionally, healthy young men who were long term anabolic steroid abusers have similar prostate volume compared to age-matched controls (Jin et al., 1996). Also, the administration of supra-physiologic concentrations of testosterone does not increase prostate volume or BPH risk (Cooper et al., 1998). Since DHT is the major androgenic stimulus for prostate growth Walsh et al investigated if DHT is increased in BPH, but they observed that DHT levels were not higher in BPH than in normal prostate (Walsh et al., 1983). Nevertheless, chronic androgen deficiency can be associated with reduced prostate size (Jin et al., 2001). Although a role for androgens in causing BPH is enormously questionable it is accepted that they play at least a permissive role.

In 1970s animal models of BPH demonstrated a synergistic effect between estrogens and androgens in inducing glandular prostatic hyperplasia in castrated dogs (Walsh and Wilson, 1976; DeKlerk et al., 1979) and this led to the hypothesis that estrogens also could have a role in etiology of BPH in men. Posterior epidemiological studies, have shown that with advancing age plasmatic androgens decrease but estrogen levels remain constant or decrease slightly (Gray et al., 1991; Roberts et al., 2004). This results in an increased estrogen-to-androgen ratio in plasma, and some authors suggest that this imbalanced ratio could trigger de development of BPH (Nicholson and Ricke, 2011). Many studies have revealed a relationship between plasma estrogen levels and the risk of BPH ((Roberts et al., 2004; Rohrmann et al., 2007; Ansari et al., 2008)). By contrast, other studies revealed that estradiol levels were slightly lower in patients with BPH than age-matched controls (Kristal et al., 2008).

Estradiol has been shown to stimulate the proliferation of stromal cells from human BPH and normal prostate and these effects were reversed by antiestrogens such as fulvestrant or tamoxifen, suggesting that estradiol increases stromal proliferation through activation of estrogen receptor (ER) (Collins et al., 1994; King et al., 2006; Ho et al., 2008; Zhang et al., 2008; Park et al., 2009). By other side, estradiol seems not to increase proliferation of epithelial cells derived from human BPH or normal prostate (King et al., 2006; Ho et al., 2008). The same observation was made *in vivo* were estradiol increased prostatic stroma volume but decreased the epithelial compartment of male rats that have been castrated and supplemented with testosterone (Daehlin et al.,1987). Taken together these results indicate that estrogens induce proliferation of prostatic stroma but has no effect in prostatic epithelium.

The different role of estradiol on stromal and epithelial cells have been hypothesized to depend on the differential expression of ERs. The ERα is mainly expressed in BPH and normal prostatic stroma and ERβ is mainly expressed in epithelium (Ehara et al., 1995; Royuela et al., 2001; Pasquali et al., 2001;

Tsurusaki et al., 2003). Functionally, animal studies demonstrated that estrogens exert its proliferative trough ERα (Zhang et al., 2008) and its apoptotic effects through ERβ (McPherson et al., 2010).

In men, estrogens are formed primarily by aromatization of testosterone to estradiol, and both normal and BPH prostates exhibit pronounced aromatase activity in stroma (Stone et al., 1986; Kaburagi et al., 1987; Matzkin et al., 1992; Hiramatsu et al., 1997) suggesting that aromatase and estrogens could have a role in the development of BPH. However, in a double-blind, placebo controlled, randomized clinical trial, patients receiving 100 or 300 mg daily of the aromatase inhibitor atamestane were found to have decreased plasmatic levels of estradiol and estrone but no improvements in clinical symptoms of BPH or a significant decrease in prostatic volume (Radlmaier et al., 1996). At the present moment it is accepted that androgens and estrogens both regulate benign prostatic growth but its fundamental role in BPH etiology was not proven.

1.5.4. Metabolic Theory

Metabolic syndrome describes a complex of disorders related to cardiovascular and metabolic aberrations comprising central obesity, dyslipidemia, arterial hypertension, insulin resistance with compensatory hyperinsulinemia and glucose intolerance. The pathophysiology of this syndrome is mainly centered on insulin resistance. A chronic, low-grade pro-inflammatory state is also characteristic of the syndrome through the generation and release of inflammatory cytokines from visceral adipose tissue and liver (Konrad et al., 2014).

Epidemiological studies found that patients with metabolic syndrome had significantly higher prostate volume than those without metabolic syndrome and the increase is even higher when specifically assessing the transition zone of the prostate (Gacci et al., 2015; Russo et al., 2015). Is has been postulated that low-grade chronic inflammation of metabolic syndrome has a determining role in inducing BPH. Two large studies have demonstrated that intraprostatic inflammation predicted BPH progression (Crawford et al., 2006, Nickel et al., 2008) and increased serum C-reactive protein have been associated with increased risk of BPH (Schenk et al., 2010).

In view of these data, some authors have suggested that metabolic syndrome influences BPH development and progression, having direct inflammatory effects within prostate (Corona et al., 2014. Vignozzi et al., 2014). The arguments for this hypothesis come from evidence demonstrating that prostatic stromal cells can secrete growth factors (mainly IL-8) in response to inflammatory stimulus such as tumor necrosis factor α, lipopolysaccharide or oxidized LDL cholesterol (Gacci et al., 2013; Vignozzi et al., 2013). Clinically, in patients surgically treated for BPH, metabolic syndrome is associated with more

severe prostatic inflammation corroborating the preclinical findings (Gacci et al., 2013; Vignozzi et al., 2013). The therapeutic effect of atorvastatin in men with LUTS and BPH was studied in two studies and none of them demonstrated the efficacy of cholesterol lowering drugs in the treatment of LUTS in men with established BPH (Mills et al., 2007; Stamatiou et al., 2008). At the present moment, the relationship between metabolic syndrome and BPH is mainly epidemiological, without strong basic or clinical scientific demonstration.

1.5.5. Inflammatory Theory

The prostate gland is an immune-competent organ characterized by the presence of a complex intraglandular immune system. From almost its formation (12th week of fetal development) the prostate is populated by T-lymphocytes and the number increases during adult life to develop the prostate-associated lymphoid tissue (PALT) (Di Carlo et al., 2007). Several epidemiologic reports have demonstrated, as previously described, the association between histological inflammation and BPH (Roehrborn et al., 2005; Nickel et al., 2008) and patients with prostatic chronic inflammation are sevenfold more likely to have BPH than patients without inflammatory infiltrates (Zlotta et al., 2014).

Many *in vitro* and *in vivo* studies have tried to clarify this epidemiologic relationship. First, it has been demonstrated that activation of T-helper1 (T_H1) and T-helper2 (T_H2) lymphocytes in prostate release several cytokines and growth factors associated with the initiation and progression of BPH (Fibbi et al., 2010). Second, prostate inflammation can activate TGF-β pathway involved in epithelial – mesenchymal transition (Funahashi et al., 2014; Funahashi et al., 2015). Third, prostatic epithelial and stromal cells are able to secrete potent prostatic growth factors such as IL-18, IL-8 and MCP-1 in response to inflammatory stimuli (Fibbi et al., 2010; Fujita et al., 2010; Hamakawa et al., 2014; Kashyap et al., 2015). All these data suggest that intraglandular immune cells and their released immunomediators can control stromal and epithelial prostatic proliferation. In the past decades, several drugs targeting prostatic inflammation have been tested for BPH, mainly, COX-2 inhibitors. In a 2013 meta-analysis summarizing the results of all studies with COX-2 inhibitors showed an improvement in symptoms and flow scores but its safety profile clearly limits their use and diffusion in clinical practice (Kahokehr et al, 2013). However, the same questions for Embryonic Reawakening hypothesis can be applied to Inflammatory theory: why BPH only appears between the third and fourth decade of life, why this occurs only in TPZ and why almost all man in the 9th decade of life have BPH.

1.6. Neuroendocrine Cells in the Prostate Gland

Prostate epithelium is composed by three types of cells: secretory cells, basal cells and neuroendocrine cells. Secretory cells are the predominant cells in prostatic epithelium, they face directly the acini lumen and produce several components of the seminal fluid such as PSA. Basal cells are in contact with basal membrane and their apical borders do not reach the lumen, they do not have secretory activity but show a great proliferative activity. Both secretory and basal cells are derived as previously described from de UGS epithelium. Neuroendocrine cells constitute a prostate epithelium subpopulation of cells whose origin and role are a great matter of investigation and debate in the last five decades (McNeal, 1988).

Neuroendocrine cells are located in a wide variety of organs, such as pituitary, pineal, lung, gastrointestinal tract, thyroid, breast and prostate gland. Curiously, most organs that develops through Branching Morphogenesis exhibit neuroendocrine cells (Kobayashi and Tata, 2018; Dor et al., 2004) . The first observation of neuroendocrine cells in prostate was made by Pretl in 1944 through the description of argyrophilic cells mainly in the urethro-prostatic region (Pretl, 1944).

The embryologic origin of prostatic neuroendocrine cells is very controversial. Two hypotheses are possible, neuroectodermic origin (neural crest) or UGS origin. Several reports have suggested a local origin of prostatic neuroendocrine cells from the basal cells (Noordzij et al., 1995; Gkonos et al., 1995). The arguments for this origin comes from the evidence that both neuroendocrine cells and secretory cells express common substances such as PSA or Serotonin. However, recently this hypothesis was challenged by de demonstration of migration of neuroendocrine cells from paraganglionic cells passing the stroma and reaching the prostate ducts (Szczyrba et al., 2017). At this moment of knowledge, it is possible that both local origin or migration to prostate could be the origin of human prostatic neuroendocrine cells.

From a histologic point of view the neuroendocrine cells are intermingled with the other epithelial cells. Two types of neuroendocrine cells exist: the “open” and “closed” type. Both types lie on the basement membrane of prostatic epithelium the open-type exhibit extensions into the prostatic lumen and the closed type does not contact acini lumen (di Sant'Agnese PA and Mesy Jensen 1984). Frequently, both types of neuroendocrine cells have dendritic processes that contact with other epithelial cells and also not rarely neuroendigs can contact with neuroendocrine prostatic cells (Santamaría et al., 2002).

Neuroendocrine prostatic cells produce a great variety of products and most of the times one neuroendocrine cell is able to produce more than just a single product. In prostate, these products are serotonin, chromogranins, protein gene product 9.5, calcitonin, calcitonin gene-related peptide, neuropeptide Y, vasoactive intestinal polypeptide, substance P, somatostatin, peptides related to

thyrotropin-releasing hormone and to thyrotropin and parathyroid hormone-related peptide (Santamaria et al., 2007). Although, the huge number of substances produced by neuroendocrine cells the role of this cells in the normal prostate physiology is virtually completely unknown.

By the peculiar cell morphology of some neuroendocrine cells through the existence of dendritic processes contacting other prostatic cells and by the fact that this cells produce several different products, it has been hypothesized from several decades that this cells can be implicated in the regulation of prostate growth.

Neuroendocrine cells can be observed in all zones of the human prostate (di Sant'Agnese et al., 1985; Abrahamsson et al., 1987; Noordzij et al., 1995). However, the majority of neuroendocrine cells are localized in prostatic transition zone in humans and in periurethral glands in the murine prostate (Santamaria et al., 2002; Cohen et al., 1993; Noordzij et al., 1995; Laczkó et al., 2005). The transition zone is the prostate region where BPH originates, so a putative role of neuroendocrine cells in the etiology of BPH was considered from long time.

Several studies from 1974 to 2002 compared the number of neuroendocrine cells in the normal transition zone and in transition zone with BPH (Azzopardi et al., 1971; Kazzaz et al., 1974; Cockett et al., 1993; Abrahamsson et al., 2000; Noordzij et al., 1995; Martín et al., 2000). All the studies addressing this question obtained the same answer: the neuroendocrine cells in BPH are significantly reduced or absent in BPH compared to normal prostate. The last study published in 2002 consistently observed that neuroendocrine cells were greatly diminished in number or completely lost from most adenoma nodules and the authors concluded that neuroendocrine cells does not seem to be related to the development of BPH (Islam et al., 2002). Since 2002 the interest for the neuroendocrine cell role in BPH drastically diminished because all the authors believe that neuroendocrine cells should have a stimulatory effect over prostatic growth and if they are decreased or absent in BPH they should not be implicated in BPH formation.

1.7. Serotonin in the Prostate Gland

Serotonin is a biogenic amine with very different functions in the human body such as, regulation of mood and cognition, hemostasis, immune function, vascular tonus control, intestinal physiology among others (El-Merahbi er al.,2015). The function of serotonin is executed through activation of one of the several serotonin receptors (5-HTR). These receptors are classified in several families: 5-HTR1, 5-HTR2, 5-HTR3, 5-HTR4-7 which are characterized by different signal transduction mechanisms (Nichols and Nichols, 2008). The synthesis of serotonin has a limiting step that is dependent on the function of the

enzyme tryptophan hydroxylase (TPH). Two types of TPH exists, one expressed only in central nervous system, TPH2, and another one expressed only in peripheral organs, TPH1 (Walther et al.,2003). The existence of two different isophorms of TPH (peripheral vs. central) is very conserved in mammals, birds and fishes suggesting very different roles for serotonin in central (neuronal) and peripheral (non-neuronal) tissues. In fact, serotonin does not cross blood-brain barrier reflecting the importance of its regulation at the central and peripheral tissues (El-Merahbi et al., 2015).

Quantitatively, the most important product of neuroendocrine cells of the human prostate is serotonin (Davis, 1987), however, its function in normal prostate physiology is unknown. The serotonin producing neuroendocrine cells in human prostate are mainly located in transition zone (Santamaria et al., 2002, Cohen et al., 1993; Noordiz et al., 1995; Laczko et al., 2005) and in murine prostate they are exclusively observed in excretory periurethral ducts (Rodríguez et al., 2003; Rodríguez et al., 2005). The interesting parallelism between localization of serotonin producing neuroendocrine cells in humans (transition zone) and rat (periurethtral region) suggest at least a topological similarity between human transition zone and murine periurethral region and by this fact murine prostate could be theoretically used to understand the function of serotonin in the physiology of prostate gland.

The first study addressing the role of serotonin in BPH was published in 1993 by Cockett et al. They observed that serotonin was significant decreased in BPH comparatively to normal prostate (Cockett et al., 1993) but since there until now all the studies of the function of serotonin in prostate focus on its role in prostate cancer, mainly through the study of its effects in different cell lines derived from prostate cancer. Interestingly, a recent study evolving 950 man, aged 69-81 years, investigate the relationship between LUTS, several components of metabolic syndrome, endocrine and inflammatory factors and BPE. In this study, a decrease in plasmatic serotonin levels were the most powerful predictor of LUTS and BPE indicating that low serotonin level could be involved in the pathophysiology of LUTS/BPH (Haghsheno et al., 2015).

Because serotonin and neuroendocrine cells are strongly decreased in BPH and because they are mostly present in prostatic transition zone (the zone of origin of BPH) its role in the regulation of benign prostatic growth should be elucidated.

1.8. Androgen Receptor in Normal Prostate and Benign Prostatic Hyperplasia

ARs are strongly expressed in prostate and they are determinant for the formation of prostate gland. Both testosterone and DHT can readily bind to AR, however normal prostate gland development and growth is dependent mainly on the ligation of DHT to AR. It is hypothesized that testosterone and DHT could exert different functions via the same receptor by mechanisms not fully understood and this could explain this peculiar dependence of prostate development and growth from DHT in detriment of other androgens (Davey and Grossmann, 2015).

Although is consensual accepted the importance of androgens for the development of BPH, is completely unsolved the paradox of declining testosterone with aging and at the same time the emergence of BPH in aging human male.

Based on several accumulated evidences, Morgentaler proposed in 2009 the saturation hypothesis for prostate growth dependence of testosterone. Saturation hypothesis propose that androgens have a finite, limited ability to stimulate prostate tissue, malignant or benign. The hypothesis confirm that prostate tissue requires androgens for optimal growth, however, it can only use a relatively small amount, beyond which additional androgen is merely excess. The saturation point is well below physiologic concentrations, which explains why manipulation of serum testosterone into or out of the castrate range produces large changes in prostate biology, whereas normal prostate appears completely indifferent to variations in serum testosterone from the near-physiologic to supra-physiologic range (Morgentaler and Traish, 2009).

Saturation Hypothesis, highlights the importance of AR in prostate growth more than androgen level per se. In BPH, AR are expressed in both epithelial and stromal cells (Kyprianou and Davies, 1986), and the expression of AR is significantly increased in transition zone with BPH comparatively to normal prostate (Kyprianou and Davies, 1986; Monti et al., 2013; Izumi and Chang, 2014).

Interestingly several lines of evidence suggest an interaction between serotonin and androgens in several aspects of male sexual behavior. While androgens are fundamental for stimulation of male sexual behavior, serotonin has an opposite role. In this regard, serotonin impair ejaculatory/orgasmic function, inhibit erectile function and male sexual behavior (Berger et al., 2009; Wilson and Davies, 2007; Murray et al., 2004). At least in sexual behavior the inhibitory function in the formation of male sexual behavior (induced by testosterone) seems to be dependent of AR down-regulation. This suggests the existence of a link between serotonin and testosterone trough regulation of AR at least in human brain (Dakin et al., 2008).

Based in all the previous evidence exposed before we think that the link between neuroendocrine cells, serotonin and androgen receptor in the regulation of prostatic growth deserves to be explored in an experimental approach.

Aims

The main goal of this proposal is to understand the role of Serotonin in the regulation of Benign Prostatic Growth.

The specific aims of this project are:

1. To study the function of Serotonin and 5-HTR in prostate branching morphogenesis
2. To investigate the interaction between Serotonin and AR during prostate branching morphogenesis
3. To study the function of Serotonin and 5-HTR in human prostatic cells from normal prostate and BPH.
4. To investigate the interaction between Serotonin and AR in human prostatic cells from normal prostate and BPH.
5. To test *in vivo* the effects of TPH1 ablation over prostate growth.
6. To test *in vivo* the effects of tryptophan over prostate growth

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CHAPTER 2 | Experimental Work

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Results

Serotonin regulates prostate growth through androgen receptor modulation

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Serotonin regulates prostate growth through androgen receptor modulation

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Aging and testosterone almost inexorably cause benign prostatic hyperplasia (BPH) in Human males. However, etiology of BPH is largely unknown. Serotonin (5-HT) is produced by neuroendocrine prostatic cells and presents in high concentration in normal prostatic transition zone, but its function in prostate physiology is unknown. Previous evidence demonstrated that neuroendocrine cells and 5-HT are decreased in BPH compared to normal prostate. Here, we show that 5-HT is a strong negative regulator of prostate growth. *In vitro*, 5-HT inhibits rat prostate branching through down-regulation of androgen receptor (AR). This 5-HT's inhibitory mechanism is also present in human cells of normal prostate and BPH, namely in cell lines expressing AR when treated with testosterone. In both models, 5-HT's inhibitory mechanism was replicated by specific agonists of *5-Htr1a* and *5-Htr1b*. Since peripheral 5-HT production is specifically regulated by tryptophan hydroxylase 1(Tph1), we showed that *Tph1* knockout mice present higher prostate mass and up-regulation of AR when compared to wild-type, whereas 5-HT treatment restored the prostate weight and AR levels. As 5-HT is decreased in BPH, we present here evidence that links 5-HT depletion to BPH etiology through modulation of AR. Serotoninergic prostate pathway should be explored as a new therapeutic target for BPH.

Benign prostatic hyperplasia (BPH) is one of the main causes of non-neurogenic lower urinary tract symptoms (LUTS) in the aging male[1,2]. The underlying mechanism responsible for BPH is not understood, and only elucidating the etiology of BPH will increase our ability to treat or even prevent its development.

Currently the most accepted hypothesis for the etiology of BPH is, that proposed by McNeal, in which BPH results from the reawakening of inductive potential in adult prostatic stroma in a specific prostatic region defined as transition zone[3–5]. This hypothesis claimed that the adult prostatic epithelium retains the ability to respond to inductive stromal signaling with new ductal branching morphogenesis[6,7]. However this hypothesis does not respond to the critical question of why this reawakening of human adult prostatic stroma occurs.

While there is no BPH without testosterone[8], testosterone levels decrease with age[9,10] and no direct correlation between testosterone concentration and prostate volume has been established yet[11]. Moreover, it is widely accepted that physiologic concentrations of testosterone provide an excess of testosterone for optimal prostatic growth suggesting that testosterone is not the etiologic factor responsible for BPH[12]. On the other hand, several reports have documented an up-regulation of the androgen receptor (AR) in BPH tissue, unveiling a potential role for AR in BPH etiopathogenesis[13–15].

The neuroendocrine prostatic cells secrete various neuroendocrine factors with 5-HT being one of the most abundant. The peculiar morphology of some neuroendocrine cells with dendritic processes extending to lumen and projections surrounding the epithelial-stroma interface justify the hypothesis that neuroendocrine products, namely 5-HT, could regulate prostate growth[16]. Notably, neuroendocrine prostatic cells are mainly located in the transition zone of the normal human prostate[17], where BPH originates[4]. However, comparing BPH tissue with normal transition zone (without BPH) the number of neuroendocrine cells is extraordinarily decreased[18–20]. Also

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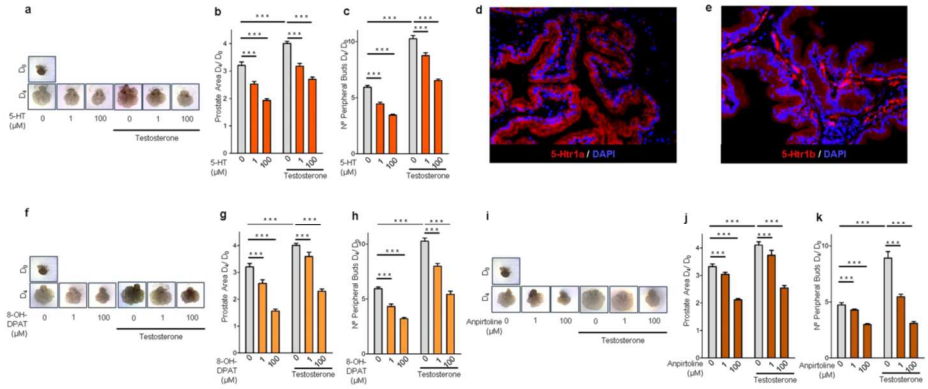

Figure 1. 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist inhibit prostate branching morphogenesis. (**a**) Photographs of representative VPs at D_0 and at D_4 of culture treated with different 5-HT concentrations. (**b**) Morphometric analysis of the effect of 5-HT on VPs area and (**c**) number of peripheral buds ($n \geq 12$ VPs per group). (**d**) Immunofluorescence analysis of *5-Htr1a* and (**e**) *5-Htr1b* expression in the rat prostate. (**f**) Photographs of representative VPs at D0 and at D4 of culture treated with different 8-OH-DPAT concentrations. (**g**) Morphometric analysis of the effect of 8-OH-DPAT on VPs area and (**h**) number of peripheral buds ($n \geq 12$ VPs per group). (**i**) Photographs of representative VPs at D0 and at D4 of culture treated with different anpirtoline concentrations. (**j**) Morphometric analysis of the effect of Anpirtoline on VPs area and (**k**) number of peripheral buds ($n \geq 12$ VPs per group). Error bars indicate s.e.m. $^{***}p < 0.001$; two-way ANOVA and Bonferroni *post hoc* test. VPs, ventral prostate explants; D_0, day 0; D_4, day 4; 5-HT, serotonin.

5-HT was shown to be significantly depleted in BPH tissue[19]. Furthermore, a recent study in a large cohort of Scandinavian men revealed that LUTS are associated with benign prostate enlargement and to decreased plasmatic 5-HT concentration[21]. These findings suggest a potential link between prostatic 5-HT depletion and BPH etiology; however, the function of 5-HT in regulation of benign prostate growth has never been studied.

We hypothesized that 5-HT had an inhibitory function over benign prostate growth and that suppression of prostatic 5-HT production could be responsible for benign prostatic growth. The aim of this study was to define the role of 5-HT in the regulation of benign prostatic growth and to test the pharmacologic modulation of the prostatic serotoninergic system as a new pharmacological target for BPH.

Results

5-HT, 5-Htr1a, and 5-Htr1b specific agonists inhibits rat ventral prostate branching through AR down-regulation. The new epithelial gland formation observed in BPH is normally seen only during prostate branching morphogenesis[22]. For this reason, we first tested the hypothesis that 5-HT could regulate prostate growth using *in vitro* cultures of rat ventral prostate explants (VPs) from P1 newborns. During 4 days in culture, 5-HT supplementation induced a significant dose-dependent inhibition of rat VPs growth (Fig. 1a), as expressed by decreased area (Fig. 1b), as well the number of peripheral explant buds (Fig. 1c). In medium conditions without additional testosterone supplementation, inhibitory effect of 5-HT over VPs growth was maximal at 100 μM where a reduction of 40% in prostate area D_4/D_0 ($p < 0.001$) and a reduction of 42% in the number of peripheral buds D4/D0 ($p < 0.001$) was observed in comparison to the control group (0 μM 5-HT). As expected, testosterone supplementation of VPs exerted a strong stimulatory effect on prostate branching morphogenesis, mainly in the number of peripheral buds (Fig. 1c), but again, 5-HT at 100 μM reduced 33% the prostate area D_4/D_0 ($p < 0.001$) and 36% the number of peripheral buds D4/D0 ($p < 0.001$) in comparison to control group (0 μM 5-HT + testosterone).

From all 5-HT receptors, *5-Htr1a* and *5-Htr1b* were the most extensively studied in the regulation of malignant prostate growth[23,24], so we tested if these receptors could contribute to the 5-HT inhibitory function in normal prostate growth. By immunofluorescence, we found that both receptors are strongly expressed in rat prostate but with a slightly different distribution pattern with *5-Htr1a* predominantly expressed in prostate epithelium (Fig. 1d), while *5-Htr1b* being expressed both in epithelium and stroma (Fig. 1e). To determine the contribution of both receptors in 5-HT inhibition of prostate branching morphogenesis, VPs were treated with drugs that specifically activate *5-Htr1a* or *5-Htr1b*. The selective *5-Htr1a* agonist 8-OH-DPAT, (Fig. 1f,g and h) and the selective *5-Htr1b* agonist, anpirtoline, (Fig. 1i, j and k) induced a significant dose-dependent inhibition of VPs growth. The inhibitory effect was maximal in VPs supplemented with testosterone and treated with 100 μM of anpirtoline, where a reduction of 39% in prostate area D_4/D_0 ($p < 0.001$) and a reduction of 66% in the number of peripheral buds D4/D0 ($p < 0.001$) was observed in comparison to the control group (0 μM anpirtoline + testosterone).

Since, androgens are a major prostatic stimulatory factor, we asked if the 5-HT inhibitory effect was related to the AR stimulatory pathway. By western blot analysis we showed that testosterone supplementation induced

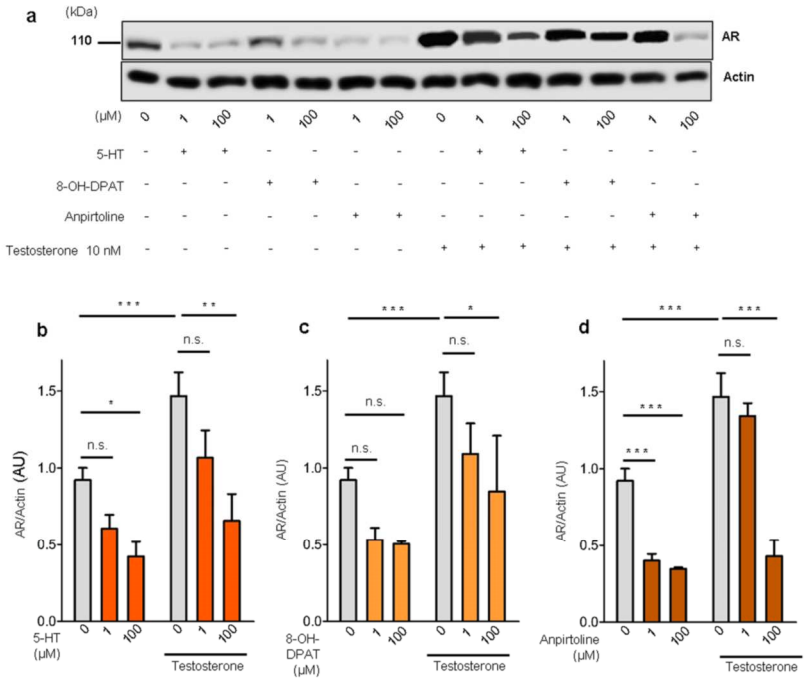

Figure 2. 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist down-regulates AR expression in rat ventral prostate. (**a**) Western blot analysis of AR expression in prostate explants treated with increasing doses of 5-HT, specific 5-Htr1a agonist, 8-OH-DPAT, and specific 5-Htr1b agonist, anpirtoline. (**b**) Quantification of AR protein in VPs treated with different concentrations of 5-HT in medium conditions without or with testosterone supplementation, $n \geq 3$ (each sample contained a pool of 4 VPs). (**c**) Quantification of AR protein in VPs treated with different concentrations of 8-OH-DPAT in medium conditions without or with testosterone supplementation, $n \geq 3$ (each sample contained a pool of 4 VPs). (**d**) Quantification of AR protein in VPs treated with different concentrations of Anpirtoline in medium conditions without or with testosterone supplementation, $n \geq 3$ (each sample contained a pool of 4 VPs). Error bars indicate s.e.m. *n.s.* non-significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; two-way ANOVA and Bonferroni *post hoc* test. VPs, ventral prostate explants; AR, androgen receptor; AU, arbitrary units; 5-HT, serotonin.

AR up-regulation, but 5-HT treatment significantly decreased AR expression either with or without testosterone supplementation (Fig. 2a and b) suggesting that the inhibitory function of 5-HT could be related to inhibition of the AR pathway. Similarly, both the selective *5-Htr1a* agonist 8-OH-DPAT, (Fig. 2a and c) and the selective *5-Htr1b* agonist, anpirtoline, (Fig. 2a and d) induced a significant AR down-regulation, more evident in anpirtoline treated VPs. Taken together these results indicate that *in vitro* 5-HT inhibits rat prostate growth through *5-Htr1a* and *5-Htr1b*, by down-regulating AR.

5-HT, 5-Htr1a or 5-Htr1b specific agonists inhibit growth of androgen sensitive human benign prostate cells through AR down-regulation. Next, we asked if this mechanism is also present in human prostate. With this purpose we performed *in vitro* 5-HT treatment of different human cell lines from epithelium of BPH (BPH-1), normal prostate epithelium (PNT1A) and normal prostate stroma (WPMY-1). We found that 5-HT significantly reduced cell viability of BPH-1 and WPMY-1 namely in the presence of testosterone but without changing PNT1A cell viability (Fig. 3a,b,c and Supplementary Fig. 1). The inhibitory effect of 5-HT was maximal in BPH-1 cells supplemented with testosterone. Under these conditions, 100 μM of 5-HT decreased cell viability by 35% compared to control (0 μM 5-HT + testosterone) ($p < 0.001$).

Next, we tested if the growth inhibitory function of 5-HT in androgen human prostate cells BPH-1 and WPMY-1 was mediated by *5-Htr1a* and *5-Htr1b*. First, we showed that both receptors are expressed in the three human prostate cell lines (Fig. 3d) and that their specific agonists significantly inhibited cell viability (Fig. 3e–j and Supplementary Fig. 1) almost exclusively in the presence of testosterone, but again only in BPH-1 and WPMY-1, without any inhibitory effect in PNT1A cells.

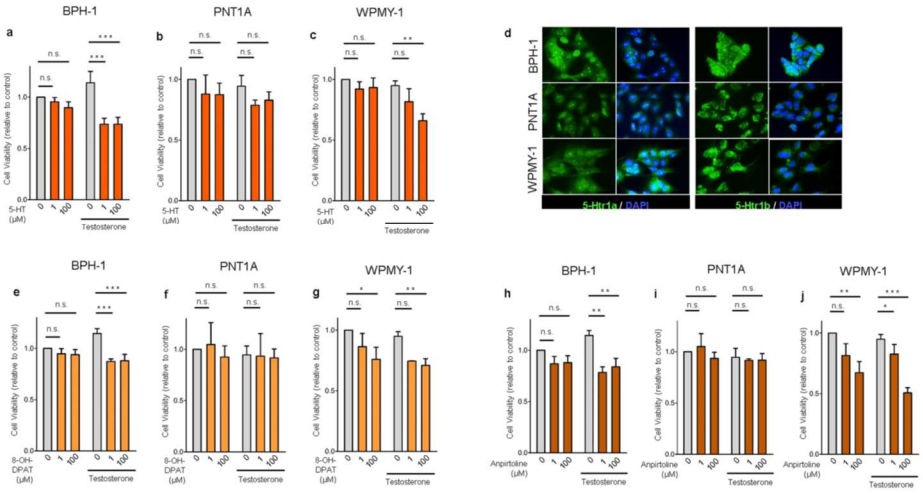

Figure 3. 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist inhibits cell viability in BPH-1 and WPMY-1 human prostatic cells without any effect in PNT1A cells. (**a**,**b**,**c**) Effect of 5-HT on cell viability analyzed by MTS assay in BPH-1, PNT1A and WPMY-1 cells. (**d**) Immunofluorescence analysis of *5-Htr1a* and *5-Htr1b* expression in BPH-1, PNT1A and WPMY-1 cells. (**e**,**f**,**g**) Effect of *5-Htr1a* specific agonist, 8-OH-DPAT, and (**h**,**i**,**j**) *5-Htr1b* specific agonist, Anpirtoline, on cell viability analyzed by MTS assay in BPH-1, PNT1A and WPMY-1 cells. The data are expressed relative to control condition (0 μM 5-HT without testosterone supplementation) and were reproduced in at least three independent experiments. Error bars indicate s.e.m. *n.s.* non-significant; 5-HT, serotonin; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; two-way ANOVA and Bonferroni post hoc test.

Additionally, Ki-67 staining confirmed that proliferation of BPH-1 and WPMY-1 cells supplemented with testosterone was significantly reduced by 5-HT treatment (Fig. 4a and c), while PNT1A cells proliferation was not affected (Fig. 4b). Similarly, both specific agonists of *5-Htr1a* and *5-Htr1b* strongly inhibited cell proliferation (Fig. 4d–i) but again only in BPH-1 and WPMY-1 cells.

Next, we investigated if this inhibitory function of 5-HT, *5-Htr1a* and *5-Htr1b* specific agonists was related to changes in the AR pathway. We observed that testosterone induced an up-regulation of AR in both BPH-1 and WPMY-1 cells. 5-HT, *5-Htr1a* and *5-Htr1b* specific agonists inhibited the AR up-regulation induced by testosterone in both BPH-1 and WPMY-1 cells (Fig. 5b). Regarding 5-HT effect in AR down-regulation this was very significant in WPMY-1 cells after testosterone supplementation ($p < 0{,}001$) while in BPH-1 cells a non-significant down-regulation of AR was observed (Fig. 5c and d). Also for both *5-Htr1a* and *5-Htr1b* specific agonists only Anpirtoline induced a significant down-regulation of AR in both BPH-1 and WPMY-1 cells (Fig. 5d,e,g,h). Additionally, by immunofluorescence analysis we observed that expression of AR after testosterone treatment was decreased in BPH-1 cells treated with 5-HT, *5-Htr1a* and *5-Htr1b* specific agonists (Supplementary Fig. 2).

Remarkably, the absence of inhibitory action of 5-HT, *5-Htr1a* and *5-Htr1b* specific agonists on PNT1A cells viability and proliferation, even in presence of testosterone, co-existed with a complete absence of AR expression in these cells (Fig. 5a). These data strongly argue that 5-HT's inhibitory function on growth of benign human prostate cells is related with the suppression of the AR pathway.

***In vivo* ablation of peripheral 5-HT synthesis in mice induces benign prostatic growth.** 5-HT synthesis is initiated by tryptophan hydroxylase (Tph). Tph type 1 (Tph1) and 2 (Tph2) regulate 5-HT production in non-neuronal and neuronal tissues, respectively[25,26]. The majority of 5-HT in the body is produced by Tph1. In fact, $Tph1^{-/-}$ mice exhibit very low levels of circulating 5-HT, while brain serotonin is not affected[25]. Based on our *in vitro* findings which suggest that 5-HT has a strong inhibitory action on prostate growth through down-regulation of AR, we used $Tph1^{-/-}$ mice to evaluate the effect of peripheral 5-HT depletion on mouse prostate gland growth. Remarkably, $Tph1^{-/-}$ mice exhibited a significantly 37% higher prostate-to-body weight ratio compared to wild-type at 20 weeks ($p < 0.001$) (Fig. 6a and b) without changes in body weight (Supplementary Fig. 3a). Interestingly, histology of the prostate gland revealed that $Tph1^{-/-}$ mice exhibit areas of hyperplasia in epithelium and stroma (Fig. 6a, lower panel). To determine if 5-HT treatment could revert higher prostate mass in $Tph1^{-/-}$ mice, we performed intraperitoneal injections of 5-HT at 10 consecutive days. 5-HT treatment resulted in significant mass reduction in prostate gland compared to levels similar to the wild-type (Fig. 6c and d) again without affecting animal weight (Supplementary Fig. 3b). Next, we asked if the higher prostate mass in $Tph1^{-/-}$ mice was associated with different expression of AR. We could not demonstrate significant differences

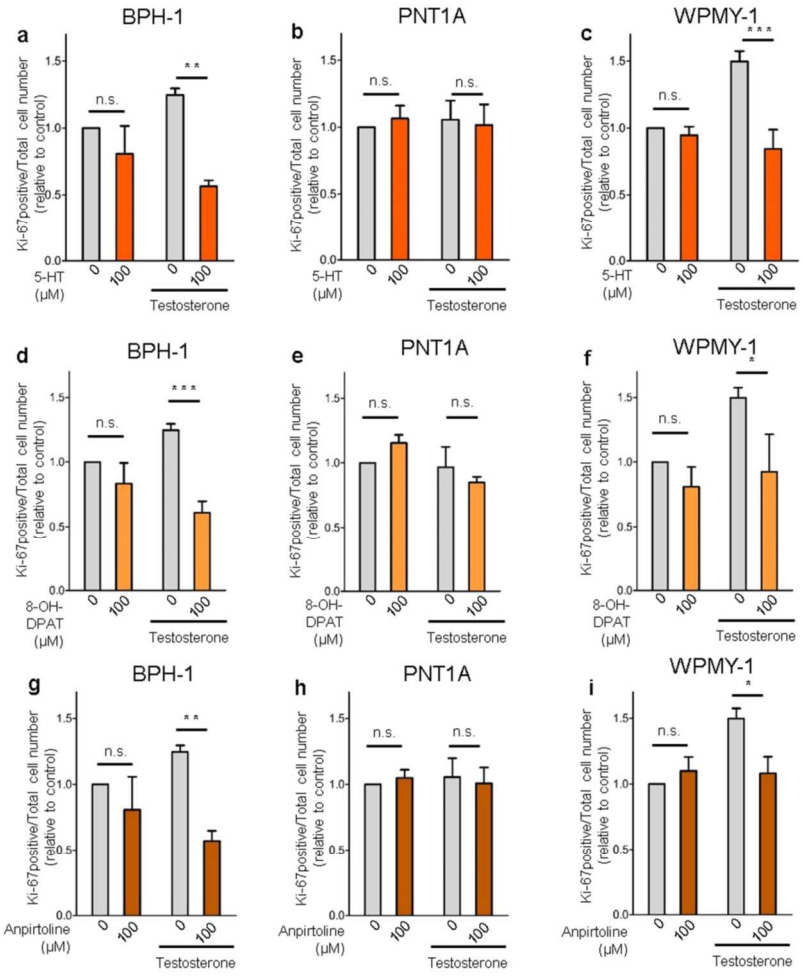

Figure 4. 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist inhibits cell proliferation in BPH-1 and WPMY-1 human prostatic cells without any effect in PNT1A cells. (**a**,**b**,**c**) Effect of 5-HT, (**d**,**e**,**f**) *5-Htr1a* specific agonist, 8-OH-DPAT, and (**g**,**h**,**i**) *5-Htr1b* specific agonist, Anpirtoline, on cell proliferation of BPH-1, PNT1A and WPMY-1 cells as quantified by Ki-67 positive cells/total cell number ratio. The data are expressed relative to control condition (0 μM 5-HT without testosterone supplementation) and were reproduced in at least three independent experiments. Error bars indicate s.e.m. *n.s.* non-significant; 5-HT, serotonin; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; two-way ANOVA and Bonferroni post hoc test.

of AR expression by western blot analysis in total prostate (data not shown), however by immunofluorescence dorsolateral prostate of $Tph1^{-/-}$ mice appeared to express more AR (Fig. 6e, lower panel). So, we investigated and demonstrated by qRT-PCR that the dorsolateral prostate of $Tph1^{-/-}$ mice has increased levels of AR mRNA expression, while 5-HT treatment partially restores it to levels to wild-type mice (Fig. 6e, upper panel), reinforcing our hypothesis.

It is well established that castration induces a strong reduction in size of the prostate gland as well as in seminal vesicles, while testosterone supplementation makes both organs return to normal size[27,28]. Interestingly, also seminal vesicles of $Tph1^{-/-}$ mice were significantly larger than the ones of wild-type (Fig. 6f) suggesting that 5-HT could regulate androgen sensitivity not only in prostate gland but also in seminal vesicles. Again, we observed that the mass of seminal vesicles was partially restored by 5-HT treatment in $Tph1^{-/-}$ mice (Fig. 6g).

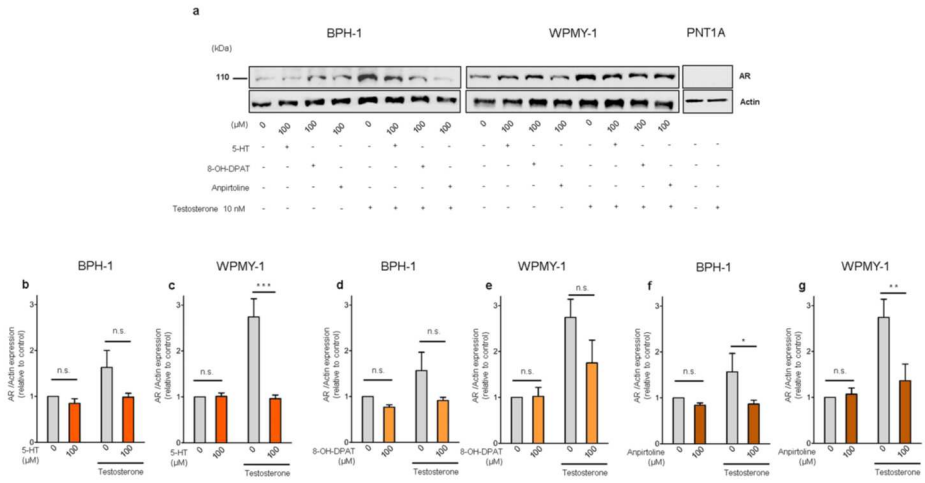

Figure 5. 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist down-regulates AR expression in human prostatic cells. (**a**) Western blot analysis of AR expression in the three cell lines after 5-HT, 8-OH-DPAT and anpirtoline treatment. (**b**) Quantification of AR in BPH-1 and (**c**) WPMY-1 cells after 5-HT treatment in medium conditions without or with Testosterone supplementation. (**d**) Quantification of AR protein levels in BPH-1 and (**e**) WPMY-1 cells after 8-OH-DPAT treatment in medium conditions without or with Testosterone supplementation. (**f**) Quantification of AR protein levels in BPH-1 and (**g**) WPMY-1 cells after Anpirtoline treatment in medium conditions without or with Testosterone supplementation. The data are expressed relative to control condition (0 μM 5-HT without testosterone supplementation) and were reproduced in at least three independent experiments. Full, uncropped gel images are shown. Error bars indicate s.e.m. *n.s.* non-significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; two-way ANOVA and Bonferroni *post hoc* test. AR, androgen receptor; 5-HT, serotonin.

Lastly, we tested if wild-type mice challenged with 5-HT treatment continue responding to 5-HT's inhibitory action, and we demonstrated that both prostate gland (Fig. 6h) as well as seminal vesicle mass (Fig. 6i) were reduced while animal weight was not affected (Supplementary Fig. 3c).

Discussion

Currently, the etiology of BPH is unknown. However, it is accepted today that BPH is a consequence of aging and the simultaneous presence of testosterone[3]. BPH almost universally affects human males and a significant part of men will develop bothersome LUTS because of benign prostate enlargement. Although some of these men respond to current medical treatment (mainly α_1-adrenoreceptors antagonists and 5 α-reductase inhibitors) a large portion continues to need a surgical procedure to treat resistant LUTS or have even more serious complications of BPH[29], creating the emerging necessity for novel therapies.

In this study, we investigated the function of 5-HT in the regulation of non-malignant prostatic growth. Here, we demonstrated for the first time in several *in vitro* and *in vivo* models that 5-HT is a powerful negative regulator of prostatic growth through down-regulation of AR. We found that *5-Htr1a* and *5-Htr1b* are strongly expressed in the rodent prostate gland as well in human benign prostate cells, and that both receptors could mediate the inhibitory action of 5-HT on prostate growth.

Our *in vitro* and animal findings lead us to propose a new mechanism to explain the development of BPH in humans (Fig. 7). Our proposed model explains how the depletion of neuroendocrine cells and serotonin observed in prostatic transition zone with aging[18–20], could be the etiologic factor responsible for the initiation and progression of BPH. In our model, the depletion of serotonin induces an up-regulation of androgen receptor in the prostatic transition zone leading to the stimulation of benign prostatic growth in this specific prostatic region.

Our neuroendocrine model for the etiopathogenesis of BPH would resolve an intriguing question about the crucial participation of androgens in the development of BPH. Previous studies have showed that testosterone does not increase with aging and some studies have even reported that plasmatic testosterone is decreased in the aging human male[9–11]. In a similar way, intraprostatic testosterone, in particular DHT, are not increased in BPH comparatively to normal prostate[30] and even the administration of testosterone in supra-physiologic concentrations to eugonadal men does not induce the development of BPH[31,32]. This data were the basis for the saturation model of prostate growth proposed by Morgentaler *et al.* suggesting that prostate gland growth is extraordinarily sensitive to low androgen concentrations (near the castrate range) but insensitive to androgen concentrations above a certain saturation point[12]. The critical point, in saturation model is that the rate-limiting step for prostatic growth, which is the concentration of AR. Recent studies demonstrated that AR is in fact up-regulated in stroma

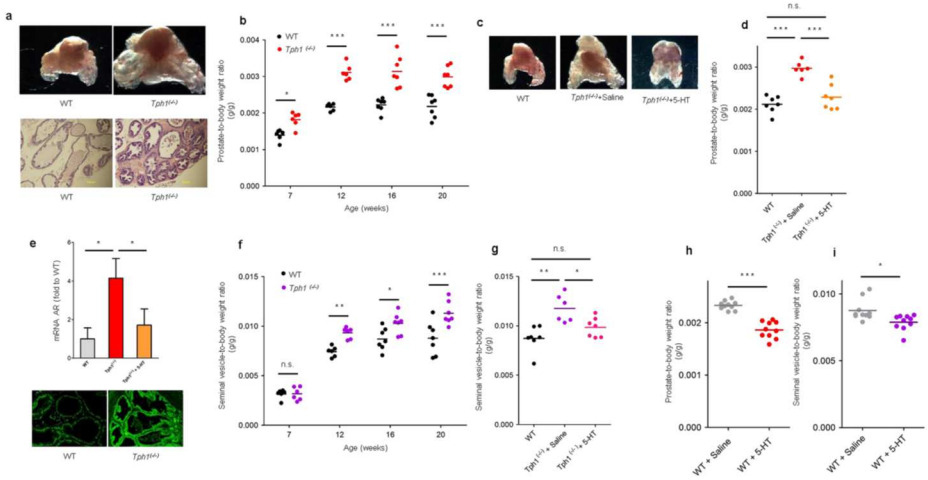

Figure 6. Genetic deletion of Tph1 increases prostate gland mass. (**a**) Representative photographs of prostates from 20 week-old wild-type and *Tph1*$^{-/-}$ mice (Top). Images from H&E staining of wild-type and *Tph1*$^{-/-}$ prostates ($n=5$ per genotype) (Bottom). (**b**) Prostate-to-body weight ratio of *Tph1*$^{-/-}$ mice compared to wild-type at different ages ($n=6$–7 for wild-type and *Tph1*$^{-/-}$ mice, for each time point). (**c**) Representative photographs of prostates and (**d**) prostate-to-body weight ratio from 20 week-old wild-type, *Tph1*$^{-/-}$ treated with saline and *Tph1*$^{-/-}$ treated with 5-HT ($n=7$ WT; $n=6$ *Tph1*$^{-/-}$ +Saline; $n=7$ *Tph1*$^{-/-}$ + 5-HT). (**e**) qRT-PCR for AR expression in dorsolateral lobe of 20 week-old wild-type, *Tph1*$^{-/-}$ treated with saline and *Tph1*$^{-/-}$ treated with 5-HT ($n=4$ wild-type; $n=4$ *Tph1*$^{-/-}$ +Saline; $n=3$ *Tph1*$^{-/-}$ + 5-HT) (Top). Immunofluorescence analysis of AR expression in dorsolateral prostate of WT and *Tph1*$^{-/-}$ mice (200x) (Bottom). (**f**) Seminal vesicle-to-body weight ratio of *Tph1*$^{-/-}$ mice compared to wild-type mice at different ages (n = 6–7 for wild-type and *Tph1*$^{-/-}$; mice, for each time point). (**g**) Seminal vesicle-to-body weight ratio from 20 week-old wild-type, *Tph1*$^{-/-}$ treated with saline and *Tph1*$^{-/-}$ treated with 5-HT ($n=7$ WT; $n=6$ *Tph1*$^{-/-}$ + Saline; $n=7$ *Tph1*$^{-/-}$ + 5-HT). (**h**) prostate-to-body weight ratio and (**i**) seminal vesical-to-body weight ratio in wild-type mice treated with Saline compared to wild-type mice treated with 5-HT during 10 consecutive days (n = 10 for each group). *n.s.* non-significant; AR, androgen receptor; 5-HT, serotonin; *$p<0.05$; **$p<0.01$; ***$p<0.001$; (B,F) two-way and (D,G) one-way ANOVA and Bonferroni *post hoc* test. (H,I) Student *t* test.

and epithelium of BPH tissue comparatively to normal prostate, implicating AR in etiophatogenesis of BPH[13–15]. Our findings seem to provide an explanation for this current view about the participation of androgens in the development of BPH. In this way, the loss of neuroendocrine cells and serotonin in prostatic transition zone up-regulates the AR and then permits the development of BPH, even with a decreased plasmatic concentration of androgens observed in the aging male.

Our first *in vitro* experimental approach focused on the function of 5-HT in the regulation of rat prostate branching morphogenesis. Because BPH is the result of new branching morphogenesis, this model permits the study the influence of 5-HT exactly in the mechanism by which BPH develops and progresses. Here, we demonstrate that 5-HT strongly inhibit the branching morphogenesis of the prostate gland through down-regulation of AR. In fact, other organs like the prostate which have a development process of branching morphogenesis, such as the mammary gland, 5-HT also have demonstrated a development inhibitory action[33].

Although new in the prostate gland, the serotoninergic inhibitory mechanism through down-regulation of AR, is well known in the brain. In fact, the complete masculinization of the brain is dependent of a perinatal surge of testosterone and a simultaneous decrease in hypothalamic 5-HT concentrations[34]. In the brain, it has been demonstrated that regulation of 5-HT concentration is crucial for normal sexual differentiation, where 5-HT down-regulates AR[35,36]. In agreement with our findings, Sayed *et al.* demonstrated that dapoxetine decreased AR expression and prevents testosterone-induced BPH in rats[37]. However, both in brain and prostate the full mechanism responsible for this down-regulation remains to be elucidated.

The most described 5-HT receptors in prostatic cells, are the *5-Htr1a* and *5-Htr1b*. Therefore, we characterize its expression in rat prostate for the first time. As we demonstrated both *5-Htr1a* and *5-Htr1b* are strongly expressed in rat prostate and the activation of these receptors resulted in a significant inhibition of prostate branching morphogenesis, which also occurred through down-regulation of AR. In human prostatic cells the function of 5-HT, *5-Htr1a*, and *5-Htr1b* have been previously studied but only in malignant cells[23,24]. These previous reports showed that 5-HT has a proliferative effect in several malignant cell lines through *5-Htr1a* and *5-Htr1b*. Curiously, this stimulatory effect was mainly evident in androgen-insensitive cells[23]. Here we studied, for

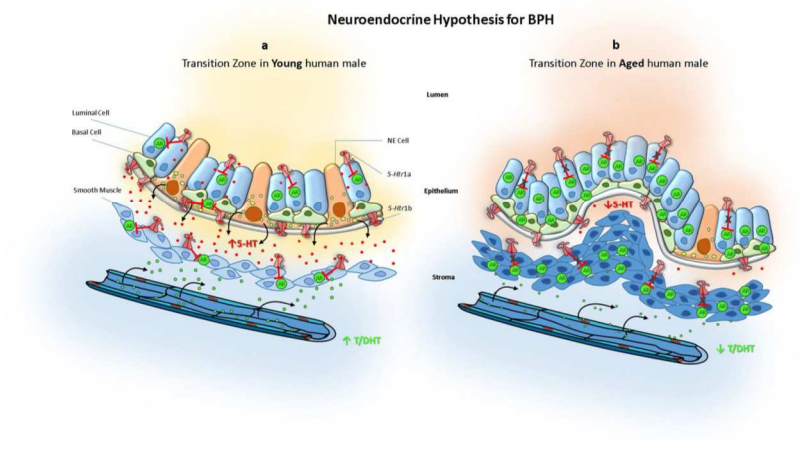

Figure 7. Neuroendocrine hypothesis for etiopathogenesis of benign prostatic hyperplasia. (**a**) In young human male, prostate transition zone is enriched with 5-HT producing neuroendocrine cells. Serotonin is secreted to the epithelium-stroma interface and through activation of *5-Htr1a* and *5-Htr1b*, both in epithelium and stroma, the expression of AR is decreased. Yet, more testosterone is delivery to prostate, down-regulated AR limits benign prostate growth. (**b**) In aged human male, transition zone loses 5-HT producing neuroendocrine cells causing a depletion in local 5-HT. As a consequence *5-Htr1a* and *5-Htr1b* release their inhibition over AR expression. Although with aging the delivery of testosterone to the prostate is decreased the up-regulation of AR induces the development of BPH. 5-HT, serotonin; AR, androgen receptor; DHT, Dihydrotestosterone; NE, neuroendocrine; T, testosterone.

the first time, the function of 5-HT, *5-Htr1a*, and *5-Htr1b* in human benign prostatic cells. We demonstrated that 5-HT inhibits proliferation of androgen sensitive benign prostatic cells, and this inhibitory function was associated to a down-regulation of AR. This different growth function of 5-HT in benign and malignant cells remains unexplained but the predominant stimulatory effect of 5-HT in androgen-insensitive malignant cells suggests that castration resistance could change the phenotypic response of prostatic cell to neuroendocrine products.

Finally, to test *in vivo* our mechanistic approach to the etiopathogenesis of BPH, we genetically ablated the peripheral production of 5-HT. Using Tph1 $^{-/-}$ mice we demonstrated that prostatic 5-HT depletion induces benign prostatic growth. The inhibition of peripheral 5-HT synthesis in mice, through genetic deletion of Tph1, simulates a decrease in the prostate transition zone 5-HT observed in the aging male. This led us to propose that the decrease in neuroendocrine cells and 5-HT in the human transition zone could contribute to the development of BPH. The increased mass of *Tph1*$^{-/-}$ prostates was associated to an up-regulation of AR in dorso-lateral prostate samples suggesting that, at least in part, the excessive prostatic growth in *Tph1*$^{-/-}$ could be attributed to AR up-regulation.

In conclusion, our findings suggest that 5-HT is a strong negative regulator of prostate growth through AR down-regulation. As 5-HT is decreased in BPH, we present here evidence that links 5-HT-producing neuroendocrine cell depletion to BPH etiology. Therefore, this new described serotoninergic inhibitory pathway over benign prostatic growth should be explored as a new target for BPH treatment.

Methods

Ethics and animal work. Mice and rats were maintained in accordance with the guidelines of "*Guide to the Care and Use of Experimental Animals*" National Academy of Science, and the EU Directive 2010/63/EU. This study was approved by the Animal Ethics Committee of the Institution were the study was performed (SECVS 003/2016) and by the National Competent Authority for Animal Protection (DGAV 0421/000/000/2016).

Drugs. 5-HT and testosterone were purchased from Sigma-Aldrich (St Louis, Missouri). The *5-Htr1a* specific agonist, 8-OH-DPAT and the *5-Htr1b* specific agonist, Anpirtoline, were purchased from Tocris-Bioscience (Bristol, UK).

Rat ventral prostate cultures. Newborn male *Sprague-Dawley* rats were sacrificed 24-hours after birth. Ventral prostate lobes were microdissected using a stereomicroscope (Leica MZ6, Switzerland) and processed for organ culture. Organ culture was performed as previously described[38]. Briefly, rat ventral prostates (VPs) from P1 newborns were cultured for 4 days at 37 °C in a humidified atmosphere of 5% CO_2. Medium and VPs were transferred to porous membranes (Millicell CM filters, Millipore Corp., Bedford, Massachusetts) in 12 well plate

for floating explant cultures. Each VPs were dipped into 500 µl of 1:1 mixture of DMEM and Ham's F-12 nutrient supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin, 10 µg/mL transferrin and 10 µg/mL insulin. Media were replenished at 48 hours of culture. Branching morphogenesis in all groups was monitored daily by a stereomicroscope and photographs were taken at day 0 and day 4. The number of peripheral buds was manually counted and the prostate tissue area was measured in ImageJ using the beProstate plugin (Version 1.0) (developed by Biomedical Engineering Solutions Research Group, Life and Health Sciences Research Institute, University of Minho; available at http://www.besurg.com/sites/default/files/beProstateApp.zip). The differences between day 0 (D0: 0 hours) and day 4 (D4: 96 hours) of culture, were expressed as D4/D0 ratio. A total of 427 VPs were cultured divided in three experimental groups: 5-HT, 8-OH-DPAT and anpirtoline. For each experimental group a dose-effect approach was used. Furthermore, each experimental group was cultured either with or without testosterone supplementation of media ([testosterone] = 10^{-8} M).

Human prostate cell lines cultures. Three human cell lines were used: PNT1A and WPMY-1 were obtained from American Type Culture Collection (ATCC, Manassas, Virginia) and BPH-1 which was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). All the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM 1x, high glucose; Gibco, Invitrogen, Grand Island, New York) supplemented with 10% FBS (Gibco, Invitrogen, Grand Island, New York) and 1% penicillin/streptomycin solution (DMEM-10), at 37 °C and 5% CO_2. For the viability assay, the cells were plated into 96-well plates at a density of 3×10^3 cells per well and allowed to adhere overnight in DMEM medium containing 10% FBS. Subsequently, the cells were treated with increasing concentrations of 5-HT, 8-OH-DPAT and Anpirtoline diluted in 0.5% FBS culture medium, with or without testosterone (10^{-8}M) supplementation. After 72 hours of incubation, cell viability was quantified using CellTiter 96 Aqueous Cell Proliferation Assay (MTS) (Promega, Madison, Wisconsin). The mean percentage of viable cells relative to the vehicle alone (considered as 100% viability) was determined, and the final results were expressed in relation to the control (adjusted to 1). For the proliferation assay, we evaluated the number of Ki-67 positive cells by immunofluorescence. The cells were plated in glass coverslips placed into 12-well plates at a density of 5×10^4 cells per well, and allowed to adhere overnight. Subsequently, the cells were treated with 5-HT (100 µM), 8-OH-DPAT (100 µM) and Anpirtoline (100 µM) diluted in 0.5% FBS culture medium, with or without testosterone (10^{-8}M) supplementation. The total number of cells and the Ki-67 positive cells were manual counted using a fluorescence microscopy (BX16; Olympus). The ratio of Ki-67 positive cells per total number of cells was determined, and the final results were expressed in relation to the control (adjusted to 1).

Tph1$^{-/-}$ Mice and *in vivo* studies. Only male mice were used for *in vivo* experiments. They were housed in specific pathogen-free conditions in a room maintained at a constant temperature of 23 °C on a 12-h light-dark cycle. Food and water were provided *ad libitum*. All treatment groups were age matched and randomized to treatment at the initiation of an experiment. The researchers performing the experiments were blinded to experimental groups during all testing. Animals were excluded from analysis if signs of fight with skin lesions were present. *Tph1*$^{-/-}$ mice on a C57BL/6 background were provided by M. Bader (Max Delbrück Center for Molecular Medicine, Berlin, Germany). For assessment of the morphological evolution with age both male wild-type and *Tph1*$^{-/-}$ mice were sacrificed at different time points (at least 6 animals for both groups at each time-point): 7, 12, 16 and 20 weeks-old. For pharmacological studies, wild-type and *Tph1*$^{-/-}$ mice with 19 week-old were treated daily with intraperitoneal injections of 0.9% saline or 5-HT (100 mg/Kg) during 10 consecutive days (at least 6 animals for each group). Mice were sacrificed and prostate tissue (all lobes combined) and seminal vesicles were micro dissected away from other urogenital and fat tissues. Total prostate was weighted immediately after dissection. The right lobes were separated from the left and processed for histology or western blotting. For histologic analysis hemi-right prostate was fixed in 10% PFA, processed and embedded in paraffin and stained with hematoxylin and eosin (H&E). Hemi-left prostate was separated in three lobes (ventral, dorsolateral and anterior) and processed to qRT-PCR.

Immunofluorescence analysis. Immunofluorescence for AR, Ki-67, *5-Htr1a* and *5-Htr1b* was performed on formalin-fixed and paraffin-embedded rat ventral prostates or in human prostate cell lines. Briefly, deparaffinized and rehydrated slides were submitted to adequate heat-induced antigen retrieval for 20 min at 98 °C with 10 mM citrate buffer (pH 6.0). Regarding cell lines, all of them were plated in glass coverslips placed into 12-well plates at a density of 5×10^5 cells per well, and allowed to adhere overnight. Then, the cells were fixed in cold methanol by 5 minutes at −20 °C. In both paraffin and cell, for block unspecific ligations the cells/tissues were incubated with a solution of PBS containing 10% FBS for 30 minutes at room temperature followed by incubation with a primary antibody against AR (1:1000 dilution; sc-816: Santa Cruz Biotechnology, Santa Cruz, California), Ki-67 (1:100 dilution, AP10244C; Gennova, Sevilla, Spain), *5-Htr1a* (1:100 dilution; sc-10801: Santa Cruz Biotechnology, Santa Cruz, California) and *5-Htr1b* (1:100 dilution; sc-28937: Santa Cruz Biotechnology, Santa Cruz, California). The cells/tissues were then washed in a PBS solution with 0.5% FBS and incubated with a goat anti-rabbit antibody conjugated with FITC for cells and with TRITC for tissues (dilution 1:500, Life Technology, Carlsbad, California) for 1 hour at room temperature in the dark. Finally, the cells were counterstained with 40,6-diamidino-2-phenylindole (DAPI). The images were obtained using a fluorescence microscopy (BX16; Olympus).

Western blot analysis. Western blot analysis for androgen receptor (AR) was done in both VPs, mouse prostate tissue and in human cell lines. All the samples were properly processed for western blot analysis and lysed in a buffer containing 50 mM Tris pH 7.6–8, 150 mM NaCl, 5 mM EDTA, 1 mM $Na3VO_4$, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and 1/7 of Protease cocktail inhibitors (Roche). Western blotting was done

using standard 10% SDS-PAGE gels, loading 20 µg of protein per lane. For AR detection a specific antibody was used (1:1000 dilution; sc-816: Santa Cruz Biotechnology, Santa Cruz, California). β-Actin was used for loading control (1:500 dilution; sc-1616; Santa Cruz Biotechnology, Santa Cruz, California). After incubation with appropriate secondary antibodies, they were detected by chemiluminescence (Thermo Scientific Pierce ECL Western Blotting) in ChemiDoc™ XRS + System (Bio-Rad). Quantification of western blot results was done using the band densitometry analysis, performed with ImageJ software.

RNA extraction and qRT-PCR. Total RNA was isolated from the dorsolateral prostate of different groups with Trizol (Invitrogen, Carlsbad, California). Then, after quantification using the NanoDrop®, 500 ng of total RNA was reverse transcribed into first strand cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California). Primers used to measure the expression levels of AR was designed using the Primer3 software, on the basis of the respective GenBank sequence. All accession numbers and primer sequences are available on request. The reference gene for hypoxanthine guanine phosphoribosyl transferase (*Hprt*) (accession number from GenBank: NM_013556) was used as an internal standard for the normalization of the expression of selected transcripts. qRT-PCR was performed on a CFX 96TM real time system instrument (Bio-Rad Laboratories, Hercules, California), with the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany), using equal amounts of RNA from each one of the samples. Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at the expected temperature.

Statistics. Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism by Student's *t* test or ANOVA where appropriate. A Bonferroni *post hoc* test was used to test for significant differences revealed by ANOVA. Statistical significance was confirmed at $p < 0.05$.

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Author Contributions
E.C.D., A.M., O.M., P.M., A.C., C.N.S., R.M., N.A. and M.B. performed the experiments. E.C.D., R.A., and J.C.P. analysed and interpreted the data. E.C.D., E.L. and J.C.P. discussed and planned the experiments. E.C.D. and J.C.P. supervised the project. E.C.D. and J.C.P. wrote the manuscript.

Additional Information
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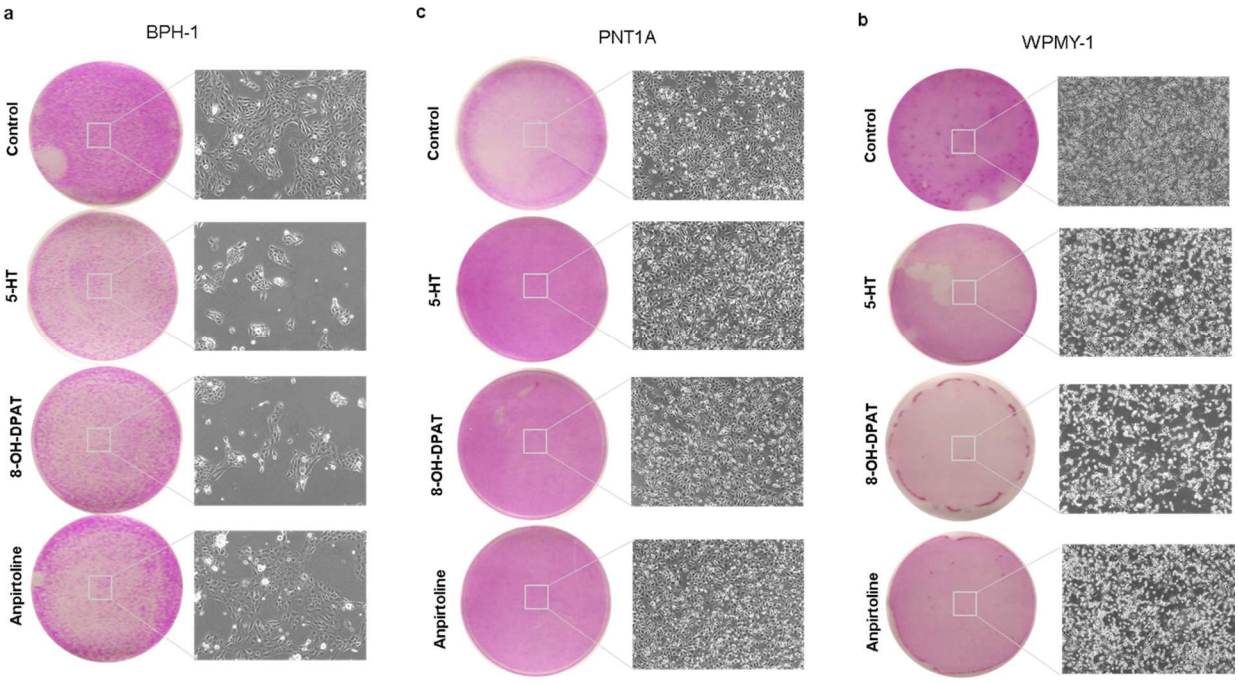
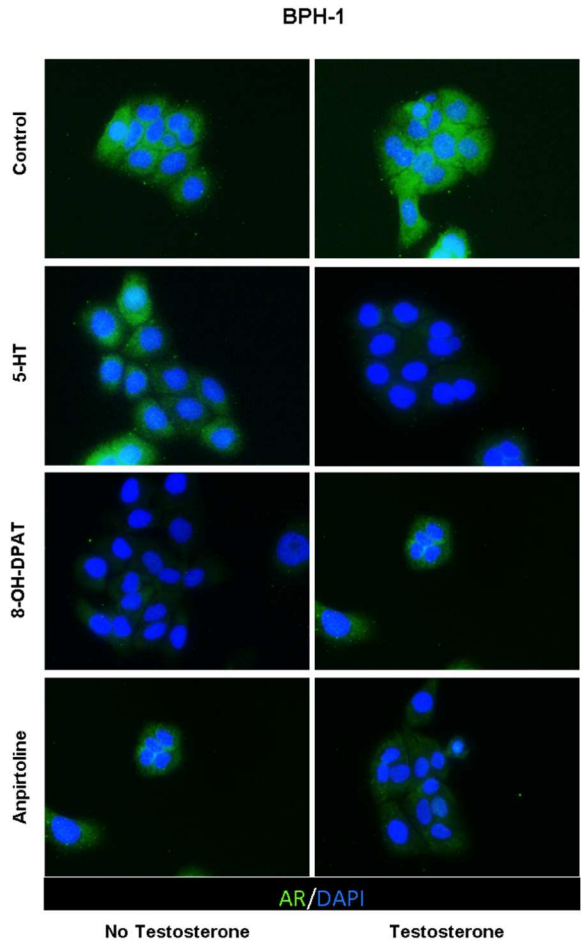
Supplementary Information

Supplementary Figure 1



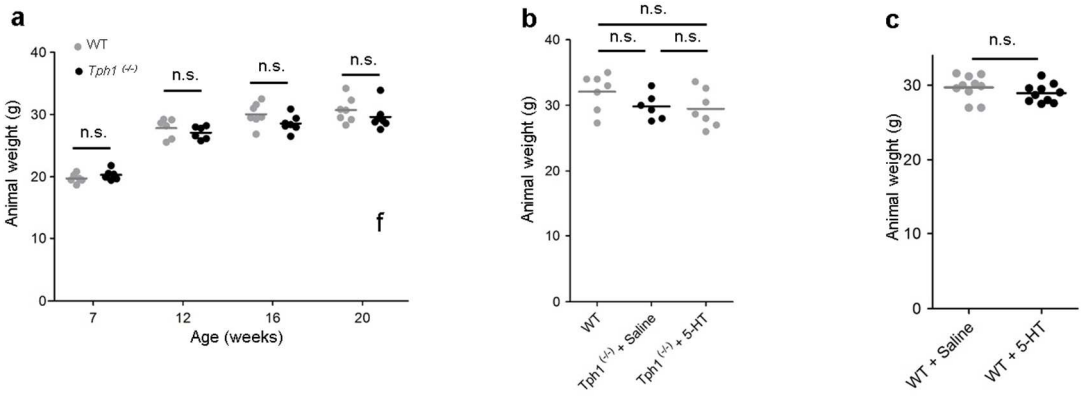
Supplementary Figure 1. Effect of 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist on cell viability and morphology of human prostatic cells treated with testosterone. Effect of 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist on cell viability and morphology after 72 hours of treatment in BPH-1 **(a)**, PNT1A **(b)** and WPMY-1 cells **(c)**. Representative example of three independent experiments, 1 field of Microscope, 400X.

Supplementary Figure 2



Supplementary Figure 2. Effect of 5-HT, *5-Htr1a* specific agonist and *5-Htr1b* specific agonist on AR expression in BPH-1 cells. Immunofluorescence analysis of AR expression after 5-HT, 8-OH-DPAT and Anpirtoline treatment in medium conditions without and with testosterone supplementation. AR, androgen receptor; 5-HT, serotonin.

Supplementary Figure 3



Supplementary Figure 3. Genetic deletion of Tph1 and 5-HT treatment does not affect animal weight. **(a)** Weight of wild-type and *Tph1*$^{-/-}$ mice at different ages. **(b)** Weight of wild-type, *Tph1*$^{-/-}$ treated with Saline and *Tph1*$^{-/-}$ mice treated with 5-HT during 10 consecutive days **(c)** Weight of wild-type treated with Saline and wild-type mice treated with 5-HT during 10 consecutive days. n.s., non-significant.

Influence of Tryptophan-Rich Diet on Prostate Growth

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Alexandra Rocha, Alice Miranda, Nuno Morais,
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(Manuscript submitted) (2021)

The influence of tryptophan-rich diet on prostate growth

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Abstract

Despite the high prevalence of Benign Prostatic Hyperplasia (BPH) in human males the etiology of this disease remains largely unknown. Recently, we demonstrated that serotonin inhibits benign prostatic growth through down-regulation of androgen receptor (AR). Since tryptophan is the amino acid precursor of serotonin, we hypothesized that modulating the plasmatic levels of serotonin through a tryptophan-rich diet we could inhibit prostatic growth.

In this study C57BL adult mice were divided in 2 groups: group 1 was fed with a normal diet and the group 2 was fed with a tryptophan-rich diet, for a 3-month period. After the sacrifice of the mice, whole prostate gland was dissected and weighted. Next, we ran an ELISA, to quantify intra-prostatic serotonin, and a Western Blot analysis to evaluate AR expression in prostate.

Here, we report that mice fed with a tryptophan-rich diet for 3-months have a decreased prostatic weight and a remarkable increase in intra-prostatic serotonin concentration comparatively to mice fed with normal diet. Furthermore, AR expression in prostatic tissue from mice who underwent tryptophan supplementation seems to be reduced comparatively to normal diet group. These results suggest that diet modulation through enrichment of tryptophan should be explored to treat BPH.

Introduction

With aging and in the presence of testosterone almost all Human males develop Benign Prostatic Hyperplasia (BPH). BPH is a nonmalignant growth of prostatic gland and represents the microscopic evidence of stromal and epithelial hyperplasia. This pathologic process occurs exclusively in the Transition Zone of the human prostate gland and approximately 50% of men in the sixth decade and 90% of men by the ninth decade of life exhibit histologic evidence of BPH[1]. More importantly, BPH is responsible for the development of lower urinary tract symptoms (LUTS) and those directly associated to BPH can affect about 20% of men in the fourth decade of life and almost 50% of men in their 80s demonstrating the great burden of this disease for Human male[2].

During several decades, the etiology of BPH was associated directly to the influence of androgens in prostate gland. In this way, the etiology of BPH was attributed to a stimulus acting for a very long period of time on prostate gland, and was presumed that the stimulus was of testicular origin since the disease does not occurred in the absence of the male gonad. The central role for androgens in the development of BPH was concluded from basic and clinical observations that castration significantly improved LUTS associated with BPH[3]. Additionally, the observation that genetic deficiency of 5α-reductase causes abnormal prostatic development and prevents BPH highlighted the importance of DHT in prostate growth[4]. Despite the consensual acceptance of androgens role in prostate development and growth, it is completely unclear why BPH develops at a stage of life (after third decade) when plasmatic levels of androgens are gradually decreasing.

In 1970s, McNeal observed that BPH occurred almost exclusively in a new prostatic zone that he called Transition Zone. McNeal proposed that BPH results from a “reawakening”, in transition zone, of inductive potential in adult prostatic stroma resulting in formation of new branching morphogenesis in the Transition Zone of the prostate gland, challenging the concept of androgens exclusivity in the genesis of BPH[5]. However, the Embryonic reawakening theory does not explain the cause of adult stroma reawakening later in life (between the third and fourth decade of life) and also does not elucidate why this reawakening occurs exclusively in the prostatic Transition Zone.

Neuroendocrine cells can be observed in all zones of the human prostate. However, the majority of neuroendocrine cells are localized in prostatic transition zone in humans and in periurethral glands in the murine prostate. Because Transition Zone is the prostate region where BPH originates and neuroendocrine cells are mainly observed in this region it was plausible an association between both[6]. In this regard, several studies investigated and compared the number of neuroendocrine cells in the normal

transition zone and in transition zone with BPH. All these studies have demonstrated that the neuroendocrine cells in BPH are significantly reduced or absent in BPH compared to normal prostate [7-9].

Serotonin is a biogenic amine with very different functions in the human body such as, regulation of mood and cognition, hemostasis, immune function, vascular tonus, intestinal physiology among others[10]. Quantitatively, the most important product of neuroendocrine cells of the human prostate is serotonin[11], however, its function in normal prostate physiology is unknown. Because serotonin is a major product of neuroendocrine cells and theses are decreased in Transition Zone with BPH it seems possible a putative interaction between serotonin and BPH. In fact, serotonin has been shown to be significantly decreased in BPH comparatively to normal prostate[8]. Recently, we demonstrated in several in vitro and in vivo models that serotonin strongly inhibits benign prostatic growth through androgen receptor (AR) down-regulation and we proposed the neuroendocrine hypothesis for the etiology of BPH[12]. Since tryptophan is the amino acid precursor of serotonin, we hypothesized that modulating the plasmatic levels of serotonin through a tryptophan-rich diet we could inhibit prostatic growth.

Results

Influence of tryptophan-rich diet on prostate weight. To test if a diet rich in the amino acid tryptophan (precursor amino acid in the synthesis of serotonin) could modulate normal prostate growth we fed mice for 12 weeks with a tryptophan rich-diet (n=9) or with a normal diet (n=8) and we compared prostate size and prostate to body-weigh-ratio in both groups. First, we observed that total animal weight was not affected by de different diets (p> 0,05) (Figure 1A). Second, we observed that a tryptophan rich-diet significantly decreased total prostate weight by approximately 15% (average total prostate weight of 0,082 g in mice fed with tryptophan rich-diet (n=9) *vs.* 0,070 g in mice fed with normal diet, p= 0,0186) (Figure 1B). Additionally, we normalize the total prostate weight to animal weight and we observed also a significant decrease of 25% in prostate-to-body weight ratio in mice fed with tryptophan rich-diet vs. mice fed with normal diet (average prostate-to-body weight ratio of 0,0018 g/g in mice fed with tryptophan rich-diet (n=9) *vs.* 0,0024 g/g in mice fed with normal diet (n=8), p= 0,0088) (Figure 1C). Lastly, also the seminal vesicle-to body weight ratio was decreased approximately 20% in tryptophan-rich diet group vs. normal diet group (average prostate-to-body weight ratio of 0,0089 g/g in mice fed with tryptophan rich-diet (n=8) *vs.* 0,0109 g/g in mice fed with normal diet, p = 0,07) (Figure 1D).

Influence of tryptophan-rich diet in intra-prostatic serotonin. To test if modulating tryptophan content in diet we could modulate intra-prostatic serotonin concentration we quantified by ELISA the intra-prostatic serotonin concentration in mice fed for 12 weeks with a tryptophan rich-diet (n=9) or with a normal diet (n=8). Tryptophan-rich diet significantly increased by near 250% serotonin concentration in prostate gland in comparison with mice fed with normal diet (average intra-prostatic serotonin concentration of 0,62 µg/mg in mice fed with tryptophan rich-diet (n=9) *vs.* 0,25 µg/mg in mice fed with normal diet (n=8), p = 0,0003) (Figure 2A).

Additionally, we correlate the total prostate weight of all mice [fed with tryptophan-rich diet and normal diet (n=17)] with concentration of intra-prostatic serotonin and we observed a negative correlation between both although non-significant [r(17) =-0,447, p=0,07] (Figure 2B).

Influence of Tryptophan-rich diet in Androgen receptor expression. Previously, we have demonstrated that serotonin regulates benign prostatic growth through modulation of AR, so we test if the tryptophan rich-diet induced increase in intra-prostatic growth and the decrease in prostatic weight was also associated to down-regulation of AR. Figure 3 compares the expression of AR in mice fed with

normal diet (n=7) comparatively to mice fed with a tryptophan rich-diet (n=7). Tryptophan rich-diet seems to decrease the prostatic expression of AR, and, in some animals fed with tryptophan rich-diet the expression of AR is almost completely absent.

Discussion

The etiology of BPH is currently incompletely understood, however, it is general accepted the crucial role of aging and the simultaneous presence of testosterone in its development and progression[1]. BPH almost universally affects human males and a significant part of them will develop bothersome LUTS as a consequence of benign prostate enlargement. Even with the current pharmacological treatment a large portion of men continues to need a surgical procedure to treat LUTS or more serious complications of BPH[14], creating the emerging necessity for novel pharmacologic options to treat this almost universal condition.

Recently, we described that serotonin is a negative regulator of prostatic growth through down-regulation of AR and we found that trough agonistic modulation of *5-Htr1a* and *5-Htr1b* we could mimic the inhibitory action of serotonin on prostate growth. These findings lead us to propose a new mechanism to explain BPH etiology: The Neuroendocrine Theory[12]. Our proposed theory explains how the depletion of neuroendocrine cells and serotonin observed in prostatic transition zone with aging[7-9], could be the etiologic factor responsible for the initiation and progression of BPH. In our model, the depletion of serotonin induces an up-regulation of AR in the prostatic transition zone leading to the stimulation of benign prostatic growth in this specific prostatic region.

Although our findings suggest that serotoninergic prostatic system should be explored as a new therapeutic target to treat or even prevent BPH, several questions about the pharmacokinetics and safety of serotonin or *Htr1a* and *5-Htr1b* agonists preclude its use in human trials of men with BPH[10].

The synthesis of serotonin has a limiting step that is dependent on the function of the enzyme tryptophan hydroxylase (TPH)[15]. Two types of TPH exists, one expressed only in central nervous system, TPH2, and other expressed only in peripheral organs, TPH1[16]. The existence of two different isophorms of TPH (peripheral vs. central) is very conserved among species suggesting very different roles for serotonin in central (neuronal) and peripheral (non-neuronal) tissues. In fact, serotonin does not cross blood-brain barrier reflecting the importance of its regulation at the central and peripheral organs[17,18]. Peripheral TPH1 converts the amino acid tryptophan in serotonin, so, theoretically we could modulate peripheral production of serotonin through the modulation of tryptophan dietary ingestion.

Several reports have examined the safety of tryptophan ingestion in humans and they observed that although it increases serotonin production and concentrations in the gut, blood, and brain, as well as melatonin production and release throughout the body (mainly in the gut and blood), the accumulated data suggest that such increases are not predictive of adverse effects[19].

In this study, first we observed that a tryptophan-rich diet could significantly decrease prostatic weigh in normal mice. This is in accordance with our previous results in which depletion of peripheral serotonin induced prostatic growth in Tph1 knockout mice and the administration of serotonin restored prostatic mass to wild-type levels[12]. Second, for the first time at the best of our knowledge we observed that the dietary supplementation with tryptophan induced a strong increase in intra-prostatic serotonin. This is important because, previous reports have suggested that increase in plasmatic serotonin could induce a decreased testosterone production in testis, and the decrease in testosterone could be responsible theoretically by the decreased in prostate gland weight. Although we do not have investigated plasmatic testosterone concentration in function of the type of diet, the robust increase in intra-prostatic serotonin in mice fed with a tryptophan rich diet, not only suggests a very active serotoninergic prostatic system but also indicates that the decrease in prostatic weight can be caused directly by the increase in intra-prostatic serotonin induced by tryptophan-rich diet, instead of a decrease of testicular testosterone production.

Recently, we have demonstrated that normal prostate mice have high serotonin levels and this concentration seems to be independent of androgens. But by other side castration of mice induces a tremendous increase in plasmatic serotonin and testosterone replacement normalizes the plasmatic serotonin levels[21]. This curious interaction between plasmatic androgens and serotonin raised the question that also extra-prostatic serotonin production could be influenced by androgens and that this extra-prostatic modulation could be used to regulate prostatic growth. Independently of the way by which prostatic serotonin increases after tryptophan supplementation (intra-prostatic vs. extra-prostatic production) this supplementation induces a decrease in prostate size.

Previously have been demonstrated that dapoxetine (a selective serotonin reuptake inhibitor) in part through AR modulation attenuates testosterone-induced prostatic hyperplasia in rats[22] and that *Htr1a* and *5-Htr1b* agonists inhibit normal prostate development and growth [12], however both therapeutic approaches are not free from adverse lateral events. Modulation of intra-prostatic serotonin trough tryptophan supplementation is natural and with a more favorable profile of adverse events.

Finally, we observed that the prostate gland of mice fed with tryptophan-rich diet seems to had a decreased expression of prostatic AR, and in some mice, this expression was almost completely absent. This is in agreement with our previous report showing that serotonin decreased prostatic growth through down-regulation of AR[12] and suggests that at least in part the inhibitory effects of serotonin is trough down-regulation of AR. As proposed by Morgentaler et al. the regulation of prostatic growth is explained by the saturation hypothesis[23]. In this model the limiting step for prostate growth is not the concentration of

testosterone but the expression of AR in prostate gland. In the current BPH treatment no drug explores the down-regulation of AR for the inhibition of benign prostatic growth. The tryptophan supplementation could be not only a natural and safe approach for BPH treatment but also a new inhibitory mechanism and potential more effective treatment for BPH.

Conclusion

In conclusion, here, we show that a tryptophan-rich diet increases intra-prostatic serotonin concentration and inhibits prostate growth through AR down-regulation. This is in accordance with our previous proposed Neuroendocrine theory for BPH etiology. Therefore, the modulation of prostatic serotoninergic inhibitory pathway over benign prostatic growth, through dietary supplementation with tryptophan should be explored as a new therapeutic approach for human BPH.

Methods

Animals and Experimental Design

C57BL/6 male mice were used for in vivo experiments. They were housed in specific pathogen-free conditions in a room maintained at a constant temperature of 23°C on a 12-h light-dark cycle. Food and water were provided ad libitum. All treatment groups were age matched and randomized to treatment at the initiation of an experiment. The researchers performing the experiments were blinded to experimental groups during all testing. Animals were excluded from analysis if signs of fight with skin lesions were present. Mice were divided in two groups: control group mice were fed on a normal diet (0,03% of tryptophan content) and experimental group were fed on a tryptophan rich-diet (3% of tryptophan content), during a 12-week period. Mice were sacrificed and prostate tissue (all lobes combined) and seminal vesicles were micro dissected away from other urogenital and fat tissues. Total prostate was weighted immediately after dissection. Total prostate was then homogenized for determination of serotonin by ELISA assay and for AR expression by western blotting.

Elisa Assay

The prostatic 5-HT content was measured using the commercial IBL Serotonin ELISA kit. Briefly, prostatic tissue was homogenized on ice, using a pellet pestle cordless motor (Kontes Glass; Vineland, NJ, USA) and a lysis buffer (CelLytic MT; Sigma-Aldrich) that contained a protease inhibitor cocktail (Sigma-Aldrich), as previously described in other tissues[13]. Subsequently, the tissue was further homogenized by sonication (Vibra Cell, SONICS; Newtown, Connecticut, USA) at an amplitude of 30 for 30 seconds at pulse of 2. The homogenate was centrifuged at 300 G for 15 minutes, and then the supernatant was collected and stored at -80°C. Prostatic 5-HT content was measured in the supernatants using the commercial IBL Serotonin ELISA kit (RE59121). All procedures were performed according to manufacturer's instructions.

Western blot analysis

The samples were properly processed for western blot analysis and lysed in a buffer containing 50 mM Tris pH 7.6-8, 150 mM NaCl, 5 mM EDTA, 1 mM $Na3VO_4$, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and 1/7 of Protease cocktail inhibitors (Roche). Western blotting was done using standard 10% SDS-PAGE gels, loading 20 μg of protein per lane. For AR detection, a specific antibody was used (1:1000 dilution; sc-816: Santa Cruz Biotechnology, Santa Cruz, California). -Tubulin was used for loading control (1:2000 dilution; Cell Signal Technologies). After incubation with appropriate secondary antibodies, they

were detected by chemiluminescence (Thermo Scientific Pierce ECL Western Blotting) in ChemiDoc™ XRS+ System (Bio-Rad). Quantification of western blot results was done using the band densitometry analysis, performed with ImageJ software.

Statistical analysis

Statistical analysis was performed using SPSS software version 22 (SPSS; Chicago, IL, USA) and graphs were made using GraphPad Prism® version 6 (San Diego, California, USA). Statistical analysis used Student's t-test or Pearson's bivariate correlation where appropriate. $p < 0.05$ was considered significant.

Author contributions

ECD, PM, RSM, AR, AM, NM, SA, NA and MB performed the experiments. ECD, BC, RL and JCP analysed and interpreted the data. ECD, PM and JCP discussed and planned the experiments. ECD and JCP supervised the project. ECD, BC, RL wrote the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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Figure 1

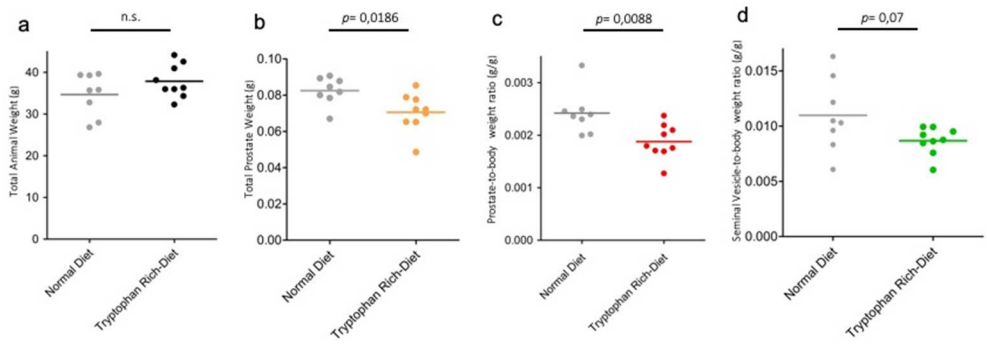

Figure 1. Tryptophan Rich-Diet decreases prostate gland weight.

(a) Total Animal Weight of mice fed with Normal Diet (n= 8) vs. mice fed with Tryptophan Rich-Diet (n= 9). (b) Mice fed with Tryptophan Rich-Diet during 3 months have a significant decrease in prostate weight compared to mice fed with Normal Diet. (c) Mice fed with Tryptophan Rich-Diet during 3 months (n= 9) have a significant decrease in prostate-to-body weight ratio compared to mice fed with Normal Diet (n= 8). (d) Mice fed with Tryptophan Rich-Diet during 3 months (n= 9) have a significant decrease in Seminal Vesicle-to-body weight ratio compared to mice fed with Normal Diet (n= 8). *n.s.* non-significant; (a, b, c and d) Student *t* test.

Figure 2

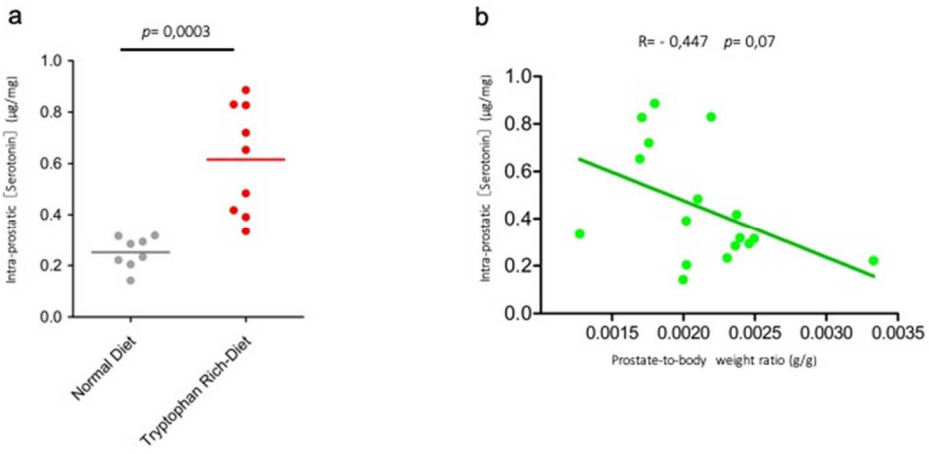

Figure 2. Tryptophan Rich-Diet increases intra-Prostatic Serotonin Concentration.

(a) Mice fed with Tryptophan Rich-Diet during 3 months (n= 9) have a significant increase in intra-prostatic Serotonin concentration compared to mice fed with Normal Diet (n= 8). (b) Pearson Correlation between intra-Prostatic Serotonin concentration and Prostate-to-Body weight ratio

Figure 3

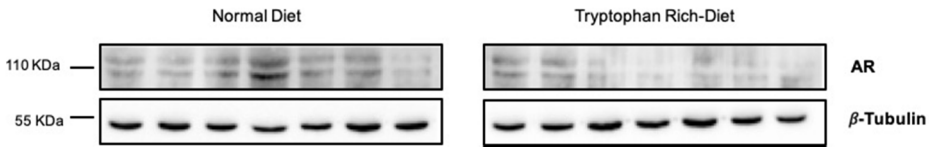

Figure 3 AR expression in mice prostate.

Western blot analysis of AR expression in mice fed with Normal Diet (n= 7) *vs.* mice fed with Tryptophan Rich-Diet (n= 7). AR, Androgen Receptor

CHAPTER 3 | Discussion and Conclusions

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General Discussion

3.1. Feedback Mechanisms in the Biology of Organ Size Determination

One of the biggest fundamental mysteries in biology is how organs know when to stop growing. In the last decades, accumulated evidence has suggested that the size of an organ is largely determined by the work it has to do, suggesting a link between size and function. The capacity of an adult organ to growth to meet its physiological needs, depends on its ability to multiply by hyperplasia the functional units of which it is composed, being the functional unit the smallest structure capable of executing the organ-specific function (Goss, 1966). The body´s most vital organs (heart, lungs, kidneys, and brain) lack the post maturation ability to multiply its functional units, but other organs, most notably exocrine glands, have an almost unlimited capacity of regeneration because they never lose the ability to replicate its functional units through hyperplasia (Goss, 1966).

From the physiology, we know that the ultimate goal of body, organs, cells and even sub-cellular elements is to maintain the Homeostasis. The body preserves the homeostasis always trough feedback mechanisms that requires at least four elements. First, a sensor monitors the function (parameter), second the sensor needs to compare the parameter with a reference internal value called set-point, third the mechanism should produce an output signal, and fourth, the output signal should modulate an effector mechanism and normalize the function (Boron and Boulpaep, 2016). This feedback mechanism its true for all functions of the human body such as arterial pressure, oxygen or carbon dioxide content in the blood, glomerular filtration rate, blood glucose, corporal temperature, and most classically to hormone levels.

One of the strategies that organs use to control its size is based on the assessment of its own function. The most classical example of this kind of regulation is liver regeneration after partial hepatectomy in mammals (Michalopoulos, 2010). Both embryonic and adult liver rapidly recover mass following cellular loss either through hypertrophy and hyperplasia.

Remarkably, the regenerated liver after partial hepatectomy neither exceeds nor falls short of its original size. This regulatory feedback mechanism of liver regeneration is known as “hepatostat”. As an example, in liver, under homeostatic conditions, a stable extrahepatic bile acid pool (Huang et al., 2006; Naugler, 2014) leads to the production of FGF15 in intestinal epithelial cells (Naugler et al., 2015), the circulating FGF15 binds to FGFR4 on hepatocytes and promotes activation of Hippo pathway, restricting the hepatic growth. After hepatectomy, the pool of extrahepatic bile acids is altered, causing a decrease

in production of FGF15, and an ultimate inactivation of Hippo pathway in hepatocytes and induction of hepatic growth (Patel et al., 2016). After normalization of liver size, the pool of extra-hepatic bile acids is normalized and the liver growth stops (Ji et al., 2019). This entero-hepatic feedback mechanism in regulation of organ size is an extraordinary example as the organ size is regulated in an attempt to maintain function homeostasis (Merrell and Stanger, 2019).

3.2. Hypothesis for a Feedback Mechanism Regulating Prostate Growth

In comparison to other body fluids the prostatic secretions have extremely high concentrations of citric acid, zinc, potassium and several proteins such as PSA and other Kalikreins, acid phosphatase and lactate dehydrogenase (Lilja et al., 1988). The prostatic secretion is mainly produced in epithelial cells lining the acini of the peripheral zone of the prostate. After its production, in the acini, the prostatic ducts drain the fluid directly to the posterior side of prostatic urethra. It is important to note that there are three types of prostatic glandular tissue. First, the mucosal glands are located around the urethra (periurethral glands), they are few in number and originate the prostatic transition zone. The second group of glands are the submucosal glands, localized external to the periurethral glands, in the central zone, and, the third type the principal glands that are the most abundant and correspond to the peripheral zone. Interestingly, the mucosal glands drain separately alongside the urethral lumen, and the ducts of submucosal and principal glands drain just in the posterior urethra (Santamaría et al., 2007). Although the mucosal glands are few (5% of glandular tissue), they have a strategic localization, around all the urethra, coincident with the drainage of the all prostatic fluid (McNeal, 1988).

At the cellular level one of the most important differences between the transition zone of the prostate and the other zones is the great number of neuroendocrine cells present in normal (young) transition zone (Cohen et al., 1993). This neuroendocrine cells lie in the normal epithelium intermingled with the most frequent epithelial cells. Neuroendocrine cells, lie on the basement membrane of the epithelium, and an important part of them are "open to the acinar lumen. Frequently, these cells also exhibit dendritic processes that extends to the neighboring epithelial cells (Santamaria et al., 2007).

The morphology of neuroendocrine cells and its strategic predominant location in the periurethral (transition) zone strongly suggests that those cells and this small glandular transition zone could be involved in sensing and tuning the prostatic fluid.

The prostate gland is unique among the other human body organs, because it is permanently dependent of androgens for its normal size and function and in absence of androgens the prostate gland regresses in size and in function. However, after puberty the prostate gland remains constant until the

adulthood life, in spite of the constant stimulation of androgens. This fact suggest that the determination of prostate size is not only dependent of the "positive "effect of androgens over the prostatic growth but certainly a "negative" regulator should exist stopping the excessive prostatic growth and maintaining the normal adult size of the gland and the normal function.

Based on the facts presented before its plausible to conceive the thesis that the regulation of prostate size and growth could be dependent of a mechanism that keeps the function of the organ homeostatic. In this hypothesis, the continuously stimulation of prostate gland by androgens stimulate the secretory activity of epithelial cells. This cells secrete its fluid to the acinus of the gland and then to the ducts that flow to the prostatic urethra. The neuroendocrine cells predominantly located in the periurethral glands of the transition zone could taste and sense the fluid, compare the quality of the fluid with an intrinsic set-point and mount an effector response mediated by its neuroendocrine products that adjust the quality of the fluid keeping the homeostasis of the gland function. Should be noted that this effector response could have, at least theoretically, an acute phase (nervous mediated) in which the individual epithelial cells adjust its individual secretory capacity and a chronic phase (nervous/paracrine mediated) in which the function is adjusted through cellular hyperplasia.

3.3. Hypothetic Role of a Prostatic Feedback Mechanism in Benign Prostatic Hyperplasia Etiology

Androgens play a determinant role in the growth of epithelial and stromal cells growth during all the life of the prostate gland. In prostate, the enzyme 5α-reductase type 2, expressed mainly in stromal cells, converts the testicular produced testosterone into DHT. In fact, 90% of prostatic androgens are in the form of DHT, that has several times more potent androgen activity than testosterone, and this occurs because the reduction of testosterone greatly increases the affinity of DHT to AR (Swerdloff et al., 2017).

As previously reviewed, human prostate development and growth occurs in three waves. The first (from fetal live to birth) and second (from the beginning of puberty until adult life) waves are clearly dependent of androgens. However, the third wave of prostatic growth, that starts at the middle life and continues with the aging, involves only the transition zone of the prostate and is coincident with the decrease in plasmatic testosterone suggesting that androgens are not the cause of this wave of benign prostatic growth. Additionally, no correlation exists between prostate size and plasmatic levels of testosterone and, curiously, in hypogonadic adult patients treated with testosterone the prostate gland actually decreases in size (Jin et al., 2001). Because the main intraprostatic androgen is DHT would be possible that an increase in DHT with aging could be responsible for the prostate growth, however several

reports have demonstrated that DHT is not increased in BPH and in some of them is actually decreased (Walsh et al., 1983; van der Sluis et al., 2012). This strongly suggests that androgens are not the cause of BPH. In fact, the actual view is that the prostate is relatively insensitive to plasmatic testosterone variations in eugonadal or even mild hypogonadal men, because AR in prostate cells is saturated by relatively low androgen levels (in the near castrate range). As described by Morgentaler and Traish and known as "the saturation hypothesis" (Morgentaler and Traish, 2008).

The "saturation hypothesis" highlight the importance of AR in the stimulation of prostatic growth. Several reports demonstrated that the expression of AR is higher in transition zone of BPH comparatively to normal transition zone. Additionally, the comparisons between expression of AR in BPH and normal peripheral zone revealed a 3-fold increase in AR expression in BPH (Kyprianou et al., 1986; Monti et al., 2001; Jiang et al., 2011; Nicholson et al., 2013). Also, in brown Norway rats (the only murine that spontaneous hyperplasia of the prostate) with aging there is an almost 3-fold increase of AR in hyperplasic prostate (Banerjee et al., 2001).

Taken together all this previous research, we first theoretically hypothesize in the construction of this project that the prostatic function, like all human functions, is regulated with the ubiquitous mechanism of feedback. Our hypothesis was that androgens stimulate AR to induce production of prostatic fluid (parameter). Prostatic fluid is tasted by neuroendocrine cells (sensor) that mount an effector mechanism (secretion of neuroendocrine products) that regulates the androgen stimulatory effect through modulation of AR.

As previously described, by an unknown reason, the neuroendocrine prostatic cells are significantly decreased in BPH and its major secretory product serotonin is also substantially decreased in hyperplasic transition zone. In this sequence, we construct the thesis that the decrease or absence of neuroendocrine cells with aging in transition zone could be implicated in the genesis of BPH in humans by dysregulating the feedback mechanism of prostatic growth. With aging, even with a decrease in plasmatic androgens, the decrease of neuroendocrine cells in transition zone increases the expression of AR and sensitizes the transition zone to the stimulatory action of androgens and the consequence is BPH of the transition zone.

To demonstrate if this thesis was plausible we design the experimental work to first, study the function of serotonin in regulation of branching morphogenesis of the prostate (the mechanism by which BPH development occurs); second, to study if serotonin modulates the AR; third, to study the function of serotonin in human BPH; and fourth determine the in vivo effects of the ablation of prostatic serotonin.

3.4. Serotonin is a new inhibitor of prostate branching morphogenesis through AR down-regulation

The most accepted contemporary explanation for BPH is the reawakening theory proposed by McNeal. Seminal in this hypothesis, is the proposal that prostatic transition zone stops distal ductal branching morphogenesis during the ontogeny of the prostate, because its intimate relationship with the periurethral smooth muscle sphincter that extends distally from the bladder neck (McNeal, 1990). In fact, studies made by Gerald Cunha et al. demonstrated that periurethral smooth muscle could have an inhibitory action over prostate development (Thomson et al., 2002). In embryonic reawakening theory, with aging, the prostate regains its capacity of branching morphogenesis, specifically in transition zone, because of a putative reawakened induction mechanism derived from periurethral smooth muscle.

Besides this assumption, several reports, most notably that presented by Youg Xue have demonstrated that in fact the development of BPH occurs trough new epithelial branching morphogenesis in prostatic transition zone (Xue et al., 2001).

Experimentally the most accepted model to study the prostatic branching morphogenesis is through *in vitro* cultures of prostatic explants. Because of this we choose to study the function of serotonin in regulation of prostatic growth in ventral prostate explants from newborn male rats cultured *in vitro,* during four days, in two different medium conditions: without or with supplementation of culture medium with testosterone. In both medium condition, we observe a very potent and dose-dependent inhibitory effect of serotonin over prostatic branching morphogenesis. This result is crucial, because at the best of our knowledge, was the first description of the serotonin (and neuroendocrine cells) role in prostate physiology and more specifically in prostate growth. All the previous descriptions of serotonin and neuroendocrine cells in prostate was just numeric and quantitative, this was a qualitative result different from all the previous reports.

From the 80s, neuroendocrine cells and serotonin have been implicated in the regulation of benign prostatic growth, perhaps mediating the interaction between stroma and epithelium. The first report, in 1986 by Abrahamsson et al demonstrated the presence of neuroendocrine cells in normal prostate and BPH and observed that by far the most predominant type of product of these cells was serotonin. In this study the authors suggested that serotonin immunoreactive cells was higher in hyperplastic prostate glands than in normal prostate (however just two cases of normal prostate were used in this study) (Abrahamsson et al., 1986). However, after this report, three additional reports quantified the neuroendocrine cells in BPH (cocket et al., 1993, Martin et al., 2000; Islam et al., 2002) comparatively to normal prostate. All the three studies observed a great decrease in neuroendocrine cells and its

products most notably serotonin in BPH. Because classically the neuroendocrine products are described in physiology and pathology as mitogens the observation that neuroendocrine cells were consistently diminished in BPH made some authors to conclude that: "neuroendocrine cells do not appear in acquired tissue within BPH nodules as the nodules develop. Thus, the distribution of NE cells does not seem to be related to the development of BPH." (Islam et al., 2002).

However, the observation that neuroendocrine cells could have an inhibitory role in prostate gland growth was made for the first and only time in 2001 by Xue et al. In this work the authors observed that in the budding tips of epithelial branching segments the expression of proliferative markers was highest but in this localization, no neuroendocrine cells were observed. They observed also that epithelial proliferation in the direct vicinity of neuroendocrine cells was lower than in areas distant from these cells. They concluded that neuroendocrine cells could have an inhibitory effect on proliferation of epithelial cells (Xue et al., 2001).

Although serotonin is classically seen as a mitogen, has been reported that serotonin could have also inhibitory actions in several organs. In liver (also a branching organ) for example, serotonin inhibits the growth of biliary tree in the course of chronic cholestasis and this effect is mediated trough *Htr1a* and *5-Htr1b* receptors (Marzioni et al., 2005). In mammary gland (branching organ) serotonin is an important regulator of gland involution after milking cessation. The accumulation of milk, increases TPH1 expression and higher levels of serotonin are produced and secreted to alveolar lumen. High concentration of serotonin in alveolar lumen induces the involution process trough the initiation of apoptosis in epithelial cells. Furthermore, Matsuda et al. showed that exposure of mammary explants from late pregnant mice to serotonin induced a loss of mammary morphological differentiation and a high number of apoptotic bodies. In contrast, they demonstrated that reducing the 5-HT amount in the explants by treating them with a 5-HT receptor blocking agent (Methysergide) or a suppressor of TPH1 (p-chlorophenylalanine), resulted in maintenance of mammary differentiation (Matsuda et al., 2004).

To support a function for serotonin over prostatic growth, obviously, the next experimental approach was to study the expression of serotonin receptors in prostate gland. During the last three decades 15 different serotonin receptors were described, which are grouped into four families based on intracellular signaling mechanisms. In BPH, one single report has previously described the existence of both *Htr1a* and *5-Htr1b* in benign prostatic tissue, but without any reference to cellular specification (epithelial vs. stroma) (Dizeyi et al. 2004). However, the most studied expression of serotonin receptors in prostate was in malignant cell lines of prostate cancer namely in PC-3, DU145 and LNCaP and the most well characterized receptors in these cells were also *Htr1a* and *5-Htr1b*. Based on this previous reports we

investigated and demonstrated for the first time at the best of our knowledge that *5-Htr1a* and *5-Htr1b* were strongly expressed in rat prostate in both epithelium and stroma. The strong expression of both receptors is an important argument about the importance of the function of serotonin in prostate biology.

To discriminate the function of each single receptor in prostatic growth we modulate the activity of both *Htr1a* and *5-Htr1b* in ventral prostate. We observed that the specific agonists of *Htr1a* (8-OH-DPAT) and the specific agonist of *5-Htr1b* (anpirtoline), both strongly inhibited the prostatic branching morphogenesis. This results highlighted the importance of both receptors in the inhibitory action of serotonin.

Our next objective was to study if the inhibitory action of serotonin could be related to the modulation of AR as our hypothesis about the feedback mechanism of prostatic growth regulation hypothesized. We observed that serotonin and the specific agonists of *Htr1a* and *5-Htr1b* downregulates in a dose dependent manner the expression of AR.

To understand how serotonin could regulate AR is important to discuss all the possibilities for this regulation. AR is a member of the family of nuclear receptors and they act as a ligand (androgen) dependent transcription factor. In the absence of androgens, AR is localized in the cytoplasm complexed with heat-shock proteins. Binding of androgens dissociates AR from heat-shock proteins and translocate AR to the nucleus as a homodimer. In the nucleus, the complex androgen-AR binds to specific DNA sequences, known as androgen response elements. Additionally, to its dependence from androgens the transcriptional activity of the androgen-AR homodimer is modulated by several proteins known as coregulators (Davey and Grossman, 2016).

Regulation of AR can occur at several stages. The first type of AR activity regulation is trough modulation of AR expression. In this setting several types of regulatory systems exists. First, the promotor of AR gene is strongly dependent of ligation of Sp1 to enhance AR transcription, in the absence of Sp1 the transcription of AR is down-regulated. Second, the NF1 site of AR promoter has been reported to downregulate AR mRNA expression and the increase in nuclear factor-B also downregulates AR. Third, AR can be regulated at post-transcriptional level through modulation of both stability and translation efficiency of the AR mRNA. Lastly, AR can be regulated at the protein level throughout modulation of protein half-life and degradation (Lee. et al., 2003).

Here we observed that at protein level, serotonin trough *Htr1a* and *5-Htr1b* downregulates AR expression but we do not elucidate the cellular mechanisms responsible for this down-regulation. This is an important aspect that deserves consideration in future experimental work.

Biologically, the most important functions of androgens and AR three: 1. development of male reproductive system and male secondary sexual characteristics, 2. stimulation of spermatogenesis and 3. brain masculinization in male-typical behaviors and bone physiology (Matsumoto et al., 2013).

Although the regulation of AR expression by serotonin in prostate gland is a completely new finding in male reproductive biology, has been previously very well documented that brain masculinization is dependent upon a perinatal surge in testosterone and a concomitant transitory decrease in hypothalamic serotonin. This decrease in serotonin is necessary for up-regulation of AR and the activation of serotonin receptors in brain influence sexual differentiation through modulation of AR expression (Wilson et al., 2007; Murray et al., 2004; Dakin et al., 2008).

3.5. Serotonin is a new inhibitor of human Benign Prostatic Growth through AR down-regulation

Preclinical models to study human BPH are almost limited to *in vitro* or *in vivo* studies using BPH cells. We used three different cell lines do determine if the serotoninergic inhibitory pathway in rat prostate branching morphogenesis was also active in human cells. We choose two cell lines of human BPH, both androgen sensitive, one from epithelial cells (BPH-1) and the other one from stromal cells (WPMY-1). The third cell line that we used was an epithelial cell line from normal prostate but androgen insensitive (since does not express AR).

As observed in rat prostate, we showed for the first time at the best of our knowledge, that all cell lines express both *Htr1a* and *5-Htr1b*, highlighting the putative role of serotonin in prostatic cells physiology. In functional studies, we observed that serotonin inhibits cell viability of BPH-1 and WPMY-1 cells (both androgen sensitive) but only if we treat this cells with testosterone. This emphasizes that the mechanism of inhibition of cell viability induced by serotonin is linked to androgen pathway. More interestingly to confirm this association was the observation that PNT1A cells (androgen insensitive) viability was not affected by serotonin. In generic terms the decrease in cell viability (analyzed by MTS assay) could be caused by decreased cellular proliferation or by increased cellular death. To clarify if serotonin induced decreased cell viability in androgen milieu BPH cells was caused by decreased cellular proliferation we study the expression of Ki-67 in all conditions. We observed clearly that BPH-1 and WPMY-1 cells have decreased cellular proliferation after treatment with serotonin but only if these cells were induced to increase its proliferation by testosterone. Again, proliferation of PNT1A cells was not affected either by testosterone or serotonin supplementation. We think that this approach, studying both androgen

sensitive and insensitive cells give a clearer picture of the interaction between serotonin and androgen pathway.

In human benign/normal prostatic cells the role of serotonin has never been studied before. However, several reports have addressed the role of serotonin in malignant prostatic cells.

The first report was published in 1994 by Abdul et al. and the study does not address directly the function of serotonin in malignant prostatic cells but the effect of three serotonin-uptake inhibitors (6-nitroquipazine, zimelidine and fluoxetine). They observed that all serotonin-uptake inhibitors decreased cell growth of all three different cells studied (LNCaP, PC-3 and DU-145) and they confirmed that the uptake of serotonin in this cells was strongly inhibited by all three drugs. Because of this, the authors concluded that serotonin would have a stimulatory role over malignant prostatic cells growth (Abdul et al., 1994).

However, the action of serotonin is mediated by its ligand properties over extracellular domain of serotonin receptors. In fact, serotonin uptake inhibitors increased extracellular serotonin and more is available to activate serotonin receptors. If serotonin uptake inhibitors inhibit prostatic malignant cell growth this could be at least theoretically caused by the direct inhibitory action of serotonin.

After this report, other studies have directly investigated the function of serotonin in malignant prostatic cells. The studies demonstrated that mainly in androgen independent (castration-resistant) prostate cancer cells the serotonin increased cellular proliferation (Dizeyi et al., 2005; Siddiqui et al., 2006; Dizeyi et al., 2011; Shinka et al., 2011). Independently of the effects of serotonin in malignant prostatic cells, and the fact that this effect was mainly observed in androgen-insensitive malignant cells suggests that castration resistance could change the phenotypic response of prostatic malignant cell to serotonin and neuroendocrine products. Additionally, a similar finding is observed in mammary gland, where serotonin inhibits benign mammary gland growth but stimulates malignant mammary cells (Pai et al., 2009).

Giving strength to our finding that serotonin inhibits androgen sensitive human BPH cells was the demonstration that the specific agonists of both Htr*1a* and *5-Htr1b* also inhibit the cell viability and proliferation exclusively in androgen sensitive cells and after testosterone stimulation of these cells.

Taken together, this results strongly argue that the inhibition of benign prostatic cells growth by serotonin is intersected with the stimulatory androgen pathway. In fact, we demonstrated that human cells of BPH epithelium and stroma are very sensitive to testosterone, and similar to previous reports testosterone increases AR expression in these cells. However, after serotonin and anpirtoline (*5-Htr1b* agonist) treatment the up-regulation of AR induced by testosterone was abolished. Even 8-OH-DPAT (*5-*

Htr1a agonist), although not statically significant, induced down-regulation of AR after testosterone treatment in androgen-sensitive cells.

These findings in human cells of BPH suggests that serotoninergic pathway either through modulation of serotonin levels or through the use of agonistic drugs of *5-Htr1a 5-Htr1b* could have the potential role to treat and prevent human BPH.

3.6. The *in vivo* depletion of prostatic serotonin increases prostatic size

Serotonin is synthesized in a two-step enzymatic pathway in which de amino acid tryptophan is first converted into 5-hydroxy-tryptophan by the enzyme tryptophan hydroxylase (Tph) and in a second step 5-hydroxy-tryptophan is converted in serotonin by the enzyme aromatic L-amino acid decarboxylase. Interestingly, the limiting step in production of serotonin is the tissue expression of Tph (Mohammad-Zadeh et al., 2008).

Almost all serotonin in the human body is produced by enterochromaffin cells of the gut, since these cells express the highest levels of Tph. In the classical view serotonin was produced by the gut and then was incorporated in platelets and transported to different organs by platelets (Mohammad-Zadeh et al., 2008). In 2003, genetic ablation of Tph in mice revealed that the peripheral concentration of serotonin was strongly decreased but curiously the brain serotonin was unaffected. This finding unrevealed the existence of a second form of Tph in neuronal tissue known today as Tph2. Importantly the gene deletion of Tph2 does not affect peripheral serotonin but brain serotonin was almost absent (Walther et al., 2003). These findings were the basis for the concept of two independent serotoninergic pathways (central vs peripheral) separated by the brain-blood barrier. Since that the peripheral role of serotonin function was considerably expanded.

The majority of blood serotonin is synthesized by Tph1 enterochromaffins cells in the duodenum and enters in circulation packed in dense granules of thrombocytes. However, the genetic ablation of Tph1 led to the discovery of complete serotoninergic systems in unexpected localizations such as pancreas, mammary gland, liver, lung and pineal gland (Mohammad-Zadeh et al., 2008). This led to speculation that within an organ the local production of serotonin could be regulatory for organ functions (independent from gastrointestinal serotonin).

As we hypothesized, the depletion of neuroendocrine cells in prostatic transition zone could be responsible for the overgrowth of this specific region of the prostate. To mimic the scenario observed in the aged human prostate the best approach was to study the effect of genetic ablation of Tph1 over the prostate growth. We observed that from very early in mice life the prostate gland of *Tph1*$^{-/-}$ was significantly

increased in size comparatively to wild type mice and at 20 weeks of age the *Tph1*$^{-/-}$ mice exhibited a 37% increase in prostate size (comparatively to wild-type).

This result demonstrates that at least physiologically serotonin is a negative regulator of prostate growth. In the regulation of prostatic growth as previously described the androgens are the main positive stimuli for prostate growth. Also, as we stated before it is not plausible to assume that the steady size of prostate gland is just dependent on the androgen action. In the absence of a negative regulator of prostate growth the constant stimulation of prostate by androgens will induce an indefinitely growth of the gland, so in our opinion is obvious, at least theoretically, the existence of negative regulators of prostate growth to keep the steady equilibrium in organ size. At the best of our knowledge we describe the first negative regulator of normal prostate growth and we demonstrated that in its absence the prostate gland equilibrates its size at a "new" increased size.

An important question is why the prostate gland does not increase even more in size with prostatic serotonin depletion. We presume that probably other neuroendocrine products could compensate in the absence of serotonin, and probably serotonin is not the only negative regulator of prostate growth. In addition, *Tph1*$^{-/-}$ are not completely depleted from peripheral serotonin. Even in simultaneous *Tph1*$^{-/-}$ and *Tph2*$^{-/-}$ 10% of plasmatic serotonin is present suggesting that other enzymes could contribute to the alternative production of serotonin, most notably the enzyme phenylalanine hydroxylase.

To give robustness to our findings we treat *Tph1*$^{-/-}$ mice with serotonin and almost restore the prostatic size. Additionally, even wild-type mice treated with serotonin for ten consecutive days demonstrate a decrease in prostate size comparatively to wild type.

The increase in prostatic size in *Tph1*$^{-/-}$ mice was associated to an up-regulation of androgen receptor in mouse dorsolateral-prostate. However, we were unable to demonstrate that in all prostate gland, at protein level, the AR was up-regulated. We could speculate that specific regions of prostate gland could respond differently to serotonin but this question was not answered in our experimental work.

3.7. Increase in diet intake of Tryptophan decreases prostate size

Our findings presented and discussed before strongly suggests that intrinsic prostatic serotoninergic pathway could be explored as a new disease-specific target for treatment of BPH. We could explore, based in our results, this system in different ways. First, we could treat the disease specifically with serotonin, however some drawbacks exist for this approach. The most important of this is that serotonin as to be administered intravenously or intraperitoneally what is almost impracticable in the clinical practice. By other side, the acute administration of serotonin could have acute side effects the most important of that are resumed in the serotonin syndrome characterized by neuromuscular and autonomic hyperactivity.

Second, we could try to treat the disease with the specific agonists of *5-Htr1a 5-Htr1b*, however both drugs that specifically activate these receptors could have potential important side effects in central nervous system.

A third approach would be to modulate the production of serotonin trough a high dietary intake of the precursor amino acid tryptophan. In the synthesis of serotonin, tryptophan hydroxylase, at normal concentration of tryptophan, is only approximately half-saturated with substrate (Mohammad-Zadeh et al., 2008). Because of this we could increase the substrate (tryptophan) and increase the production of serotonin without the necessity to pharmacologically modulate Tph activity.

The increase in production of serotonin in body could have several potential side effects. However, several reports have shown that acutely and chronic ingestion of oral doses of tryptophan between 1 and 15 g have not reported any relevant adverse event in gastrointestinal system (gastrointestinal pain, diarrhea, or constipation and only occasional nausea has been documented in doses higher than 7,5 g in a single dose), in cardiovascular system (in fact tryptophan decreases arterial blood pressure) or central nervous system (Fernstrom, 2012).

Based on these facts, in our last aim, we demonstrated that a diet enriched in tryptophan strongly increases the prostatic serotonin content and decreases prostate size. At the best of our knowledge, this was the first demonstration that trough diet we could significantly modulate prostatic serotoninergic system. In mice, this dietary modulation did not induce any visible adverse effects confirming the safety of this approach. Also in this model, it seems that the increase in intraprostatic serotonin and decrease in prostate size were associated with apparent decrease in AR expression.

3.8. The Neuroendocrine Hypothesis for Etiopathogenesis of Benign Prostatic Hyperplasia.

The almost universal development of BPH in humans is intriguing. Nowadays, two things are definitive for the development of BPH: aging and the presence of testosterone. Also, very clear today, is the fact that testosterone per se is not the cause of the disease. Another aspect is also unquestionable: BPH only develops in prostatic transition zone with the aging process. Because of this aspect, the most important question is what happens to transition zone with aging, or putting in other words, what are the differences between the young and aged prostatic transition zone (besides the obvious increase cellular proliferation - BPH). This was the main question that move this thesis. From the previous accumulated knowledge one aspect was clear: the young transition zone is the local where neuroendocrine cells are localized in prostate and with aging and development of BPH in transition zone the neuroendocrine cells are lost.

Because the prostate gland is an organ that depends from a tonic daily stimulation produced by testosterone it seems also very logic that something should act also on a daily basis to restrict the overgrowth and keep the prostate size "normal".

From other organs that like prostate have the capacity to growth after maturation, is each time clearer that the regulation of organ size is dependent of its function, and the corollary of this is that the organs compensate the inadequate function with modulation of its growth/size.

Together these observations led us to construct the thesis that, in prostate gland, neuroendocrine cells could act as sensors of prostatic function and mount an effector response to correct deviations in normal function to keep the homeostasis of the organ, and as part of this effector response they could modulate the prostatic growth induced by androgens. Since, in transition zone with aging (BPH), the AR seems to be up-regulated we complete our thesis with the postulate that the neuroendocrine cells modulate androgen action trough regulation of AR.

The results of our experimental work led us to propose a new hypothesis for the etiopathogenesis of BPH that we call "The Neuroendocrine hypothesis". In this hypothesis, the decrease in neuroendocrine cells in prostatic transition zone with aging, decreases the production of serotonin, and trough less activation of serotonin receptors, the androgen receptor is up-regulated and androgens induce more growth resulting the development of BPH.

Obviously, some questions remain unsolved to give the full picture of this hypothesis. The most important two questions that we need to elucidate in the future are: why neuroendocrine cells decrease with aging and what is sensitized by neuroendocrine cells. We believe that both questions are connected.

The prostate gland is the only organ of the human male that does not contribute nothing to the normal function of the organism, instead all its function its reserved to maintain and perpetuate the species (and not the single male organism) trough optimization of human reproduction. This fact, probably fine-tuned the regulatory mechanism of prostatic function and even small losses of function (somewhere in the middle-aged men) are probably detected and a very effective compensatory response is mounted to normalize the prostatic function. As part of this compensatory response, the transition zone of the prostate gland will suffer cellular hyperplasia because is in this region that besides a niche of adult prostatic stem cells. If we understand what is sensed, and what in prostatic fluid is lost with aging probably we will explain why neuroendocrine cells decrease in transition zone and we will give a complete picture of our Neuroendocrine Theory.

Future Directions

The major objectives of this thesis were to gain knowledge in the etiology of BPH and with that to find new effective ways for prevention and treatment of BPH. In the near future, our main purpose is to elucidate the hypothetical benefits of treatment with tryptophan in patients with clinical BPH. To accomplish that objective, we wait at the present moment the approval from the Portuguese regulatory national authorities to proceed to a clinical trial entitled "TryptoBPH - Proof-of-concept study to evaluate the safety and efficacy of tryptophan in patients with BPH".

TÍTULO

TryptoHBP – Prova-de-conceito e avaliação da segurança e eficácia do triptofano em pacientes com HBP.

ENQUADRAMENTO

A Hiperplasia Benigna da Próstata (HBP) é uma das doenças mais prevalentes e uma das principais causas de sintomas do trato urinário inferior (STUI). Apesar de alguns homens responderem ao tratamento médico (principalmente antagonistas dos 1α adrenorreceptores e inibidores da 5 α-redutase), uma grande proporção de pacientes continua a precisar de um procedimento cirúrgico para tratar os STUI resistentes ou mesmo para complicações mais sérias da HBP, criando a necessidade de novas terapias farmacológicas.

OBJETIVOS

Este estudo de investigação tem como objetivo principal, avaliar o efeito do tratamento com triptofano sobre os STUI, em pacientes com HBP. São também objetivos deste estudo avaliar o efeito do triptofano sobre o fluxo máximo de urina, o volume da próstata a função erétil e a qualidade de vida devido a sintomas urinários.

DESENHO DO ESTUDO

Trata-se de um ensaio clínico aleatorizado, controlado, para avaliar o efeito do triptofano sobre os STUI, em pacientes com HBP.

O presente ensaio clínico irá incluir 2 grupos de estudo. Os participantes elegíveis serão aleatorizados, num rácio de 1:1, pelo grupo experimental (5-hidroxitriptofano, 100mg, três vezes ao dia) e grupo controlo (tansulosina 0.4mg, uma vez ao dia). A fase de tratamento para ambos os grupos terá a duração de 6 meses. A fase de tratamento é seguida por um período de acompanhamento de segurança (*follow-up*) de 6 meses.

Prevê-se incluir no estudo um total de 70 participantes, 35 por braço do estudo.

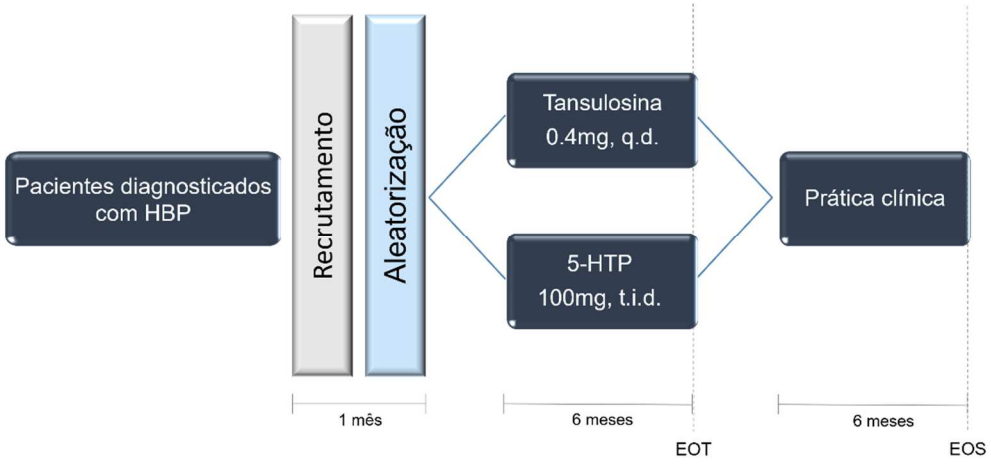
Este estudo tem previstas 7 visitas, das quais 4 decorrem durante a fase de tratamento.

PARÂMETROS DE AVALIAÇÃO DO ESTUDO (*ENDPOINTS*)

O objetivo principal do estudo é demonstrar a superioridade do triptofano em relação à prática clínica na melhoria dos STUI associados à HBP. Tal será medido por uma mudança da linha de base na pontuação total (questões 1–7) do questionário *International Prostate Symptom Score* (IPSS).

Adicionalmente, existem quatro parâmetros de avaliação (*endpoints*) de eficácia secundários: a taxa de fluxo máximo da urina; a função erétil, avaliada pelo Índice Internacional de Função Erétil-5 (IIEF-5); o volume da próstata (em cc), avaliado por ultrassom transretal; e a Qualidade de Vida devido aos sintomas urinários (questão 8 do IPSS).

PLANO ESQUEMÁTICO



SELEÇÃO DOS PARTICIPANTES

CRITÉRIOS DE INCLUSÃO:

- Consentimento informado escrito;
- Pacientes do sexo masculino com HBP;
- Idade ≥50 anos;
- Com volume da próstata ≥30 cm3, avaliado por ultrassom transretal;
- Diagnosticado com STUI definido por uma pontuação total de IPSS estável ≥13 pontos.

CRITÉRIOS DE EXCLUSÃO:

- Pacientes com volume residual da bexiga pós-micção ≥250 ml;
- Pacientes com obstrução intravesical por qualquer outra causa que não seja HBP;
- História de qualquer procedimento interventivo para HBP;
- Pacientes com infeção ativa do trato urinário;
- História de infeções recorrentes do trato urinário;
- Prostatite atual ou diagnóstico de prostatite crónica;
- História de cancro da próstata ou invasivo da bexiga;
- Uso de inibidores da 5 -redutase nos últimos 6 meses;

- Fitoterapia nas 2 semanas anteriores à inclusão no estudo;
- Uso de inibidores da recaptação da serotonina ou inibidores da monoaminoxidase;
- Pacientes com insuficiência renal relevante (definida por MDRD GFR <60 mL/min/1,73 m^2);
- Pacientes com diagnóstico ou suspeita de intolerância à lactose;
- Pacientes submetidos a anestesia geral nas últimas 4 semanas;
- Incapacidade intelectual conhecida que possa condicionar a capacidade de dar consentimento informado e que, na opinião do investigador, torne a inclusão do participante no estudo inapropriada.

PRODUTO A TESTAR, DOSE E VIA DE ADMINISTRAÇÃO

O medicamento em estudo será fornecido em cápsulas de 100 mg de oxitriptano (na forma de L-5-hidroxitriptofano) como substância ativa. O medicamento do estudo é fornecido em blister de 60 cápsulas. O medicamento em estudo deve ser tomado por via oral, três vezes ao dia.

CENTRO DE ESTUDO

Centro Clínico Académico – Braga, Associação (2CA-Braga)

Hospital de Braga, EPE.

DURAÇÃO APROXIMADA DE PARTICIPAÇÃO DOS SUJEITOS NO ESTUDO

12 a 13 meses.

Conclusions

We demonstrate for the first time with experimental work that one of the functions of prostatic neuroendocrine cells is to regulate prostate growth. Its major product of secretion, serotonin, inhibits prostatic growth through down-regulation of androgen receptor. With this experimental demonstration we proposed the thesis of a neuroendocrine cause for etiology of benign prostatic hyperplasia. In our thesis that we call here "Neuroendocrine hypothesis for etiology of BPH" the depletion of neuroendocrine cells in prostatic transition zone cause an up-regulation of androgen receptor in this specific prostatic zone and as a last consequence the development of benign prostatic hyperplasia. We hope with this discover to open a new field in the prevention and treatment of the most common human male disease – BPH.

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