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MECHANISMS OF ACTION OF BOTULINUM TOXIN IN THE TREATMENT OF OVERACTIVE BLADDER

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Prólogo

"O que fazemos por nós mesmos morre conosco.
O que fazemos pelos outros e pelo mundo permanece e é imortal."

Albert Pine

Quem me conhece sabe que adoro desafios. Desde que ingressei no grupo de investigação de Neuro-Urologia de Translação no ano de 2006, o desafio foi constante e aliciante.

Por essa altura, eu era apenas uma aluna da licenciatura em Biologia a realizar estágio de final de curso sem conhecimento do caminho que se abriria à minha frente. Nos primeiros dias de trabalho no Departamento de Biologia Experimental da Faculdade de Medicina do Porto, rapidamente despertei um forte interesse pelas neurociências e pela investigação, em grande parte graças às empolgantes descrições do Professor António Avelino acerca dos seus interesses científicos.

O agradável ambiente e companheirismo sentido no dia-a-dia do departamento fez-me sentir parte de um grupo com objectivos ambiciosos e motivadores que foram fulcrais no caminho que percorri até aos dias de hoje.

Assim, uma dissertação de doutoramento apesar de possuir uma índole académica e científica, não representa apenas o resultado de várias horas de trabalho. O culminar do objectivo a que me propus é também fruto de contribuições de natureza diversa que não devem deixar de ser realçadas.

Em 2008, foi-me proposto pelo Professor António Avelino estudar os mecanismos de acção da toxina botulínica, um desafio que abracei com bastante motivação. A ele agradeço a orientação científica e todo o conhecimento transmitido, base de todo o meu trabalho. Agradeço também os seus conselhos, ensinamentos e a sua amizade.

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E porque a vida não é só trabalho...

Agradeço às minhas amigas Raquel, Ana, Sara e Loide pelas nossas conversas, pela amizade, cumplicidade e partilha de sorrisos e lágrimas. A distância nunca separou a nossa amizade, e que assim seja pelos próximos anos.

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Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efectuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados e redigi as publicações que fazem parte integrante desta dissertação.

I. Coelho A, Dinis P, Pinto R, Gorgal T, Silva C, Silva A, Silva J, Cruz CD, Cruz F, Avelino A (2010) Distribution of the high-affinity binding sites and intracellular target of Botulinum Toxin type A in the Human Bladder. Eur Urol 57:884-890

II. Coelho A, Cruz F, Cruz CD, Avelino A (2012) Spread of Onabotulinumtoxin/A after bladder injection. Experimental study using the distribution of cleaved SNAP-25 as the marker of the toxin action. Eur Urol 61(6):1178-1184

III. Coelho A, Cruz F, Cruz CD, Avelino A (2012) Effect of Onabotulinumtoxin/A on intramural parasympathetic ganglia: an experimental study on guinea pig bladder. J Urol 187(3): 1121-6

IV. Coelho A, Oliveira, R, Rossetto, O, Cruz F, Cruz CD, Avelino A. (2013) Intrathecal administration of botulinum toxin type A improves urinary bladder function and reduces pain in rats with cystitis. (Submitted).

A reprodução destas publicações foi feita com autorização das respectivas editoras.

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Abbreviations

Abbreviations used in “Introduction”, “Final considerations” and “Conclusions”

Abobot/A – abobotulinum toxin A
BDNF – brain derived neurotrophic factor
BoNT – botulinum toxin
BoNT/A – Botulinum toxin type A
BPS/IC – bladder pain syndrome/interstitial cystitis
CGRP – calcitonin gene-related peptide
DO – detrusor overactivity
FDA – food and drug administration
IDO – idiopathic detrusor overactivity
Incobot/A – incobotulinum A
IT - Intrathecal
LUT – lower urinary tract
NDO – neurogenic detrusor overactivity
NGF – nerve growth factor
NO – nitric oxide
OAB – overactive bladder
Onabot/A – onabotulinum A
PAG – periaqueductal grey matter
PMC – pontine micturition centre
SCI – spinal cord injury
SNAP-25 – synaptosomal-associated protein of 25 kDa
SNARE – soluble N-ethylmaleimide sensitive fusion attachment receptor
SP – substance P
SV2 – synaptic vesicle 2
TRPV1 – transient receptor potential vanniloid 1

Abstract

Botulinum toxin type A is successfully used in the treatment of lower urinary tract dysfunctions in which hyperactivity of the detrusor is a main symptom. The use of this toxin in neuromuscular disorders relies on its ability to cleave the SNAP-25 protein impairing the release of acetylcholine on the neuromuscular junction. This mechanism of action has been deeply studied in the skeletal muscle however, its action on the detrusor smooth muscle remained to be elucidated. Due to the complexity of urinary bladder neuronal pathways and to the multifactorial etiology of lower urinary tract disorders, many questions were still unanswered. In the present work we give new insights on the targets of action of botulinum toxin as well as its effects in the urinary bladder.

In **Publication I** a detailed description of the distribution of the receptor and the intracellular target of botulinum toxin A was performed in the human bladder. Both proteins were present in all bladder nerve types with a total co-localization. With this work we could show that all the human urinary bladder nerve fibers could be targeted by botulinum toxin A.

Furthermore, in **Publication II** we decided to investigate the exact location of botulinum toxin activity, administrating the toxin in the bladder of an animal model. We used the cleaved form of the SNAP-25 to detect the toxin action. The cleaved protein was detected and quantified in the three bladder nerve types demonstrating that all fibers are targets of the toxin. In addition we compared different administration forms of the toxin and tested the importance of some dilution factors. We observed that the volume of injection is crucial for the effectiveness of botulinum toxin A in the bladder.

Another aspect of this issue was addressed in **Publication III**. We explored the effect of botulinum toxin in the parasympathetic ganglia since in the human and guinea-pig bladders they are located within the bladder wall. Thus, intramural administration of the toxin directly affects the preganglionic synapse of these ganglia besides the effect on the postganglionic synapse at the muscle bundles.

Finally, in **Publication IV** we decided to investigate the effect of botulinum toxin A in an animal model where bladder pain and hyperactivity were present. In addition, we wanted to test the direct effect of the toxin in the spinal cord. Thus we used the cyclophosphamide-induced bladder inflammation model to analyze the effect of intrathecal botulinum toxin. With this work we found that the toxin counteracts pain behavior and bladder hyperactivity induced by cyclophosphamide. We also observed a direct effect on the expression of neuronal

activation markers that are usually increased in this model. Furthermore we detected the presence of cleaved SNAP-25 not only throughout nerve fibers of the L6 spinal cord segment but also in the urinary bladder. Overall with these results we could say that the intrathecal administration of botulinum toxin A is effective in the treatment of bladder inflammatory pain and that this route of administration may be further explored for intractable forms of pain.

Taken together, the studies comprised in the present dissertation give new insights about the mechanisms of botulinum toxin action on the detrusor muscle as well as in the central nervous system. With clinical importance, the present findings could be directly applied in the improvement of treatments for bladder dysfunctions.

Resumo

A toxina botulínica do tipo A tem vindo a ser utilizada com sucesso em disfunções do tracto urinário baixo cuja hiperactividade do detrusor é o sintoma principal. O uso desta toxina em doenças neuromusculares tem por base a sua capacidade para clivar a proteína SNAP-25 impedindo a libertação de acetilcolina na junção neuromuscular. Este mecanismo de acção tem vindo a ser estudado em profundidade no músculo esquelético contudo, a sua acção no músculo liso ainda não foi clarificada. Dada a complexidade das vias neuronais da bexiga e tendo em conta a etiologia multifactorial das disfunções do tracto urinário baixo, várias questões estão ainda por responder. No presente trabalho são fornecidos novos dados relativos aos alvos de acção da toxina botulínica assim como os seus efeitos na bexiga.

Na **Publicação I** foi efectuada uma descrição detalhada da distribuição do receptor e do alvo intracelular da toxina botulínica do tipo A na bexiga humana. Ambas as proteínas estavam presentes em todos os tipos de fibras da bexiga com total co-localização. Com este trabalho foi possível mostrar que todas as fibras nervosas da bexiga humana podem ser alvo de acção da toxina botulínica do tipo A.

Na **Publicação II** decidimos investigar a localização exacta da acção da toxina botulínica administrando-a na bexiga de um modelo animal. Utilizamos a forma clivada da SNAP-25 para detectar a actividade da toxina. A proteína clivada foi detectada e quantificada nos três tipos de fibras nervosas da bexiga demonstrando que todas as fibras eram alvos da toxina. Além disto, comparamos diferentes modos de administração da toxina e testamos a importância de alguns factores de diluição. Observamos que o volume da injecção é crucial para a eficácia da toxina botulínica do tipo A na bexiga.

Um outro aspecto deste tema foi abordado na **Publicação III**. Exploramos o efeito da toxina botulínica nos gânglios parassimpáticos tendo por base que no humano e no cobaio estes gânglios se encontram na parede da bexiga. Assim, verificamos que a administração intramural de toxina afecta directamente a sinapse pré-ganglionar destes gânglios para além do seu efeito pós-ganglionar exercido na sinapse estabelecida com os feixes musculares.

Finalmente, na **Publicação IV** decidimos investigar o efeito da toxina botulínica do tipo A num modelo animal onde a dor e a hiperactividade da bexiga estivessem presentes. Além disto, pretendemos testar o efeito directo da toxina na medula espinal. Assim, utilizamos o modelo de inflamação da bexiga induzida pela ciclofosfamida para analisar o efeito da injecção intratecal de toxina botulínica. Com este trabalho descobrimos que a toxina contraria o

comportamento nociceptivo e a hiperactividade vesical induzidas pela ciclofosfamida. Também observamos um efeito directo na expressão de marcadores de activação neuronal que estão habitualmente aumentados neste modelo. Detectamos a presença de SNAP-25 clivada não apenas em fibras nervosas dispersas pelo segmento L6 da medula espinal mas também na bexiga. Com este conjunto de resultados podemos afirmar que a administração intratecal de toxina botulínica do tipo A é eficaz no tratamento da dor inflamatória da bexiga e que esta via de administração poderá ser explorada para tratamento de casos de dor intratável.

Os estudos compreendidos na presente dissertação trazem novos detalhes sobre o mecanismo de acção da toxina botulínica no músculo detrusor assim como no sistema nervoso central. Com relevância clínica, estas descobertas poderão ser directamente aplicadas na melhoria dos tratamentos para disfunções da bexiga.

Introduction

1. The discovery of botulinum toxin

Botulinum toxins are among the most deadly natural toxins known and have been following mankind since very early times. Botulinum toxin is responsible for botulism, a deadly intoxication caused by the ingestion of food contaminated with the bacteria *Clostridium botulinum*. Since the first description of the disease, the toxin evolved from poison to therapy in a controversial as well as fascinating journey.

The first botulism-like symptoms are reported from the 10th century, when the Byzantine Emperor Leo IV banned the consumption of blood sausages after the occurrence of several deaths. However, the medical knowledge of the time was not enough to characterize the disease. Therefore, the first reports of botulism appeared only in the 18th century (1).

For centuries storage conditions used to preserve food provided ideal environments for the presence and growth of anaerobic organisms. In 1793, several cases of a fatal food poisoning appeared in the region of Wurttemberg after a decrease of the sanitary measurements triggered by the poverty during Napoleonic wars (1795-1813) (2). The first symptoms reported included gastrointestinal problems, diplopia, mydriasis and progressive muscular paralysis, which were studied by researchers of the University of Tübingen (3). In 1802, after an increasing number of deaths, the link between the consumption of sausages and a possible food poisoning reappeared and a public announcement was released to alert the population.

Between 1817 and 1820, with the spread of the disease, the German physician Justinus Kerner (1786-1862) decided to investigate the cases. He accurately described all the clinical symptoms of more than 200 victims of what we nowadays call botulism. The symptoms included disturbances of the autonomic nervous system, like mydriasis, reduction of lacrimal and salivary glands secretion, gastrointestinal and urinary bladder paralysis. Kerner stated that the toxin was being developed in sausages under anaerobic conditions, that it was interrupting the motor and autonomic nervous system transmission and that it was strong enough to be lethal even in very small doses (4). In a visionary way, Kerner proposed for the first time a possible therapeutic application for the lethal agent, being considered the godfather of botulism research (4).

So far, botulism had been accurately described even before the identification of the toxin and nothing important was added to Kerner's work until the 19th century (2). The following significant step was given by the microbiologist Emile Pierre van Ermengen who established for

the first time a correlation between several cases of intoxication and an anaerobic microorganism. Actually, he was the pioneer on the isolation of the bacteria *Clostridium botulinum* and characterization of its toxin (5).

18th century	First reports of food-borne botulism in Europe
1817-1822	**Justinus Kerner** - experiments with the causative agent of botulism, preliminary experiments with animals, systematic descriptions of its clinical effect, theoretical considerations and speculations about its therapeutical use
1870	**Müller –** referred to the "sausage poisoning" as "botulism" form the latin word "botulus" which means sausage
1895-1897	**Emile Pierre van Ermengen** - identification of *C. botulinum* as the causative agent of botulism
1910	**Leuchs –** discovery of the second serologically different botulinum toxin serotype
1919	**Georgina Burke –** designation of the two serologically different strains as botulinum toxin types A and B
1920	**Herman Sommer –** isolation and purification of botulinum toxin type A as a stable acid precipitate
1922	**Bengston and Seddon** - identification of botulinum toxin type C
1928	**Meyer and Gunnison** - identification of botulinum toxin type D
1936	**Bier** - identification of botulinum toxin type E
1941-1946	**Edward Schantz -** purification of botulinum toxin type A in a crystalline form
1949-1950	**Arnold Burgen and Vernon Brooks** - description of the temporary paralysis induced by botulinum toxin blockade of acetylcholine release from the neuromuscular junction
1960	**Moller and Scheibel** - identification of botulinum toxin type F
1968	**Alan Scott and Edward Schantz –** botulinum toxin experiments with monkeys to determine its use in strabismus
1970	**Gimenex and Ciccareli** - identification of botulinum toxin type G

Table 1: Historical timeline of the discovery and study of botulinum toxin before its therapeuthic application (2, 3).

2. The botulinum toxin family

Botulinum toxins (BoNT) are produced by *Clostridium botulinum,* a Gram-positive, spore-forming, anaerobic bacillus that is ubiquitously found in the environment (5). The toxins produced by these rod-shaped bacteria are some of the most powerful naturally occurring compounds known, with lethal doses for humans around 1 ng/kg of body weight (6).

C. botulinum produces seven immunologically distinct types of BoNTs, isolated and identified as serotypes from A to G (7). Other bacteria such as *Clostridium butyricum* and *Clostridium baratii* also produce two types of BoNTs, types E and F respectively. The seven serotypes are homologous and each one is characterized by particular toxicity properties and different molecular targets.

BoNTs are initially synthesized as inactive, single-chain polypeptides with a molecular weight of approximately 150 kDa (8). The protein is post-translationally proteolyzed by clostridial proteases expressed intra or extracelularlly to form an active dichain that possesses maximum biological activity. The active molecule is composed by a heavy chain of 100 kDa and a light chain of 50 kDa linked by a disulfide bridge and non covalent interactions associated with a zinc atom (9).

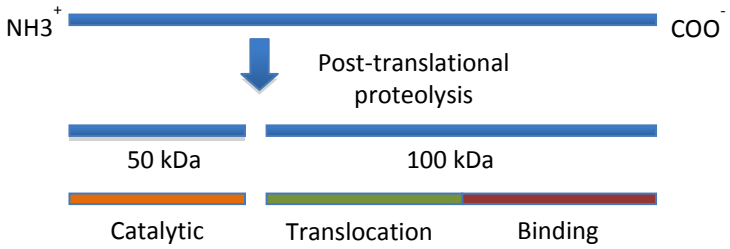

Figure 1: The single chain polypeptide of 150 kDa is proteolyzed to be on its active form. The activated dichain is composed by a light chain of approximately 50 kDa and a heavy chain of 100 kDa linked by a disulfide bridge. The light chain contains the catalytic properties and the heavy chain comprises the translocation and the binding domain. Adapted from Lacy et al (8).

The molecular structure of the active dichain reveals three distinct functional domains of similar size named as binding, translocation and catalytic domains (10, 11). The heavy chain comprises a C-terminal binding domain and an N-terminal translocation domain. The light chain is the catalytic domain located in the N-terminal portion of the molecule and is characterized by its zinc-dependent endopeptidase activity (Figure 1).

2.1 Mechanism of action

Botulinum toxins usually enter the human organism through oral ingestion of contaminated food. In its natural environment, BoNTs interact with nontoxic proteins, to form a complex progenitor toxin of 300 to 900 kDa. These non-toxic proteins increase BoNTs stability, protecting them from the acidic conditions of the gastrointestinal tract and allowing their passage through the intestinal epithelium into the blood circulation (12). Once inside the bloodstream, BoNTs travel until their target cells acting through a multi-step intoxication process composed by four stages: binding, internalization, translocation to the cytosol and SNARE cleavage (13), as detailed below.

2.1.1 Binding to the target cell

BoNTs attach to their target cells through a two-receptor phenomenon that involves a low and a high affinity receptor (14). The toxin heavy chain specifically interacts with membrane glicolipids called polysialogangliosides which are particularly abundant on the cell surface of neuronal cells (15, 16). BoNT/A is known to exclusively interact with the GT1b type of gangliosides (17). The low affinity complex toxin-ganglioside forms a mesh that accumulates the toxin on the plasma membrane surface. This mesh lingers the toxin on the cell membrane plane until it interacts with the high affinity binding site. The high-affinity receptor for BoNT/A has recently been identified as the synaptic vesicle protein 2, SV2 (18, 19), expressed in the luminal surface of synaptic vesicles (20). During exocytosis, vesicles fuse with the plasma membrane exposing their luminal side to the extracellular space. Consequently, SV2 is exposed and allowed to contact with the toxin captured by the ganglioside mesh (21). BoNT/B, on the other hand, has the ability to bind both synaptotagmin II and the ganglioside GD1a at the same time, increasing its stability to specifically affect neuronal cells (22). Toxin binding to high-affinity receptors is serotype-specific and the identity of the receptors for all BoNT serotypes is still unknown.

2.1.2 Internalization – receptor-mediated endocytosis

Internalization of botulinum toxins occurs during membrane retrieval, after exocytosis of synaptic vesicles (18, 19). According to this process, the lumen of the synaptic vesicles is temporarily exposed to the synaptic cleft, allowing the contact with the accumulated toxin. The cell membrane is then recycled and internalized to form a new synaptic vesicle, dragging the toxin attached to its receptor (23). Very active cells, like the cholinergic neurons of the neuromuscular junction, are frequently releasing acetylcholine by exocytosis. Thus, as more SV2 is exposed, more BoNT/A internalization occurs in an activity-dependent process already shown in *in vitro* studies with hippocampal neurons and in *in vivo* studies using the rat phrenic nerve (18, 23, 24).

2.1.3 Translocation

After internalization, a pH decrease inside the endosome induces a structural rearrangement of the toxin. This phenomenon increases the protein hydrophobicity, facilitating the penetration of the molecule in the lipid bilayer in a way that promotes the translocation of the light chain to the cytosol (25). Once the light chain contacts the cytosol, the disulfide bridge is broken, allowing the release of the catalytic domain into the cytosol (26). The hypothesis that botulinum toxins are released to the cytosol through a pH-induced translocation is currently accepted, however, the exact mechanism of translocation is still a matter of debate.

2.1.4 SNARE cleavage

The release of neurotransmitters involves a complex process in which stimulation of the nerve fiber causes a depolarization that activates voltage-dependent calcium channels and a consequent influx of calcium into the nerve terminal. The increase of calcium concentration causes synaptic vesicles to fuse with the plasma membrane, thereby releasing neurotransmitters at the synaptic cleft (27) (28).

Soluble N-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) proteins mediate the fusion of the synaptic vesicle with the plasma membrane and are classified as vesicle-associated SNAREs (v-SNAREs) and target membrane SNAREs (t-SNAREs) located at the plasma membrane. The v-SNARE is a protein known as synaptobrevin or vesicle-

associated membrane protein (VAMP) that is attached to the synaptic vesicle membrane via its C-terminal region. The t-SNAREs comprise two proteins located at the cell membrane: the synaptosomal associated protein of 25 kDa (SNAP-25) and syntaxin. When the synaptic vesicle approaches the plasma membrane, the three proteins interact to form the SNARE complex, which plays an essential role in exocytosis.

Once released to the cytosol, the BoNT light chains, which have zinc-dependent endopeptidase activity (29), specifically target one of the SNARE proteins (30), inhibiting vesicle exocytosis.

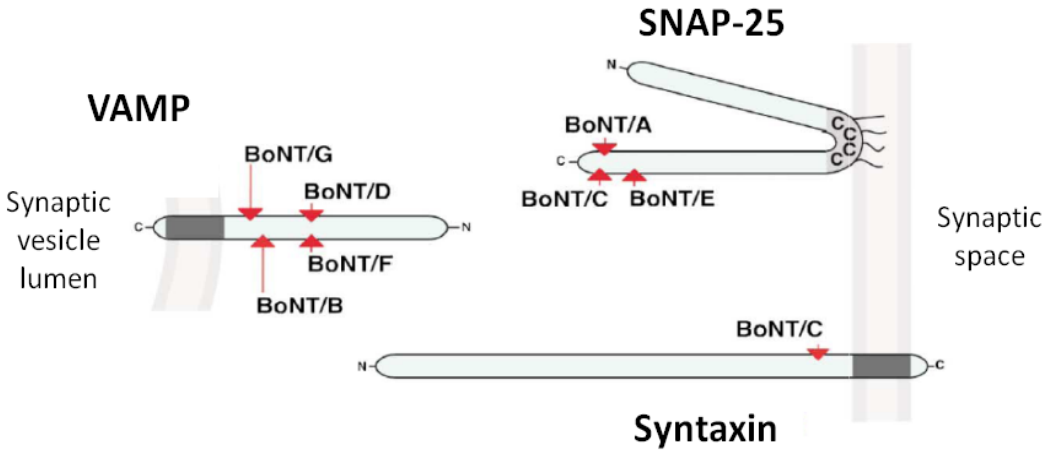

Figure 2: Intracellular targets of botulinum toxins. Once inside the cell the light chain of each botulinum toxin has the ability to cleave, specifically, one of the SNARE complex proteins. VAMP is cleaved by serotypes B, D, F and G. SNAP-25 is cleaved by botulinum toxins A, C and E. Syntaxin is cleaved only by serotype C. Cleavage of one of these proteins will inhibit the vesicle exocytosis. Adapted from Meunier *et al* (31).

Each light chain of a BoNT serotype selectively targets one SNARE protein (Figure 2). BoNT/A cleaves SNAP-25 by removing nine aminoacids from the C-terminus while Bont/E also cleaves SNAP-25 but removing 26 aminoacids (32). The other serotypes are able to cleave VAMP and syntaxin.

Cleavage of individual SNARE proteins does not prevent the SNARE complex formation, but results in a nonfunctional complex in which the coupling between Ca^{+} influx and fusion is disrupted (33). Calcium has a crucial role in the process of neurotransmitter release inhibition as the increase of calcium concentration in the synaptic terminal partially counteracts the toxin effect (31). Histological analyses showed that an accumulation of synaptic vesicles occur in the cytosolic side of the plasma membrane as a direct consequence of the proteolytic activity

described above. The toxin does not affect Ach synthesis or storage nor the electrical conduction along the nerve fiber (25, 34).

This multi-step mechanism of action illustrates the toxin's effect on its most studied target, the neuromuscular junction. Here, botulinum toxin interferes with the signaling between α and γ motor neurons and extrafusal and intrafusal muscle fibers (35, 36). Besides botulinum toxin action on the striated muscle, it is nowadays known that the toxin also acts on the smooth muscle and on other cell types through the same mechanism.

2.2 Regeneration of the neuromuscular junction after paralysis

During BoNT intoxication in the striated muscle, inhibition of exocytosis occurs and the affected nerve terminals lose their functionality. Unlike what happens during denervation, the contact between nerve terminal and muscle fiber is maintained without loss of motor axons. This process is temporary and lasts only for a few months. Histological studies showed that the recovery process happens in two steps. Initially, a compensatory nerve sprouting occurs and an extensive network of new synapses develops along with an increased vesicle recycling rate. Later, when the exocytosis of the main terminal is recovered, the previously formed branches recess and functionality returns to normal (37). The recovery time varies according to the toxin serotype and local of action.

3. Therapeutic uses of botulinum toxin

Of the seven different strains of botulinum toxin only two, serotypes A and B, are commercially available. Serotype A is commercially available as Botox (Onabotulinum toxin A – Onabot/A, produced by Allergan Inc, Irvine, CA, USA), Dysport (Abobotulinum toxin A – Abobot/A, produced by Ipsen Ltd, Slough, Berks, UK) and Xeomin (Incobotulinum toxin A – Incobot/A from Merz Pharmaceuticals, Frankfurt, Germany). Other recent BoNT/A formulations were also created as Prosigne (Lanzhou Biological Poducts, Lanzhou, China) and PurTox (Mentor Corporation, Madison, WI, USA). Serotype B is available as Neurobloc/Myobloc (Solstice Neurosciences Inc, Malvern, PA, USA) (38). All therapeutic botulinum toxin preparations are composed by the actual toxin (light and heavy chains) plus non-toxic proteins added by the pharmaceutical company (38). Each BoNT formulation has its own characteristics because isolation, extraction and purification processes are different. Concerning BoNT/A there are two currently approved methods that can be used to estimate the potency of a specific BoNT/A brand. The first method is the mouse lethal dose (LD50), which corresponds to the mass of toxin that kills 50% of the mice. A more recently approach is a cell-based assay in which human neuroblastoma cells are used to measure the concentration of toxin required to cleave 50% of the SNAP-25. However, the conversion of clinical doses of different BoNT/A brands to be used in LUT disorders were never performed. Thus, even among preparations of the same serotype, doses are not comparable and not interchangeable (39, 40).

Onabotulinum toxin A is the most studied formulation so far and comparison studies showed that its activity is stronger and has a more prolonged effect than the others (41). It has become widely known mainly for cosmetic use but it is undoubtedly considered a valuable and evolving tool in the treatment of various neurological disorders. Therefore, BoNT/A is the main focus of the present thesis and its usefulness in urology will be discussed deeper along the following chapters.

The demonstration that the inhibition of neurotransmitter release at the striated muscle is followed by a functional recovery of the neuromuscular junction as well as the improved techniques of biochemical purification of BoNTs, provided a solid basis for the rapidly growing use of these toxins in the therapy of human diseases caused by excessive acetylcholine release.

Alan Scott used botulinum toxin type A for the first time in 1968 for the treatment of human pathologies reporting the therapeutic effects in strabismus (42, 43). Since then, botulinum toxin had been used in the management of several disorders where a reversible inhibition of a cholinergic terminal activity is desired. In 1989, the Food and Drug Administration (FDA) approved the use of BoNT/A for the treatment of blepharospasm, cervical dystonia and strabismus (44). Nowadays, toxin is approved for the treatment of a variety of movement disorders like spasticity, hypersecretory disorders such as hyperhidrosis (45), gastrointestinal disorders such as achalasia (46) and many cosmetic applications such as wrinkles or facial assymetrics. The use of the toxin in the lower urinary tract will be addressed in the following section.

4. Botulinum toxin type A for lower urinary tract disorders

4.1 Lower urinary tract physiology

The lower urinary tract (LUT) components comprise the urinary bladder, the urethra and the urethral sphincter. The bladder is a hollow and elastic organ whose wall is composed by bundles of smooth muscle, named detrusor muscle, separated by connective tissue that provides the organ great compliance. The urethra and the urethral sphincter are surrounded by striated muscle, which is in part under voluntary control of the somatic nervous system.

The coordination of all these components is a complex and highly distributed process called micturition which involves multiple pathways from spinal and supraspinal levels and the peripheral nervous system. Recent findings from functional brain imaging experiments suggested the midbrain periaqueductal gray (PAG) as the main coordination area that integrates somatic, autonomic and sensory information with a crucial role in bladder control and functioning (47). PAG coordination involves modulation of the pontine micturition center (PMC) of the brain stem and the three sets of peripheral neuronal circuits that include the sympathetic, the parasympathetic and the somatic nervous system (48).

The sympathetic innervation originates at the lower thoracic level and upper lumbar segments of the spinal cord (T10 to L2), and travels through preganglionic fibers until the inferior mesenteric ganglion and pelvic ganglia. Postganglionic fibers follow the hypogastric and pelvic nerves until the bladder. Parasympathetic neurons are located in the sacral parasympathetic nucleus of the spinal cord segments S2 to S4. Their axons pass along the pelvic nerve and synapse with postganglionic nerves of the parasympathetic ganglia located

within the bladder wall. Somatic innervation arises from the ventral horn of the sacral segments S2 to S4, an area called Onuf's nucleus, and through the pudendal nerve innervates the striated muscle of the external urethral sphincter (Figure 3). Finally, sensory afferent innervation can be found in the three previous described nerves and transmit information from the LUT to the lumbosacral spinal cord through the dorsal root ganglia (pudendal and pelvic nerves) and the hypogastric nerve.

The normal micturition reflex is mediated by a supraspinal pathway that passes through relay centers of the brain. These cortical areas are activated differently during the filling or storage phase and in the emptying phase in a coordinated fashion that resembles an on-off switch (47). During the storage phase afferent impulses convey information through the pelvic nerve to the central nervous system, specifically to the PAG, which relay information to higher brain regions. The PAG-PMC output is responsible for the activation of sympathetic innervation that keeps the detrusor muscle relaxed. This is achieved by inhibition of the parasympathetic pathways at spinal and ganglion levels thus allowing the accumulation of urine. In addition, somatic innervation provides excitatory innervation to the striated muscles of the urethral sphincter, preventing bladder emptying (49). Bladder filling occurs with a relatively low increase of pressure. Once the volume of urine reaches a critical level and luminal pressure is high, bladder sensory afferent nerve fibers and its mechanoreceptors convey the information of bladder fullness to supraspinal areas, generating conscious bladder sensations. The circuits of the PAG-PMC complex are responsible for switching the neuronal circuits in order to start the voiding phase. Thus, the micturition reflex is initiated by decreasing the sympathetic activity and increasing the parasympathetic one (50). Parasympathetic stimulation of the detrusor induces its contraction and simultaneously relaxes the urethral sphincter, initiating bladder emptying. During micturition, the activity of the somatic system on the urethral sphincter is temporarily inhibited allowing its relaxation (51).

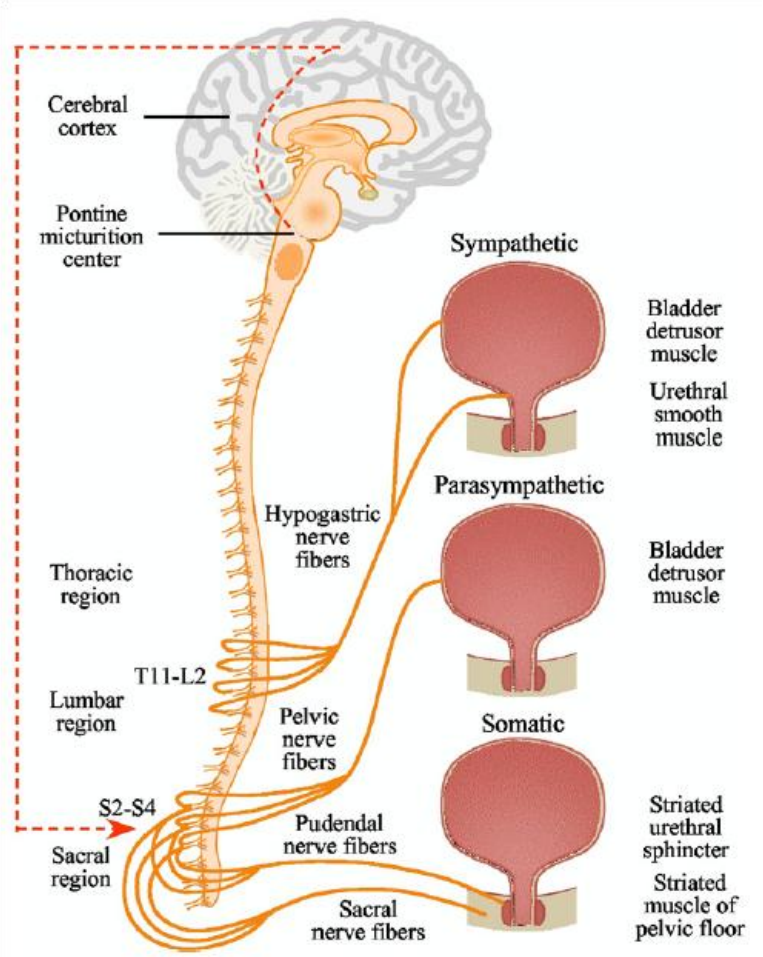

Figure 3: Schematic representation of LUT innervation. Urinary bladder is controlled by parasympathetic, sympathetic and sensory fibers which convey the information through pelvic, hypogastric and pudendal nerves, respectively. Adapted from Kanai *et al* (52).

Another bladder component that has a crucial role in the modulation of bladder activity is the urothelium. This structure is not only a passive barrier between the urine and the underlying tissues, it rather exhibits signaling properties that make it act as a sensory organ (53). Urothelial cells express nicotinic, muscarinic, adrenergic and vanilloid receptors that respond to mechanical and chemical stimuli. These cells also release ATP, prostaglandins, nitric oxide (NO), nerve growth factor (NGF) and brain-derived neurotrophic factor, substances that have excitatory and inhibitory actions on the afferent neurons. In addition, the ATP released from the urothelium can act on purinergic receptors expressed on cholinergic fibers modulating detrusor contractility.

Overall, urothelial cells exhibit specific properties that allow them to perceive chemical and mechanical changes in the environment and establishing a crosstalk between urothelium, the surrounding and intertwining nerve fibers and smooth muscle of the bladder wall.

Therefore, the normal micturition reflex is the result of a complex interaction between all LUT components in which any alteration could contribute to LUT dysfunctions (51, 54).

The development of therapeutic strategies for urinary bladder disorders is, nowadays, dependent on a better understanding of bladder physiology. Current treatments were designed aiming to restore normal bladder function. However, since the etiology of many pathologies is not completely clear, the majority of therapies simply improve LUT symptoms without any effect on the cryptogenic origin.

4.2 Applications of botulinum toxin A in the lower urinary tract

Onabotulinum toxin A has been used in the urology field since 1988, after its first injection into the external urinary sphincter, to manage detrusor sphincter dyssynergia in spinal cord injured (SCI) patients (55). A few years later, in 2000, Brigitte Schurch and co-workers described the first application into the detrusor muscle in SCI patients with successful results that demonstrated a significant increase in the mean maximum bladder capacity (56).

Since then, Onabot/A became a safe and effective tool for the treatment of lower urinary tract pathologies being used to treat urinary dysfunctions such as detrusor overactivity, overactive bladder, bladder pain syndrome/interstitial cystitis and benign prostate hyperplasia, among others, with very good outcomes even in children (57-59).

Its main urological target symptom is incontinence associated with urgency and frequency due to detrusor overactivity (60). Actually, the efficacy of Onabot/A treatment for LUT disorders is nowadays well documented and safety profiles are good. Very recent phase 3 clinical trials reported positive outcomes about Onabot/A treatment for OAB and NDO. These studies culminated on the recent FDA approval of 100U of Onabot/A for the management of idiopathic OAB and 200 or 300U for NDO. In OAB patients refractory to anticholinergics, Onabot/A significantly decreased the number of urinary incontinence episodes per day compared to placebo. Other OAB symptoms such as urgency and nocturia were also significantly reduced. Patients reported an improvement of their general quality of life in addition with positive responses on the treatment benefit scale (61, 62). Concerning NDO patients, Onabot/A benefits were observed by 2 weeks after its application, mainly concerning incontinence episodes. Other improvements were observed in the maximum cystometric capacity, maximum detrusor pressure during first involuntary detrusor contraction and in the incontinence quality of life score (63, 64). The most common side effects were urinary tract infection and urinary retention. The urinary tract infections were uncomplicated without upper

urinary tract involvement in 15.5% of patients and usually due to an increase in post-void residual volume (61-64). Urinary retention occurred in approximately 5-7% of OAB patients and 40% of NDO patients leading to the need of catheterization (61-64). Discontinuation rates are less than 2%, very low when compared to other treatments (65).

Other studies were also completed exploring its potential effect on other pathologies such as BPS/IC. The efficacy of Onabot/A was evaluated in refractory BPS/IC patients and the toxin also showed to be very effective. Pain, urgency and urodynamic parameters such as frequency, bladder volume to first pain and maximal cystometric capacity seemed to improve after toxin administration. The improvement of LUT symptoms lasted for 9 months in around 50% of patients who also reported a significant improvement in their quality of life (66).

Although many progresses have been done in the field, there is still a lack of standardized protocols for Onabot/A injection. The variety of existent studies are not consistent concerning the precise area of injection, the number of injections, the dilution volumes and even the ideal dose for different pathologies (67). In addition, some studies suggest intravesical instillation as a less invasive option for Onabot/A administration (68). Even though already widely used, this variety of protocols makes it difficult to conclude about the optimal approach for toxin administration urging the need of systematic studies.

4.3 Mechanisms of action of botulinum toxin A in the LUT

As described above, it is well documented that botulinum toxin acts on the cholinergic terminal of the neuromuscular junction, blocking the ACh release and consequently impairing the contraction of the striated muscle. On the other hand, it is nowadays clear that the toxin also acts on the smooth muscle of the urinary bladder, where a complex nerve plexus is present. The exact mechanism of BoNT/A on the modulation of this intricate bladder control is still a matter of debate and several questions need to be answered.

From a microscopic point of view, no relevant histological or ultrastructural changes were observed after treatment. After Onabot/A treatment, NDO and idiopathic OAB patients showed no TUNEL-positive cells, indicating no signs of apoptosis (69). Detrusor ultrastructure also remains intact without alterations on muscle cells structure, width of intercellular space and number or type of muscle cell junctions (70). Histological analysis showed no differences in the expression of myofibroblasts and no significant signs of inflammation or fibrosis before and after treatment of NDO and IDO patients (71, 72). Sprouting of smooth muscle terminal

axons is scarce showing a major difference when compared to the toxin effect on striated muscle (70).

In the detrusor, BoNT/A is known to decrease the release of ACh like in the striated muscle since it decreases the unstable contractions of the detrusor during IDO. Studies performed in animals showed that Onabot/A effect resulted in a selective paralysis of the low-grade contractions while still allowing the high-grade contractions that initiate micturition (73). Other studies also showed that BoNT/A could inhibit the evoked release of ACh and ATP from rat and guinea-pig bladder strips (74-76). A recent study corroborated this effect of the toxin on efferent fibers of the detrusor. The authors used spinal cord transected mice to show that Onabot/A elicits its suppressant effects on NDO by blocking neurotransmitters release from efferent nerve terminals. Interestingly, any effect was observed in terms of intrinsic contractile activity of the detrusor as well as in the intracellular Ca^{2+} transients responsible for these contractions (77).

The Onabot/A effect has been also previously described in sensory fibers (78). The biochemical mechanisms and locals of action are similar in these nerve fibers when compared to the ones observed in motor neurons. The difference is found in the pharmacological effect and it is due to the blockade of peripheral release of neuropeptides that act by sensitizing nerve terminals involved in pain perception. Bladder afferent nerve fibers express numerous receptors such as the vanniloid receptor TRPV1 (79) and the purinergic receptor P2X3 (80). P2X3 has a role in nociception, especially in inflamed tissue (81). TRPV1, a noxious stimulus integrator, has a specific role in pain perception namely thermal hiperalgesia and inflammatory pain (82). The expression of both receptors is increased in LUT disorders thus increasing bladder sensitivity (81, 82). Studies performed in human bladders showed that Onabot/A reduces the expression levels of both receptors in suburothelial nerve fibers (83). Actually, *in vitro* studies showed that BoNT/A blocks PKC-induced membrane expression of TRPV1 (84). Thus, it is expected that the decrease of both receptors induced by the toxin could suppress bladder sensory activity and prevent the activation of the micturition reflex during bladder filling.

The research about other mechanisms of action of BoNT/A has been increasing on animal models of disease especially around an antinociceptive effect of the toxin. For example, in a formalin-induced inflammatory pain model, Onabot/A was shown to reduce the formalin-evoked glutamate release from primary sensory neurons of the rat paw. In the same studies, formalin-induced Fos-like immunoreactivity in the dorsal horn, was also reduced by subcutaneous Onabot/A (85). Furthermore, in an animal model of neuropathic pain, Onabot/A induced a long-lasting antinociception induced by sciatic nerve injury (86).

Bladder sensory afferents also express neuropeptides like CGRP and SP, which are inflammatory mediators released in response to noxious stimulation. During OAB and BPS/IC, these neuropeptides are overexpressed suggesting that they have a role in the pathophysiology of these dysfunctions (87, 88). Pre-clinical studies showed that BoNT/A reduced pain responses and inhibited CGRP and SP release from bladder afferent terminals (89-91) suggesting an effect of the toxin in bladder pain. Furthermore, Onabot/A also modulates the urinary levels of neurotrophins that are usually increased in OAB and BPS/IC patients (92). Intramural injections of the toxin counteracted the increase of NGF levels associated with idiopathic and neurogenic DO as well as BPS/IC patients (66, 93). Thus the antinociceptive effect of BoNT/A has been also studied in the urology field due to its potential role in the decrease of hiperalgesia associated with LUT pathologies.

Finally, some authors suggested that BoNT/A has the capacity to affect non-neuronal cells like the urothelial ones, therefore modulating afferent nerve excitability. Indeed, BoNT/A was shown to reduce ATP and increase nitric oxide released from urothelial cells, counteracting the altered levels during conditions like chronic inflammation or spinal cord injury (94, 95). This fact might be extremely important for sensory transmission since ATP released from the urothelium seems to activate suburothelial purinergic receptors which consequently enhance bladder activity while NO release seems to decrease detrusor contractility (96). However, it is still not clear if the BoNT/A effect on these cells is direct or indirect since the presence of SV2 and SNAP-25 on the urothelium is controversial. SNAP-25 has been described in the urinary bladder after western-blot analysis of full-thickness human bladder specimens (59). So far, SNAP-25 expression in the urothelium has never been shown, although there is a recurrent mention to this finding without any image to support it (97).

Concerning SV2, a recent study described the expression of SV2 on a normal urothelial cell line (98). This aspect remains to be clarified. A possible but controversial explanation to the Onabot/A effect on the urothelium is the cleavage of SNAP-23. SNAP-23 is a homologue protein of SNAP-25 essential to the vesicle fusion machinery of non-neuronal cells (99). *In vitro* studies demonstrated that SNAP-23 is a non-neuronal target of botulinum toxin A and E in inner medullary collecting duct cells of the kidney (100). Actually, the presence of SNAP-23 was already shown in the three urothelial layers of the mouse bladder (101) but if the toxin has the ability to cleave the protein in these cells is a question that remains to be answered.

5. Botulinum toxin A in the central nervous system

Botulinum toxin, unlike tetanus toxin, is not considered to undergo retrograde transport or transcytosis across neurons, however, some studies seem to contradict that consensus. The first studies suggesting a possible central effect of BoNT/A were performed after injection of the striated muscle. A study using radiolabeled BoNT/A injected into the cat gastrocnemius muscle demonstrated the presence of radioactivity in spinal ventral roots and spinal cord segments innervating the injected muscle 48 hours after injection (102). However, these studies were never accepted as relevant since it was thought that the toxin molecule would reach the CNS without enzymatic activity. Only recently, more studies supported the central effect of the toxin (103). Studies from Matak *et al* reported retrograde transport and transcytosis of the toxin into the rat brain facial motor neuron nucleus after administration into the whisker muscles. This study showed for the first time that BoNT/A peripheral administration could access the central nervous system (104). More recently, other authors showed that after BoNT/A injection into the brain tectum, cleaved SNAP-25 was found at least two synapses away from the injection site, in rod bipolar cells and photoreceptors (105). Other studies that injected BoNT/A in more distant sites, like the sciatic nerve, also observed cleaved SNAP-25 immunoreactivity in the ipsilateral dorsal and ventral horns of lumbar segments. The same study showed that this transport was microtubule-dependent since it was blocked by colchicine (106).

Concerning LUT disorders, it should be kept in mind that the afferent signals generated by the bladder have to be processed in the CNS. Therefore, the antinociception and the general sensory impairment caused by peripheral or central administration of Onabot/A should include the impairment of neurotransmitters released from the central sensory endings, something that has never been addressed. Thus, studies regarding this aspect on LUT disorders are crucial and challenging.

Goals

The lack of information regarding the targets of botulinum toxin A in the urinary bladder was the general base of the present thesis. Thus, aiming to obtain new insights about the mechanism of action of Onabot/A in the urinary bladder, the goals of the current work were the following:

- Perform a detailed description of the distribution of the high affinity binding site and intracellular target of botulinum toxin A on the human and guinea pig bladder

- Analyze the neurochemical content of the nerve fibers affected by Onabot/A in guinea pig bladders treated with the toxin

- Evaluate the effect of Onabot/A in the parasympathetic intramural ganglia

- Evaluate the diffusion pattern of Onabot/A through the urinary bladder

- Compare the efficacy of the most common routes of Onabot/A administration

- Investigate the effect of intrathecal Onabot/A in an animal model of bladder pain

Publications

Publication I

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Distribution of the High-Affinity Binding Site and Intracellular Target of Botulinum Toxin Type A in the Human Bladder

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Abstract

Background: Botulinum toxin type A (BoNTA) has been successfully used in the treatment of refractory detrusor overactivity. The toxin is internalized after binding a high-affinity receptor, synaptic vesicle protein 2 (SV2), which is exposed in the cell membrane during the exocytosis process. In the cytoplasm, BoNTA cleaves specific sites of synaptosomal-associated protein 25 (SNAP-25), preventing the assembly of the synaptic fusion complex SNARE and blocking exocytosis.

Objective: In the present work, the distribution of SV2 and SNAP-25 was first investigated in human bladders. The neurochemistry of BoNTA-sensitive structures was then investigated using markers for parasympathetic, sympathetic, and sensory fibers.

Design, setting, and participants: Human bladders were obtained from cadaveric organ donors (age range: 19–74 yr).

Measurements: Bladder sections were processed for single or dual immunofluorescence staining with antibodies against SV2, SNAP-25, β-3 tubulin, vesicular acetylcholine transporter, tyrosine hydroxilase, and calcitonin gene-related peptide.

Results and limitations: SV2 and SNAP-25 immunoreactive fibers were distributed throughout the suburothelium and muscular layer. Double labeling showed extensive colocalization of both proteins in nerve fibers. SV2 is more expressed in parasympathetic fibers than in sympathetic or sensory fibers. No expression was found in urothelial or muscular cells. Because only normal bladders were used, this distribution should be applied with caution to pathologic bladders.

Conclusions: SV2 and SNAP-25 colocalize abundantly throughout the urinary bladder. SV2 is more abundant in cholinergic, parasympathetic fibers. These nerves are suggested to be the main target for BoNTA action in the human urinary bladder.

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1. Introduction

The off-label application of botulinum toxin type A (BoNTA) in the treatment of lower urinary tract pathologies has increased noticeably in the last 20 yr. From its initial application to treat bladder-sphincter dyssynergia, BoNTA injection in the urinary bladder is now the first option for the treatment of refractory detrusor overactivity (DO) in many centers [1–5].

The clinical use of the neurotoxin is based on its ability to block neurotransmitter exocytosis. Once internalized by nerve terminals, BoNTA undergoes a pH-dependent conformational change that causes the dissociation of its heavy and light chains. After translocation to the cytosol, the light chain enzymatically cleaves specific sites of synaptosome-associated protein 25 (SNAP-25), preventing the SNARE-mediated fusion of synaptic vesicles with the neuronal membrane and, thus, blocking neurotransmitter release [6,7].

The current model to explain preferential internalization of BoNTA by particular cell types relies on the presence of high-affinity binding sites for the toxin, namely, synaptic vesicle protein 2 (SV2), which is a membrane protein localized in synaptic and endocrine cell secretory vesicles [8]. During exocytosis, SV2 becomes exposed in the cell surface, allowing neurotoxin binding and internalization when vesicle recycling occurs [8].

The overwhelming majority of the studies concerning the mechanism of action of BoNTA were performed in striated muscle [9,10]. Thus, despite the increasing use of BoNTA for treatment of refractory DO, the distribution of SV2 and SNAP-25 was never systematically studied in the human urinary bladder. In the present study, we performed a detailed mapping of SV2 and SNAP-25 expression in the normal human urinary bladder and investigated the nature of the positive structures.

2. Materials and methods

2.1. Tissue samples

After receiving permission from of the ethics committee of Hospital S. João, 10 human bladders were collected from male cadaver organ donors aged 16, 19, 30, 38, 57 (two), 58 (two), 65, and 74 yr. Blood supply to the bladder was interrupted during the harvest procedure, between 45 and 60 min before bladder removal.

Bladders were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight at 4 °C, washed in PB, and cryoprotected for 24 h in 30% sucrose in PB. Longitudinal 20-μm thick sections from bladder wall and trigone were obtained in a Microm cryostat, slide mounted in Superfrost Plus glass slides, and stored at −80 °C for further processing.

2.2. Antibodies

The mouse anti-SV2 antibody [11] was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. Rabbit anti-SNAP-25, sheep anti–calcitonin gene-related peptide (CGRP), and mouse anti-β3-tubulin were obtained from Abcam plc (Cambridge, UK). Rabbit anti–tyrosine hydroxilase (TH) was obtained from Chemicon (Millipore [U.K.] Ltd, Watford, UK). Rabbit anti–vesicular acetylcholine transporter (VAChT) was obtained from Sigma (Sigma-Aldrich Quimica, S.A., Sintra, Portugal). The biotinylated donkey anti-mouse was obtained from Jackson ImmunoResearch (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). The biotinylated swine anti-rabbit was obtained from Dakopatts (Dako Denmark A/S, Glostrup, Denmark). Fluorochrome-labeled secondary antibodies were obtained from Molecular Probes Europe (Leiden, The Netherlands).

2.3. Immunolabeling with chromogenic substrate

After thawing, representative sections of each bladder were washed in phosphate-buffered saline (PBS). After inhibition of endogenous peroxidase activity (0.3% hydrogen peroxide, 30 min), the sections were blocked with PBS containing 0.3% Triton X-100 (PBST) with 10% normal horse serum (NHS) for 1 h, and then incubated in antibodies anti-SV2 (1:2000) or anti-SNAP-25 (1:1000) for 48 h at 4 °C. The avidin-biotin complex (ABC) horseradish peroxidase (HRP)/diaminobenzidine (DAB) reaction was performed, as previously described, using biotinylated species-specific secondary antibodies [12]. Control staining was performed by omitting the primary antibodies, followed by incubation with secondary antibodies. Representative images were collected in a Zeiss Axioscope 40 microscope using the AxioVision 4.6 software.

2.4. Double immunofluorescence labeling

After thawing, sections were permeabilized in 50% ethanol for 30 min, washed in PBS, and blocked with PBST containing 10% NHS for 1 h. Incubation with primary antibodies was performed for 48 h at 4 °C. Anti-VAChT, anti-TH, and anti-CGRP were diluted 1:4000; anti-SV2 and anti-β3-Tubulin were diluted 1:2000; and anti-SNAP-25 was diluted 1:1000 in PBST containing 2% of NHS. After several washes, the immunoreactions were visualized according to species specificity by Alexa Fluor 488-conjugated donkey anti-rabbit, Alexa Fluor 568-conjugated donkey anti-mouse, and Alexa Fluor 568-conjugated donkey anti-sheep diluted 1:1000. Controls were performed by omitting primary antibodies. Representative images were collected in a Zeiss Axioscope 40 microscope using the AxioVision 4.6 software with the appropriate filter sets.

2.5. Counting and statistical analysis

Five representative images taken from full-thickness sections of the bladder trigone were analyzed per sample. After splitting the red and green channels, immunoreactive (IR) fibers were counted by superimposing a pure color spot on each. The number of spots was then counted using the Image J software (U.S. National Institutes of Health). Statistical analysis was performed using the χ^2 test. A p value of <0.05 was considered statistically significant.

3. Results

A dense SV2 and SNAP-25 network was detected both in the suburothelium and in the muscular layer after the ABC HRP/DAB reaction (Fig. 1). In the suburothelium, IR structures formed bundles scattered in the connective tissue and became progressively thinner as they approached the bladder lumen. Some were varicose and penetrated the first layers of urothelial cells (Fig. 1A and C). In the muscular layer, the positive structures were parallel to the detrusor smooth muscle fibers (Fig. 1B and D). These structures had a morphology similar to nerve fibers and were also positive to

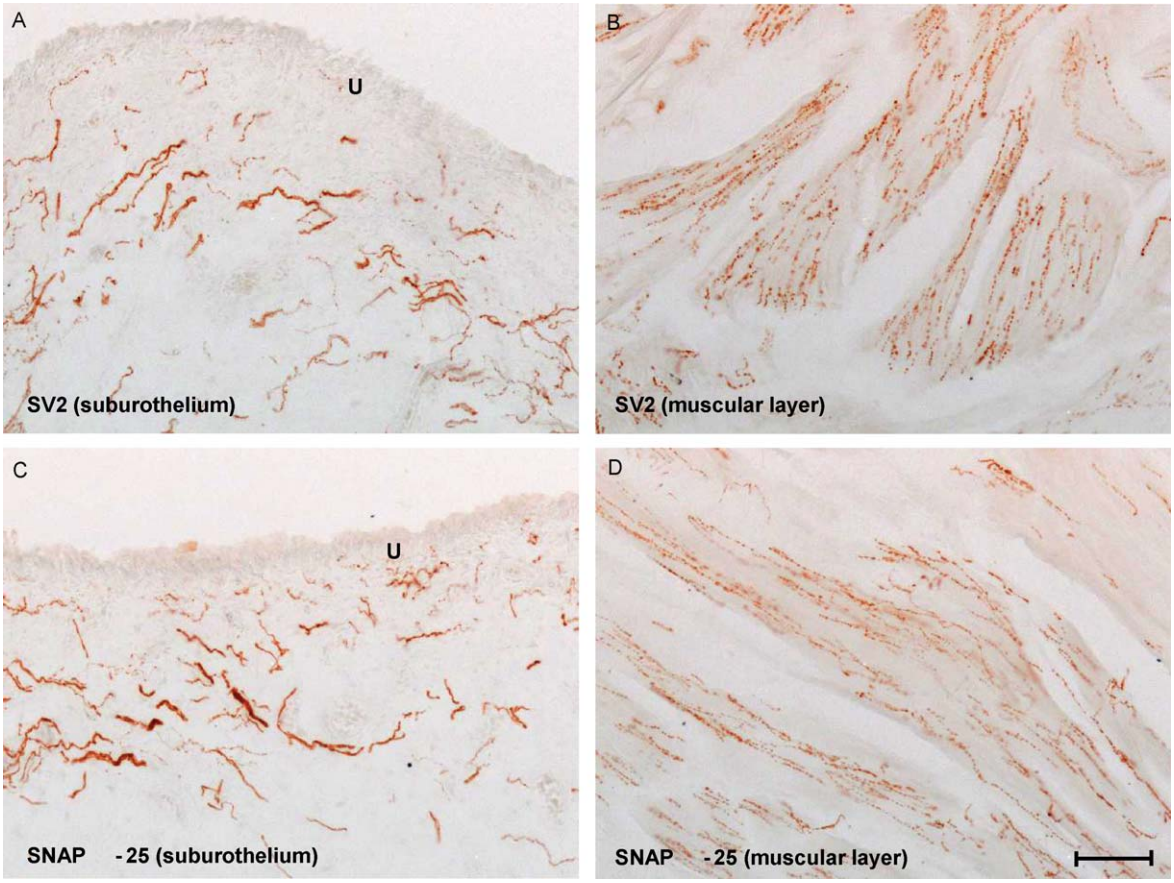

Fig. 1 – Expression of synaptic vesicle protein 2 (SV2) and synaptosomal-associated protein 25 (SNAP-25) in the suburothelium and muscular layer of urinary bladder trigone using the avidin-biotin complex (ABC) method. The expression of both proteins is very similar. Immunoreactive fibers in the suburothelium are present in the lamina propria. Some fibers can be seen penetrating the urothelium (U). In the muscular layer, they are parallel to the detrusor smooth muscle. Magnification bar: 50 μm for all images.

the pan-neuronal marker β-3 tubulin, attesting their neuronal nature (Fig. 2A). No labeling was found in urothelial or muscular cells (Fig. 1).

Double immunostaining of SV2 and SNAP-25 revealed abundant colocalization of both proteins throughout the bladder (Fig. 2B and C).

The distribution of VAChT-, TH-, and CGRP-IR fibers is shown in Figs. 3A–C and 4A–C. A dense VAChT-IR plexus of nerve fibers and varicosities was found in the suburothelium (Fig. 3A). In addition, an extensive network of VAChT-IR fibers coursed the entire thickness of the detrusor smooth muscle layer (Fig. 4A–C). TH-IR fibers were more abundant around blood vessels and were rare around smooth muscle bundles (Fig. 3B). CGRP-IR nerve fibers were sparse in the suburothelial layer (Fig. 3C) and were rarely found in other bladder areas, including the detrusor.

VAChT-, TH-, and CGRP-IR fibers and SV2-IR fibers colocalized at different degrees. Ninety-five percent of VAChT-IR fibers were also SV2-IR (Fig. 3D). This percentage was inferior in TH- and CGRP-IR fibers (69% and 58%, respectively; Fig. 3D).

The percentage of VAChT- and SV2-IR fibers did not show any major difference, regardless of whether the samples analyzed came from younger or older subjects; however, the small number of subjects in each age range precluded statistical analysis.

4. Discussion

SV2 is the high-affinity BoNTA receptor that mediates the access of the neurotoxin into neurons [8], and SNAP-25 is the SNARE-complex component that is specifically cleaved by the toxin [6,8]. To the best of our knowledge, our results are the first to show a rich SV2- and SNAP-25–IR network in the human bladder. The neuronal nature of the positive structures was confirmed by the one-to-one colocalization between SNAP-25 and the pan-neuronal marker β-3 tubulin. SV2 and SNAP-25 showed extensive colocalization, demonstrating that nerve fibers that have the capacity to internalize the neurotoxin also express the BoNTA intracellular target.

The present study also demonstrated that SV2 immunoreactivity occurs in combination with VAChT, TH, and CGRP labeling. VAChT is present in cholinergic fibers that are responsible for the transport of acetylcholine from its place of synthesis into synaptic vesicles, where is stored and from which is released. TH is the first enzyme in catecholamine biosynthesis and catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA).

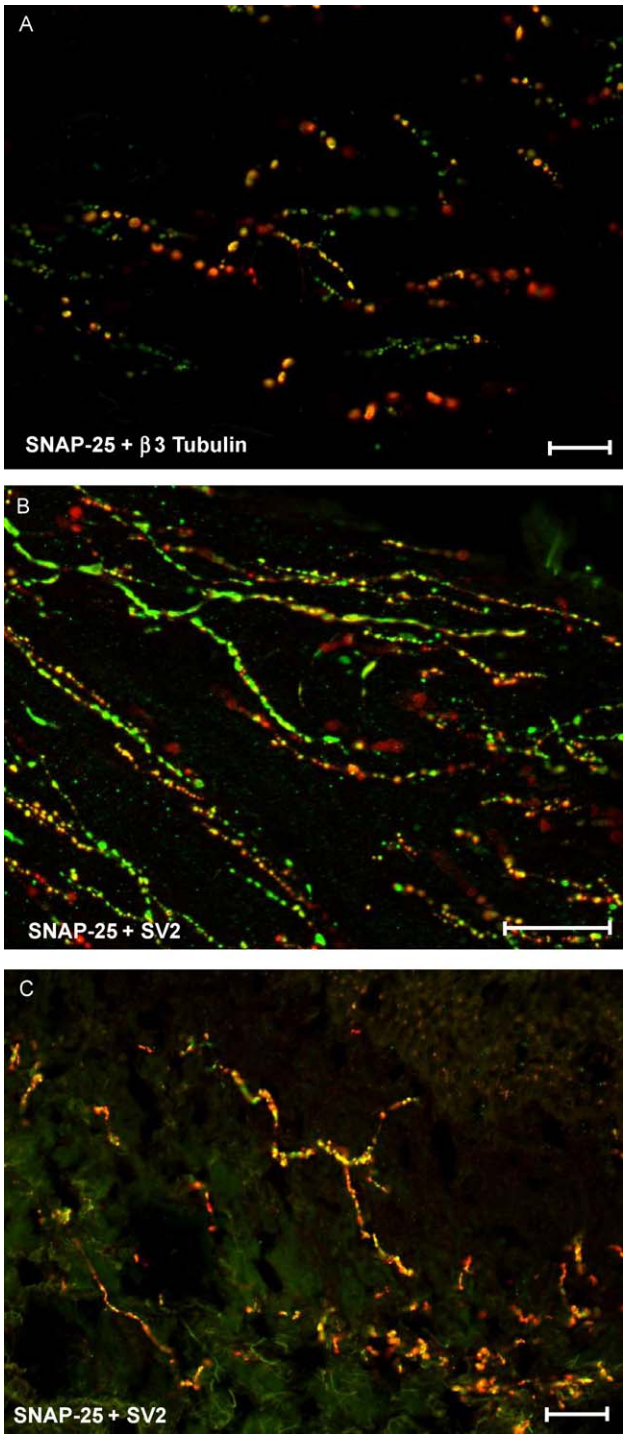

Fig. 2 – (A) Double staining for synaptosomal-associated protein 25 (SNAP-25; green) and β-3 tubulin (red) shows abundant colocalization (yellow-orange). (B,C) Double staining for SNAP-25 (green) and synaptic vesicle protein 2 (SV2; red). Numerous varicose fibers appear throughout the detrusor muscle bundles with a high degree of colocalization between them both (B) in the suburothelium and (C) in the muscular layer. Magnification bars: 20 μm.

CGRP is a neuropeptide present in small-diameter sensory fibers. Therefore, colocalization of SV2 with VAChT, TH, and CGRP labeling indicates that the high-affinity protein necessary for the internalization of BoNTA [8] is present in parasympathetic, sympathetic, and primary afferent fibers. Similar colocalization studies were impossible with SNAP-25, due to the similar species in which SNAP-25 and the other antibodies were raised. Taking into consideration that SNAP-25 and SV2 had a one-to-one colocalization, it is highly probable that SNAP-25 occurs in VAChT-, TH-, and CGRP-positive structures.

The distribution of VAChT-IR and TH-IR fibers provided in this study is similar to that described in previous papers [13,14]. In fact, a VAChT-IR plexus had been described in the detrusor and in the suburothelial layer up to the urothelial base [13], and TH fibers had been found mostly in the bladder neck [14]. The role of the VAChT plexus, in the absence of an obvious target, is unclear at the moment but may be related to the acetylcholine-induced sensitization of primary afferents previously described in other visceral organs [15]. Alternatively, other authors described suburothelial myofibroblasts in the lamina propria of the bladder, and these could constitute a possible target for these efferent fibers [16,17].

CGRP-IR fibers were surprisingly scarce in the human suburothelium. This finding contrasts with descriptions available from rodent bladders, so frequently used in preclinical experiments, in which the CGRP-positive plexus is extremely dense and occurs not only in the suburothelium but also in the muscular layer [12]. Other studies that investigated the distribution of neuropeptide containing sensory fibers in the adult human bladder [18] used small biopsy specimens, precluding a thorough analysis of fiber density.

The expression of SV2 in these three nerve subpopulations was surprisingly distinct. SV2 expression was significantly more abundant in parasympathetic fibers. This finding may be relevant because it may indicate that parasympathetic fibers are more susceptible to internalizing the neurotoxin and thus are more prone to suffer from its action. The higher colocalization of SV2 in parasympathetic fibers can be related to nerve fiber activity needed to trigger repeated contractions that are necessary to expel urine. The relationship between nerve activity and susceptibility to BoNTA poisoning has been shown in the rat phrenic nerve [19] and in the human skeletal muscle [20]. The frequent acetylcholine release would increase SV2 exposure in parasympathetic nerve terminals, increasing BoNTA binding.

Surprisingly, no SV2 immunoreaction was found in the urothelium, making difficult to predict how BoNTA can be internalized by urothelial cells. It could be possible that BoNTA penetrates those cells through low-affinity binding sites [21], but this would not explain how BoNTA could act. In fact, SNAP-25, the intracellular target, also was not detected in the human urothelium. Some studies support the effect of BoNTA on urothelial cells because its administration decreases adenosine triphosphate (ATP) release in the bladder [7,22]. However, this might constitute an indirect outcome of the neurotoxin treatment rather than a direct effect on urothelial cells. One characteristic of BoNTA administration to human bladders is a decrease in maximal detrusor pressure [2,23]. Interestingly, ATP release

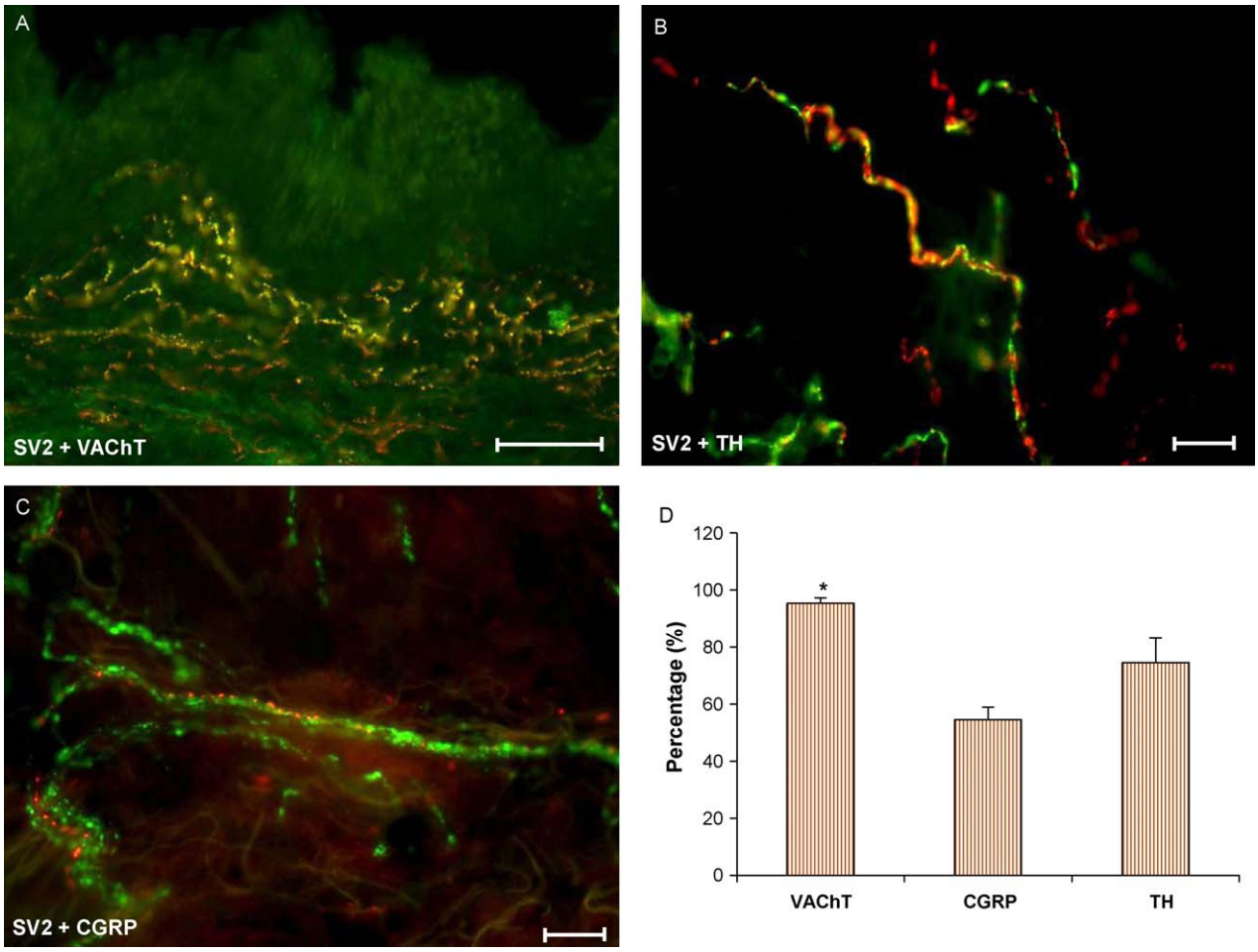

Fig. 3 – Presence of synaptic vesicle protein 2 (SV2) in (A) parasympathetic, (B) sympathetic, and (C) sensory fibers in the suburothelium of the urinary bladder trigone. SV2 is shown in red; vesicular acetylcholine transporter (VAChT), tyrosine hydroxilase (TH), and calcitonin gene-related peptide (CGRP) are shown in green. The colocalization between them is represented by yellow-orange. (D) The percentage of parasympathetic fibers (VAChT) that express SV2 is 95%, higher than the percentage of sympathetic (TH) and sensory (CGRP) fibers, at 69% and 58%, respectively. Comparison of percentages (double-labeled fibers/single labeled fibers) showed statistical significance. Quantitative data were expressed as mean plus or minus standard error of the mean. $p < 0.05$. Magnification bars: 20 μm.

from rodent urothelial cells was shown to be directly related to increasing intravesical pressure [24]. Thus, it is possible that the reduction in maximal detrusor pressure caused by BoNTA might be the reason for decreased ATP release.

Our data suggest that only half of bladder sensory fibers are susceptible to BoNTA. This finding suggests that the contribution of sensory-fiber impairment for the final effect of BoNTA in DO is less important than parasympathetic-fiber impairment. Nevertheless, this finding does not exclude an effect of BoNTA on sensory function. In fact, intravesical application of BoNTA is known to reduce noxious bladder input [7,25], although in some reports, the effect may be rather transient [3]. BoNTA inhibits CGRP, substance P, and glutamate release from afferent nerve endings [22,26]. In addition, BoNTA decreases overexpression of TRPV1 and P2X3 in the human bladder of patients with DO [1], most probably by reducing the SNARE-dependent receptor trafficking [27].

BoNTA should also have targeted sympathetic fibers, taking into consideration the high degree of colocalization of TH and SV2. Sympathetic nerve impairment by BoNTA is accompanied by a decrease in norepinephrine release [7]. The consequences of BoNTA action in these fibers, however, should be limited. In the bladder dome and body, sympathetic fibers induce bladder relaxation. Thus, sympathetic bladder nerve impairment should increase detrusor tonus, an effect not observed after BONTA administration [23].

When considering the limitations of our study, we cannot ignore that bladders collected from cadaveric organ donors were presumed to be normal after the lower urinary tract assessment carried out during the process of donor selection. It is possible, however, that some, particularly the elderly donors, could have had DO, taking into consideration the higher prevalence of this urodynamic abnormality in the general population. Furthermore, it is possible that in pathologic bladders, the nerve distribution may show profound differences from the findings described in this paper. Significant reduction of detrusor innervation occurs in the obstructed human bladder [28]. Women with DO were shown to have denser neuropeptide-positive sensory

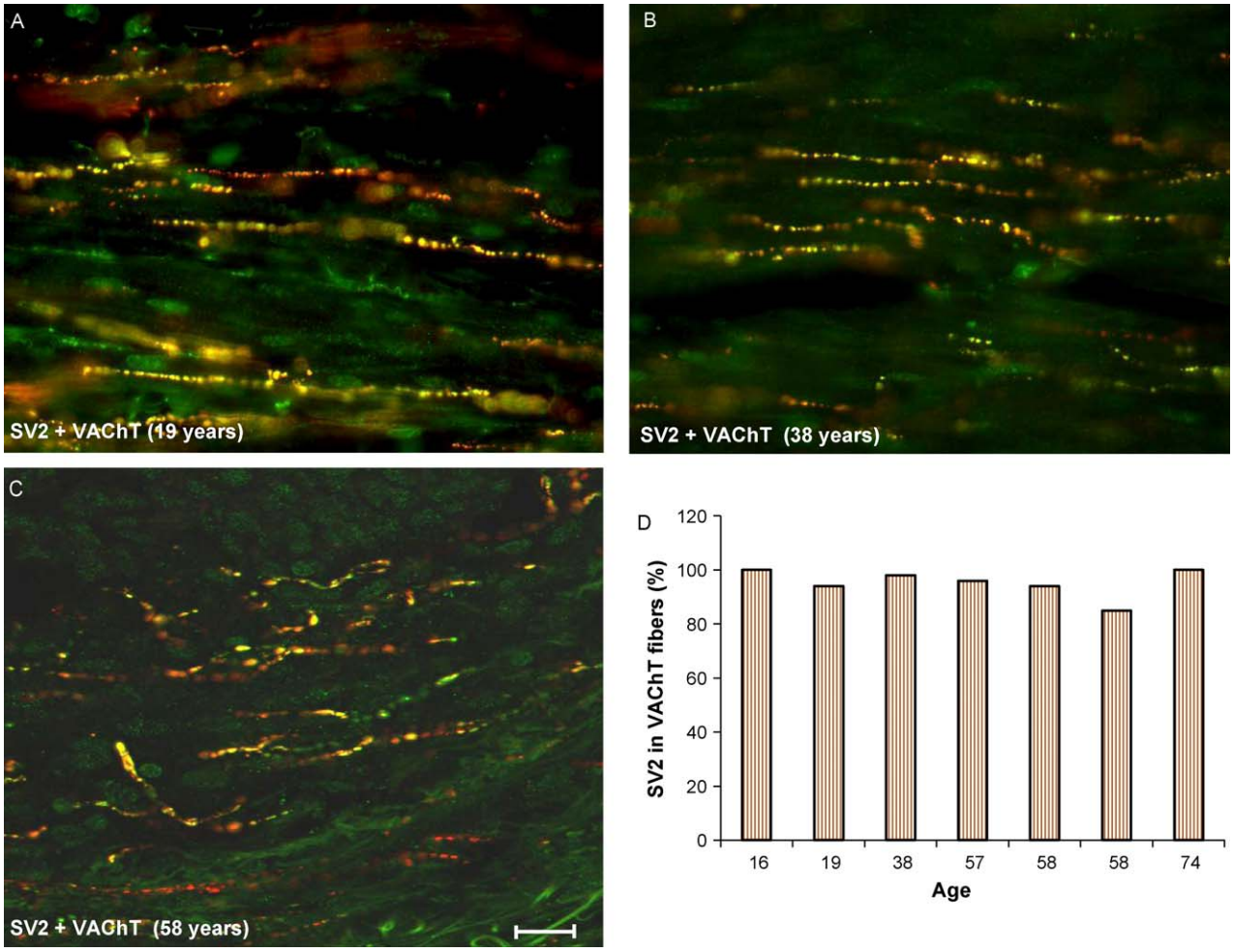

Fig. 4 – (A–C) Double immunostaining for synaptic vesicle protein 2 (SV2) and vesicular acetylcholine transporter (VAChT) in the muscular layer of bladder's trigone taken from donors with different ages. (D) Although no statistical analysis was performed, no major differences were observed in the number of fibers or in the percentage of colocalization. SV2 is shown in red, VAChT in green. The colocalization between them is shown in yellow-orange. Magnification bars: 20 μm in all images.

fibers in the suburothelium [18], and TRPV1- and P2X3-expressing sensory fibers are more numerous in patients with neurogenic DO [29]. Whether DO exposes more SV2 and facilitates BoNTA binding to parasympathetic fibers is currently unknown [8,30].

5. Conclusions

SV2 and SNAP-25 immunoreaction is observed in all nerve-fiber subtypes innervating the human bladder. SV2, the high-affinity binding site of BoNTA, is expressed in almost all parasympathetic fibers but in only half of the sensory fibers innervating the human bladder. Together with the lack of SV2 and SNAP-25 in urothelial cells, our results suggest that the main mechanism for BoNTA action injected in the bladder wall is through the impairment of parasympathetic fibers.

Author contributions: António Avelino had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Coelho, Cruz, Avelino.
Acquisition of data: Coelho, Dinis, Pinto, Gorgal, C. Silva, A. Silva, J. Silva, Cruz.
Analysis and interpretation of data: Coelho, Cruz, Avelino.
Drafting of the manuscript: Coelho, Cruz, Avelino.
Critical revision of the manuscript for important intellectual content: Cruz, Avelino.
Statistical analysis: Coelho.
Obtaining funding: Avelino.
Administrative, technical, or material support: Avelino.
Supervision: Avelino.
Other (specify)*:* None.

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References

[1] Apostolidis A, Dasgupta P, Denys P, et al. Recommendations on the use of botulinum toxin in the treatment of lower urinary tract disorders and pelvic floor dysfunctions: a European consensus report. Eur Urol 2009;55:100–20.

[2] Karsenty G, Denys P, Amarenco G, et al. Botulinum toxin A (Botox®) intradetrusor injections in adults with neurogenic detrusor overactivity/neurogenic overactive bladder: a systematic literature review. Eur Urol 2008;53:275–87.

[3] Silva CM, Cruz F. Has botulinum toxin therapy come of age: what do we know, what do we need to know, and should we use it? Curr Opin Urol 2009;19:347–52.

[4] Gamé X, Mouracade P, Chartier-Kastler E, et al. Botulinum toxin-A (Botox) intradetrusor injections in children with neurogenic detrusor overactivity/neurogenic overactive bladder: a systematic literature review. J Pediatr Urol 2009;5:156–64.

[5] Giannantoni A, Mearini E, Del Zingaro M, Porena M. Six-year follow-up of botulinum toxin A intradetrusorial injections in patients with refractory neurogenic detrusor overactivity: clinical and urodynamic results. Eur Urol 2009;55:705–12.

[6] Simpson LL. The action of botulinal toxin. Rev Infect Dis 1979;1:656–62.

[7] Chancellor MB, Fowler CJ, Apostolidis A, et al. Drug insight: biological effects of botulinum toxin A in the lower urinary tract. Nat Clin Pract Urol 2008;5:319–28.

[8] Dong M, Yeh F, Tepp WH, et al. SV2 is the protein receptor for botulinum neurotoxin A. Science 2006;312:592–6.

[9] Shen J, Ma J, Lee C, Smith TL, Tan KH, Koman LA. How muscles recover from paresis and atrophy after intramuscular injection of botulinum toxin A: study in juvenile rats. J Orthop Res 2006;24:1128–35.

[10] Jurasinski CV, Lieth E, Dang Do AN, Schengrund CL. Correlation of cleavage of SNAP-25 with muscle function in a rat model of botulinum neurotoxin type A induced paralysis. Toxicon 2001;39:1309–15.

[11] Bucley K, Kelly RB. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J Cell Biol 1985;100:1284–94.

[12] Avelino A, Cruz C, Nagy I, Cruz F. Vanilloid receptor 1 expression in the rat urinary tract. Neuroscience 2002;109:787–98.

[13] Dixon JS, Jen PY, Gosling JA. The distribution of vesicular acetylcholine transporter in the human male genitourinary organs and its co-localization with neuropeptide Y and nitric oxide synthase. Neurourol Urodyn 2000;19:185–94.

[14] Gosling JA, Dixon JS, Jen PYP. The distribution of noradrenergic nerves in the human lower urinary tract. A review. Eur Urol 1999;36(Suppl 1):23–30.

[15] Holzer P. Role of visceral afferent neurons in mucosal inflammation and defense. Curr Opin Pharmacol 2007;7:563–9.

[16] Wiseman OJ, Fowler CJ, Landon DN. The role of the human bladder lamina propria myofibroblast. BJU Int 2003;91:89–93.

[17] Roosen A, Datta SN, Chowdhury RA, et al. Suburothelial myofibroblasts in the human overactive bladder and the effect of botulinum neurotoxin type A treatment. Eur Urol 2009;55:1440–9.

[18] Smet PJ, Moore KH, Jonavicius J. Distribution and co-localization of calcitonin gene-related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder. Lab Invest 1997;77:37–49.

[19] Hughes B, Whaler BC. Influence of nerve-ending activity and of drugs on the rate of paralysis of rat diaphragm preparations by Cl. botulinum type A toxin. J Physiol 1962;160:221–33.

[20] Eleopra R, Tugnoli V, De Grandis D. The variability in the clinical effect induced by botulinum toxin type A: the role of muscle activity in humans. Mov Disord 1997;12:89–94.

[21] Rummel A, Mahrold S, Bigalke H, Binz T. The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. Mol Microbiol 2004;51:631–43.

[22] Apostolidis A, Dasgupta P, Fowler CJ. Proposed mechanism for the efficacy of injected botulinum toxin in the treatment of human detrusor overactivity. Eur Urol 2006;49:644–50.

[23] Reitz A, Stöhrer M, Kramer G, et al. European experience of 200 cases treated with botulinum-A toxin injections into the detrusor muscle for urinary incontinence due to neurogenic detrusor overactivity. Eur Urol 2004;45:510–5.

[24] Vlaskovska M, Kasakov L, Rong W, et al. P2X3 knock-out mice reveal a major sensory role for urothelially released ATP. J Neurosci 2001;21:5670–7.

[25] Vemulakonda VM, Somogyi GT, Kiss S, Salas NA, Boone TB, Smith CP. Inhibitory effect of intravesically applied botulinum toxin A in chronic bladder inflammation. J Urol 2005;173:621–4.

[26] Rapp DE, Turk KW, Bales GT, Cook SP. Botulinum toxin type a inhibits calcitonin gene-related peptide release from isolated rat bladder. J Urol 2006;175:1138–42.

[27] Morenilla-Palao C, Planells-Cases R, García-Sanz N, Ferrer-Montiel A. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. J Biol Chem 2004;279:25665–72.

[28] Gosling JA. Modification of bladder structure in response to outflow obstruction and ageing. Eur Urol 1997;32(Suppl 1):9–14.

[29] Brady CM, Apostolidis AN, Harper M, et al. Parallel changes in bladder suburothelial vanilloid receptor TRPV1 and pan-neuronal marker PGP9.5 immunoreactivity in patients with neurogenic detrusor overactivity after intravesical resiniferatoxin treatment. BJU Int 2004;93:770–6.

[30] Smith CP, Franks ME, McNeil BK, et al. Effect of botulinum toxin type A on the autonomic nervous system of the rat lower urinary tract. J Urol 2003;169:1896–900.

Publication II

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Editorial by Prokar Dasgupta on pp. 1185–1186 of this issue

Spread of OnabotulinumtoxinA After Bladder Injection. Experimental Study Using the Distribution of Cleaved SNAP-25 as the Marker of the Toxin Action

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Abstract

Background: OnabotulinumtoxinA (Onabot/A) has been used to treat detrusor overactivity disorders. The treatment is based on several injections of toxin throughout the bladder wall. However, injection protocols are not well established among clinicians, varying in dose and dilution.
Objective: Study the distribution and neurochemistry of cleaved synaptosome-associated protein of 25 kDa (cSNAP-25) after Onabot/A administration in the guinea pig bladder. In addition, we analyzed which factor, dose or volume, contributes more to the diffusion of the toxin.
Design, setting, and participants: Guinea pig bladders were treated with Onabot/A via intramural injection or an instillation.
Measurements: Bladder cryostat sections were processed for single or dual immunohistochemistry staining with antibodies against cSNAP-25, vesicular acetylcholine transporter, tyrosine hydroxylase, and calcitonin gene-related peptide. Different administration methods and doses were analyzed. Statistical analysis was performed using the chi-square test for colocalization studies after multiple injections and the *t* test for the evaluation of affected fibers after a single injection.
Results and limitations: cSNAP-25 immunoreactive fibers were abundant throughout the bladder tissue in the mucosa and muscular layer. Double labeling showed that parasympathetic fibers are more affected than sympathetic or sensory. A single Onabot/A injection is more effective if diluted in a higher volume. Onabot/A instillation in the bladder does not cleave SNAP-25 protein.
Conclusions: A single Onabot/A injection spreads the neurotoxin activity to the opposite side of the guinea pig bladder. This action is more evident when high saline volumes are used to dissolve Onabot/A. The toxin cleaves the SNAP-25 protein mainly in cholinergic but also in adrenergic and sensory fibers. In contrast with intramural injection, instillation of Onabot/A does not cleave SNAP-25 in nerve fibers.

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1. Introduction

OnabotulinumtoxinA (Onabot/A) was recently approved for the treatment of urinary incontinence in patients with neurogenic detrusor overactivity refractory to antimuscarinic therapy [1]. The recommended dose of Onabot/A, 200 U, is injected in 30 different points in the bladder wall above the trigone, each one receiving 1 ml of normal saline containing 6.7 U of toxin [2]. In clinical practice, however, other injection protocols have been used. Saline volumes vary between 0.1

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and 0.5 ml per injection site [1,3]. Ten injection sites were shown to be as effective as 30 [4]. In addition, intravesical instillation has been suggested as an alternative to bladder wall injection [5,6], although the capacity of Onabot/A to cross the intact urothelium was never clearly demonstrated. The consequences of toxin dilution, injection volume in each site, or instillation versus injection to the final effect of Onabot/A were never systematically investigated. This contrasts with the use of botulinum toxin to treat skeletal muscle disorders where different volumes were investigated, showing that larger injections resulted in increased muscle relaxation [7,8].

Onabot/A cannot be easily traced in the tissues after injection. To circumvent this problem, magnetic resonance imaging (MRI) has been used to demonstrate the presence of the injection fluid in the bladder wall [9]. The presence of the cleaved form of synaptosome-associated protein of 25 kDa (cSNAP-25), the target protein of the toxin, can be used as a reliable marker of the neurotoxin diffusion and local action. Its persistence in the bladder has been suggested as evidence of botulinum toxin action [10]. Interestingly, the presence of cSNAP-25 can be easily traced in tissues with an appropriate antibody [11,12].

In the present work, we characterize the diffusion pattern of Onabot/A in the guinea pig bladder using the expression of cSNAP-25 following different injection volumes and administration protocols. In addition, we also investigated the time course and the neurochemistry of fibers expressing cSNAP-25.

2. Material and methods

2.1. Experimental animals

Adult Dunkin-Hartley guinea pigs weighing 400–449 g (Harlan-Iberica, Barcelona, Spain) were used. All experiments were in accordance with ECCD (directive 2010/63/EU). All efforts were made to reduce the number of animals used and treat them humanely.

2.2. Antibodies and reagents

Nonterminal animal handling was performed under ketamine (6 mg/100 g body weight) and medetomidine (0.025 mg/100 g body weight) anesthesia via intraperitoneal injection. For terminal handling, an overdose of sodium pentobarbital was used. Onabot/A was obtained from Allergan USA. Sheep anticalcitonin gene-related peptide (CGRP) was obtained from Abcam (Cambridge, UK). Rabbit antityrosine hydroxylase (TH) was obtained from Millipore (Watford, UK). Rabbit antivesicular acetylcholine transporter (VAChT) was obtained from Sigma (Sintra, Portugal). Mouse anti-cSNAP-25 was obtained from R&D Antibodies (North Las Vegas, UT, USA). The biotinylated horse antimouse was obtained from Jackson ImmunoResearch (Jackson Europe, Suffolk, UK). Fluorochrome-labeled secondary antibodies were obtained from Molecular Probes (Leiden, The Netherlands).

2.3. OnabotulinumtoxinA administration

2.3.1. OnabotulinumtoxinA injection on one single injection point for studying the spread of cSNAP-25

Male animals ($n = 4$ per experimental group) were anesthetized and their bladders exposed by laparotomy. One experimental group received a single intramural injection of 2 U of Onabot/A diluted in 2 μl and another group a similar injection of 2 U diluted in 20 μl of saline. Both injections were performed in the dorsal side of the urinary bladder between the entry of the ureters, which were used as a reference point for subsequent sectioning. Bladders were collected 3 d after administration. Another four animals were injected with saline and used as controls. In this experiment, the 2-U dose was selected because, on a body weight basis, it is similar to the recommended dose for humans.

2.3.2. OnabotulinumtoxinA instillation for studying the spread of cSNAP-25

Female animals ($n = 2$ per experimental group) were chosen for intravesical instillation due to ease of catheterization. Under anesthesia, 2 ml of saline containing 50 U of Onabot/A were instilled through a catheter inserted in the urethra and kept inside the bladder for 30 min. Bladders were afterward emptied, washed, and collected 3 d later. Controls were instilled with saline.

2.3.3. OnabotulinumtoxinA injection on multiple injection points for cSNAP-25 time course and colocalization analysis

Male animals ($n = 4$ per experimental group) were anesthetized and a laparotomy performed. Ten injections of a total of 5 U of Onabot/A diluted in 200 μl of saline were performed around the entire organ. Saline-injected animals were used as controls. Bladders were collected from each group at 6 h, 12 h, 24 h, 3d, and 7 d. In this experiment, our goal was to perform an image analysis of the presence of SNAP-25 in the three bladder nerve types. Thus a 5-U dose was selected to affect the maximum number of fibers.

2.3.4. Tissue fixation

Bladders were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 4 h at 4 °C and stored in 30% sucrose in 0.1 M PB. A total of 20-μm-thick transverse sections taken from the level of the ureters were cut in a cryostat, collected in Superfrost Plus slides, and stored at −20C° until further processing.

2.3.5. Immunolabeling of cSNAP-25 with chromogenic substrate

Detection of cSNAP-25 was performed using the primary antibody diluted 1:500 for 48 h at 4 °C using the avidin/biotinylated enzyme complete-horseradish peroxidase method as previously described [13]. Controls were performed by testing the antibody in saline-injected tissue. Representative images were collected in a Zeiss Axioscope 40 microscope using AxioVision v.4.6 software.

2.3.6. Double immunofluorescence labeling

Bladder sections were permeabilized in 50% ethanol for 30 min, washed in phosphate-buffered saline (PBS), and blocked with PBST (PBS plus 0.5% Tween 20) containing 10% normal human serum (NHS) for 1 h. Incubation with primary antibodies was performed for 48 h at 4 °C. Anti-VAChT, anti-TH, and anti-CGRP were diluted 1:4000; anti-cSNAP-25 was diluted 1:500 in PBST containing 2% NHS. After several washes, antigens were visualized using Alexa Fluor 488 conjugated donkey antirabbit, Alexa Fluor 568 conjugated donkey antimouse, and Alexa Fluor 568 conjugated donkey antisheep, diluted 1:1000. Controls were performed as described earlier or by omitting primary antibodies. Images were collected using the AxioVision v.4.6 software with the appropriate filter sets.

2.3.7. Counting and statistical analysis

2.3.7.1. Bladders injected in a single point and immunolabeled with chromogenic substrate. From each single injected bladder, six cryostat sections were analyzed. Each section was totally observed and approximately 100 photos per section were taken. All cSNAP-25 immunoreactive (IR) fibers from each photo were visually identified and hand drawn using

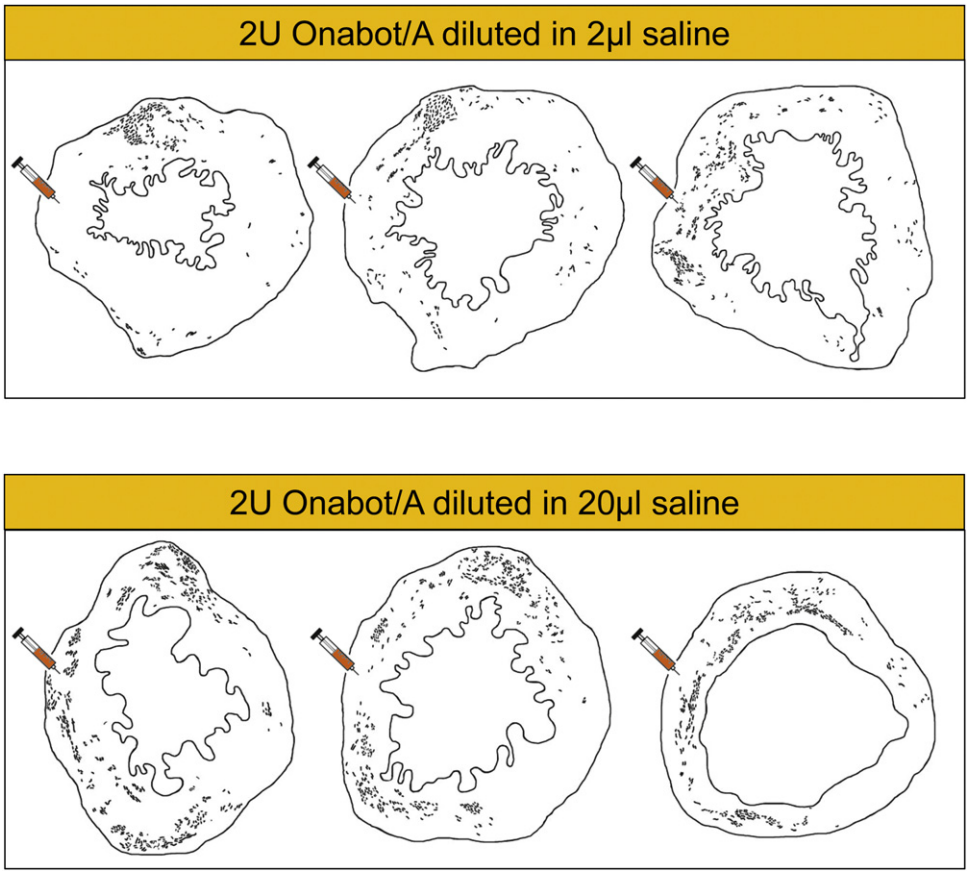

Fig. 1 – Guinea pig bladder diagrams showing the distribution of cleaved synaptosome-associated protein of 25 kDa immunoreactive fibers (dark lines). A total of 2 U of onabotulinumtoxinA (Onabot/A) diluted in 2 µl (upper diagrams) or 20 µl (lower diagrams) of saline were administered in the indicated area (syringes). Transverse sections taken at the level of the ureters were analyzed.

Adobe Photoshop CS. After digital mounting to create a single image of the entire bladder section, drawn fibers were counted. Statistical analysis was performed using the *t* test of GraphPad Prism software. Values of $p < 0.05$ were considered statistically significant.

2.3.7.2. Bladders injected in multiple points for immunofluorescence analysis. For double immunofluorescent colocalization analysis, 10 representative images per animal were analyzed as previously described [13]. Values of $p < 0.0001$ were considered statistically significant.

3. Results

3.1. cSNAP-25 distribution after single onabotulinumtoxinA injections

Three days after a single injection of 2 U of Onabot/A diluted in 2 µl of saline, scarce cSNAP-25 IR fibers were found in the bladder wall. This time point was chosen based on results concerning the time course analysis of cSNAP-25. cSNAP-25 IR fibers were present close to the injection site and occasionally at more distant areas (Fig. 1). After injecting the same amount of Onabot/A diluted in 20 µl of saline, cSNAP-25-IR fibers were more abundant and were present around the entire perimeter of the bladder, including the wall opposed to the injection site (Fig. 1). The number of cSNAP-25-IR fibers was significantly higher in the bladders that received Onabot/A diluted in a larger volume (Fig. 2). Saline-injected animals had no cSNAP-25.

3.2. cSNAP-25 distribution after intravesical onabotulinumtoxinA administration

In animals receiving Onabot/A by intravesical instillation, no cSNAP-25 IR structures were found either in the detrusor

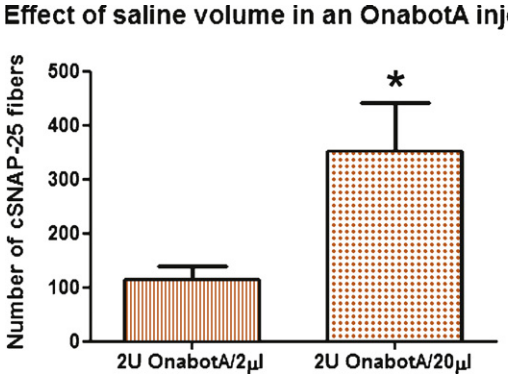

Fig. 2 – Average number of immunoreactive fibers for cleaved synaptosome-associated protein of 25 kDa (SNAP-25) observed per section after onabotulinumtoxinA (Onabot/A) intramural injection diluted in 2 µl or 20 µl of saline. Six bladder sections were analyzed per animal. The difference has statistical significance.
*** $p < 0.05$; error bars show standard deviation.**

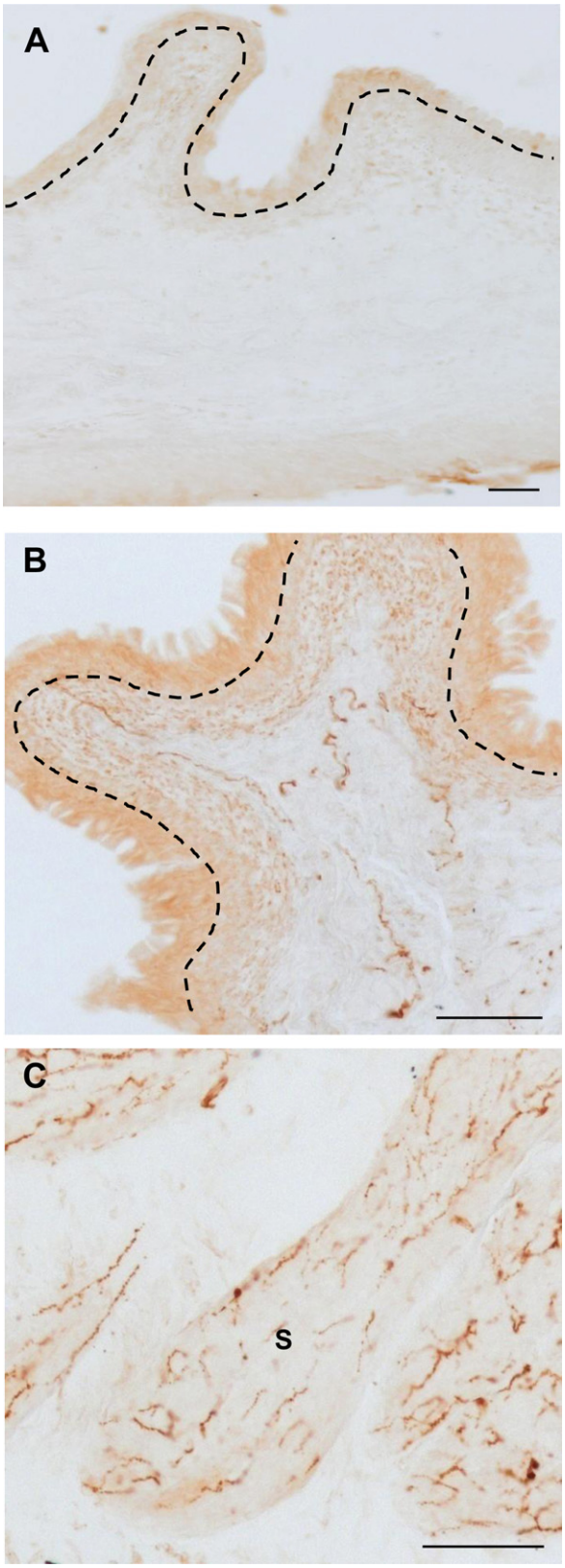

Fig. 3 – Expression of cleaved synaptosome-associated protein of 25 kDa (cSNAP-25) using the avidin-biotin method after onabotulinumtoxinA administration (A) through intravesical instillation or (B, C) through an intramural injection. Dashed lines indicate the boundary between the urothelium and lamina propria. (a) Animals that received intravesical instillation of toxin did not express cSNAP-25 either in the urothelium and mucosa or in the muscular layer. (B) In animals that received an intramural injection of toxin, immunoreactive fibers are observed in the suburothelium present in the lamina propria and in fibers penetrating the urothelium. In (C) the muscular layer, immunoreactive fibers run parallel to the detrusor smooth muscle (S). Magnification bars: 50 μm.

or in the urothelium or lamina propria (Fig. 3A). Because the results were very consistent in both animals treated, no more animals were used. In preliminary experiments, no gender differences were observed in intact SNAP-25 distribution (data not shown).

3.3. Time course of cSNAP-25 and neurochemistry of positive nerve fibers

cSNAP-25 IR fibers were absent at 6 h but could be detected in some animals 12 h after Onabot/A. cSNAP-25 IR was consistently observed at 24 h, with the number of positive fibers not increasing at 3 and 7 d. In the mucosa, IR fibers were present in the lamina propria, some of which were within or immediately below the urothelium (Fig. 3B). cSNAP-25 IR was not detected in urothelial cells (Fig. 3B). In the muscular layer, cSNAP-25 IR fibers ran along smooth muscle bundles (Fig. 3C). Saline-injected animals had no cSNAP-25.

Double labeling experiments revealed the presence of cSNAP-25 in the three bladder nerve types analyzed (Fig. 4A–4C) although in different proportions. At 24 h cSNAP-25 was expressed in 85% of VAChT-positive cholinergic fibers but only in 42% and 36% of TH-positive adrenergic and CGRP-positive sensory fibers, respectively (Fig. 4D). These numbers did not change at 3 and 7 d (Fig. 5).

4. Discussion

It is well established that Onabot/A acts by cleaving the SNAP-25 protein [14,15]. The cleaved form of the protein has already been detected in cultured cells [16] and human bladder tissues by western blot [10] and in rat bladder by immunohistochemistry [11]. Thus the detection of the cleaved form of SNAP-25 seems to be a reliable approach to study nerve fibers or cells affected by the toxin. So far, the diffusion of the toxin has been estimated by the diffusion of dyes [17] or contrast agents for MRI [9].

Onabot/A doses used for the treatment of idiopathic or neurogenic detrusor overactivity has ranged between 100 and 400 U [3]. However, the effect of different injection volumes was never systematically tested. We evaluated the diffusion of 2 U injected in one single site of the guinea pig bladder, which approximately represents an injection of 300 U in a 70-kg patient. We found that for the same dose of Onabot/A, higher injection volumes cleaved more SNAP-25 and at more distant places. In fact, after 20-μl injection volume, cSNAP-25 IR fibers were observed throughout the entire perimeter of the bladder, including the bladder wall opposed to the injection site. To the best of our knowledge, no one has ever tried to compare the diffusion of Onabot/A in the urinary bladder according to injection volume. However, our data agree with findings in the skeletal

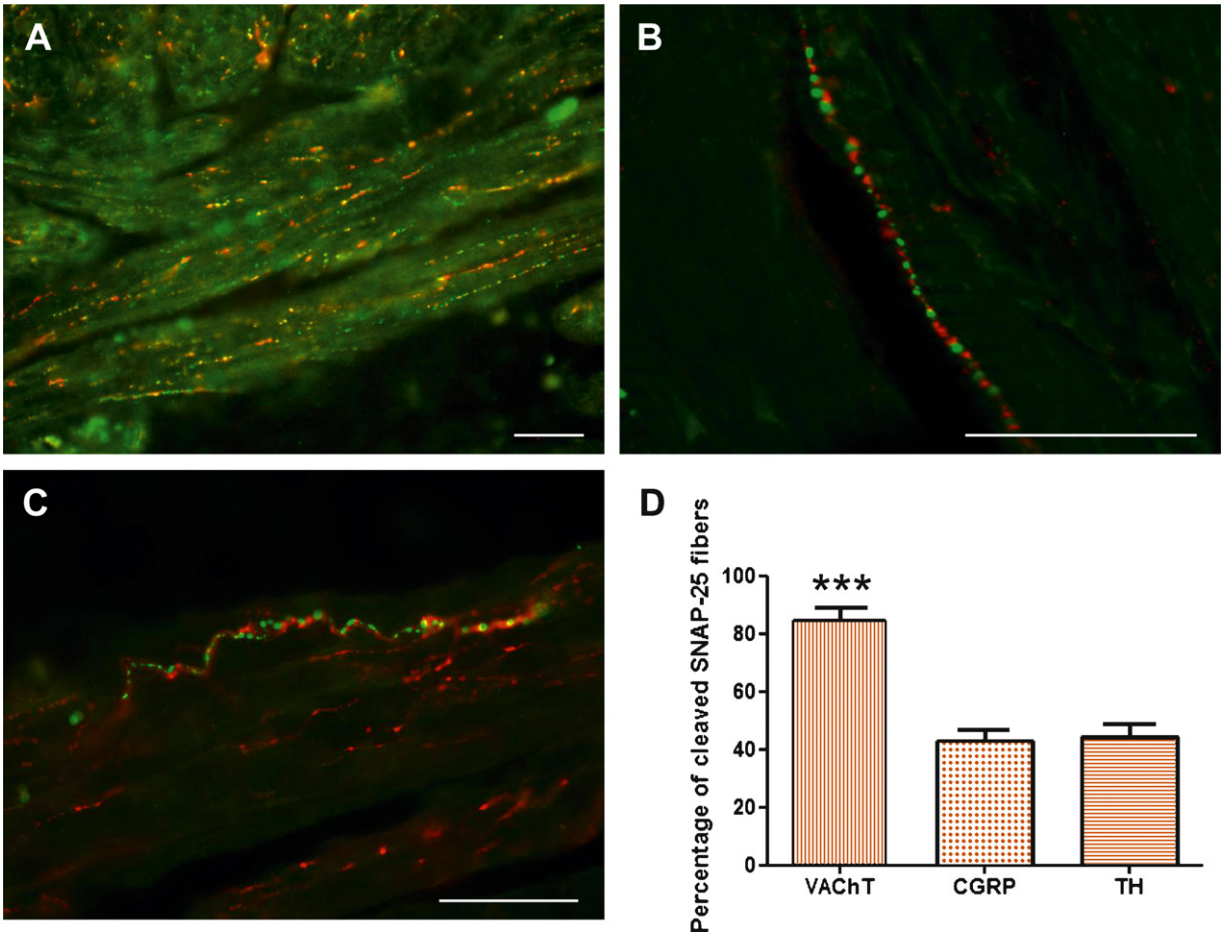

Fig. 4 – Coexpression of cleaved synaptosome-associated protein of 25 kDa (cSNAP-25) (red) with vesicular acetylcholine transporter (VAChT) (A, green), tyrosine hydroxylase (TH) (B, green), and calcitonin gene-related peptide (CGRP) (C, green) after onabotulinumtoxinA (Onabot/A) administration throughout the whole bladder. Note the extensive colocalization found between cSNAP-25 and VAChT (A, yellow/orange); (D) quantification of the coexpression.
*** $p < 0.0001$. **Magnification bars: 50 μm.**

muscle, where larger injection volumes caused more muscle relaxation, indicating a more widespread diffusion of the toxin [7,8]. One of the factors that may relate to the more distant diffusion of the toxin may be the easier spread of the solution through the connective tissue and muscle bundles.

We also undertook a detailed analysis of the bladder structures where cSNAP-25 was expressed. In accordance

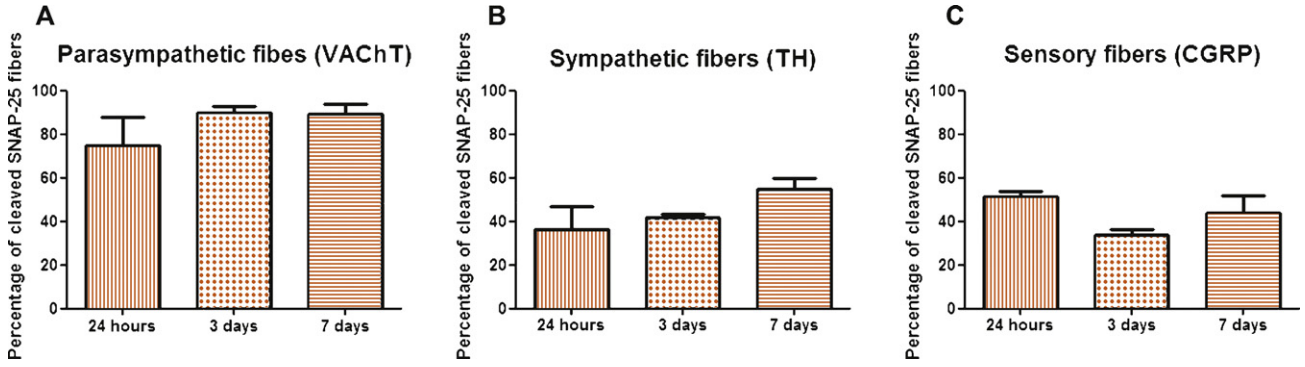

Fig. 5 – Percentage of (A) parasympathetic, (B) sympathetic, or (C) sensory fibers that also express cleaved synaptosome-associated protein of 25 kDa (cSNAP-25) at different time points after onabotulinumtoxinA (Onabot/A) intramural injection throughout the whole bladder. After 24 h, 3 d, or 7 d after (Onabot/A) injection, the number of cSNAP-25 and the percentage of colocalization is unchanged.
CGRP = calcitonin gene-related peptide; TH = tyrosine hydroxylase; VAChT = vesicular acetylcholine transporter.

with the distribution of intact SNAP-25 observed in human bladder [13], cholinergic fibers expressed the most cSNAP-25, in contrast with adrenergic and sensory fibers. At least in the guinea pig SNAP-25, cleavage is a very fast process. Within 24 h, the percentage of cholinergic, adrenergic, and sensory fibers expressing cSNAP-25 had already reached its maximum, without relevant increases at 48 or 72 h.

In contrast with intramural injections, we could not detect cSNAP-25-IR fibers following intravesical instillation of Onabot/A. However, several studies showed that this method of Onabot/A administration decreases bladder sensation in humans [18] and reduces the frequency of bladder contractions in animal models with injured spinal cords [5,19]. However, in most studies Onabot/A diffusion was improved either by urothelium disruption with protamine sulfate [19] or cyclophosphamide [5]. Onabot/A instillation in the bladder encapsulated in liposomes may eventually overcome the urothelial barrier and induce distinctive bladder effects [20].

Onabot/A administration in the bladder has already been shown to decrease the exocytosis of neuropeptides such as CGRP and substance P from afferent nerves and of neurotrophins from the urothelium [21,22]. A decreased release of nitric oxide and adenosine triphosphate from urothelial cells after Onabot/A administration to the bladder is also well demonstrated [21]. In the case of nerve terminals, the expression of synaptic vesicle protein 2 (SV2) and SNAP-25 is well documented. Regarding urothelial cells, the information is scarcer. Available data suggest that the human urothelium does not express SV2 or SNAP-25 [13]. Likewise, cSNAP-25 was absent from the guinea pig urothelium.

The most relevant findings of the present study are the importance of the volume injection to the diffusion of the toxin and the predominant localization of cSNAP-25 in cholinergic nerves. Although the latter is relevant for the toxin mechanism of action, the former is highly pertinent for clinical practice. If larger injection volumes may increase Onabot/A diffusion in the bladder, clinical trials investigating different injection volumes per site are justified to investigate a possible enhancement of the toxin effect.

The main limitation of this study was the lack of correlation between the amount of cSNAP-25 in the bladder wall with the functional effects of toxin injection. In addition, results obtained in guinea pigs may not be directly extrapolated to humans.

5. Conclusions

We showed that Onabot/A injections in the bladder wall can spread the toxin to areas very distant from the injection point. This is particularly remarkable when high volumes of saline are used to dissolve the toxin. When injected, but not instilled, Onabot/A cleaves SNAP-25 in almost all cholinergic nerves and in less than half of the adrenergic and sensory fibers. Maximal effect is observed rapidly, within 24 h, and does not increase with time.

These findings suggest that the injected volume is a key factor in the spread and action of Onabot/A in the bladder, and this may be relevant to improve the clinical effect of Onabot/A.

Author contributions: António Avelino had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Coelho, F. Cruz, Avelino.
Acquisition of data: Coelho.
Analysis and interpretation of data: Coelho, F. Cruz, Avelino.
Drafting of the manuscript: Coelho, F. Cruz, C.D. Cruz, Avelino.
Critical revision of the manuscript for important intellectual content: F. Cruz, C.D. Cruz, Avelino.
Statistical analysis: Coelho.
Obtaining funding: C.D. Cruz, Avelino.
Administrative, technical, or material support: C.D. Cruz, Avelino.
Supervision: Avelino.
Other (specify): None.

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References

[1] Mangera A, Andersson K-E, Apostolidis A, et al. Contemporary management of lower urinary tract disease with botulinum toxin A: a systematic review of Botox (onabotulinumtoxinA) and Dysport (abobotulinumtoxinA). Eur Urol 2011;60:784–95.
[2] Mangera A, Chapple CR. Use of botulinum toxin in the treatment of lower urinary tract disorders. Current status. Arch Esp Urol 2010;63:829–41.
[3] Rapp DE, Lucioni A, Bales GT. Botulinum toxin injection: a review of injection principles and protocols. Int Braz J Urol 2007;33:132–41.
[4] Karsenty G, Reitz A, Lindemann G, Boy S, Schurch B. Persistence of therapeutic effect after repeated injections of botulinum toxin type A to treat incontinence due to neurogenic detrusor overactivity. Urology 2006;68:1193–7.
[5] Vemulakonda VM, Somogyi GT, Kiss S, Salas NA, Boone TB, Smith CP. Inhibitory effect of intravesically applied botulinum toxin A in chronic bladder inflammation. J Urol 2005;173:621–4.
[6] Chuang YC, Yoshimura N, Huang CC, Chiang PH, Chancellor MB. Intravesical botulinum toxin a administration produces analgesia against acetic acid induced bladder pain responses in rats. J Urol 2004;172:1529–32.
[7] Shaari CM, Sanders I. Quantifying how location and dose of botulinum toxin injections affect muscle paralysis. Muscle Nerve 1993;16:964–9.
[8] Kim HS, Hwang JH, Jeong ST, et al. Effect of muscle activity and botulinum toxin dilution volume on muscle paralysis. Dev Med Child Neurol 2003;45:200–6.
[9] Mehnert U, Boy S, Schmid M, et al. A morphological evaluation of botulinum neurotoxin A injections into the detrusor muscle using magnetic resonance imaging. World J Urol 2009;27:397–403.

[10] Schulte-Baukloh H, Zurawski TH, Knispel HH, Miller K, Haferkamp A, Dolly JO. Persistence of the synaptosomal-associated protein-25 cleavage product after intradetrusor botulinum toxin A injections in patients with myelomeningocele showing an inadequate response to treatment. BJU Int 2007;100:1075–80.
[11] Coelho A, Cruz F, Cruz CD, Avelino A. Effect of onabotulinumtoxinA on intramural parasympathetic ganglia: an experimental study in the guinea-pig urinary bladder. J Urol 2012;187:1121–6.
[12] Restani L, Antonucci F, Gianfranceschi L, Rossi C, Rossetto O, Caleo M. Evidence for anterograde transport and transcytosis of botulinum neurotoxin A (BoNT/A). J Neurosci 2011;31:15650–9.
[13] Coelho A, Dinis P, Pinto R, et al. Distribution of the high-affinity binding site and intracellular target of botulinum toxin type A in the human bladder. Eur Urol 2010;57:884–90.
[14] Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J 1993;12:4821–8.
[15] Schiavo G, Santucci A, Dasgupta BR, et al. Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. FEBS Lett 1993;335:99–103.
[16] Dong M, Yeh F, Tepp WH, et al. SV2 is the protein receptor for botulinum neurotoxin A. Science 2006;312:592–6.
[17] Landman BWM, Capelle JW. Intravesical Botox injections in children with a idiopathic overactive bladder. Eur Urol Suppl 2009; 8:169.
[18] Krhut J, Zvara P. Intravesical instillation of botulinum toxin A: an in vivo murine study and pilot clinical trial. Int Urol Nephrol 2011;43:337–43.
[19] Khera M, Somogyi GT, Salas NA, Kiss S, Boone TB, Smith CP. In vivo effects of botulinum toxin A on visceral sensory function in chronic spinal cord-injured rats. Urology 2005;66:208–12.
[20] Chuang YC, Tyagi P, Huang CC, et al. Urodynamic and immunohistochemical evaluation of intravesical botulinum toxin A delivery using liposomes. J Urol 2009;182:786–92.
[21] Chancellor MB, Fowler CJ, Apostolidis A, et al. Drug Insight: biological effects of botulinum toxin A in the lower urinary tract. Nat Clin Pract Urol 2008;5:319–28.
[22] Pinto R, Lopes T, Frias B, et al. Trigonal injection of botulinum toxin A in patients with refractory bladder pain syndrome/interstitial cystitis. Eur Urol 2010;58:360–5.

Publication III

Coelho A, Cruz F, Cruz CD, Avelino A (2012) Effect of Onabotulinumtoxin/A on intramural parasympathetic ganglia: an experimental study on guinea pig bladder. J Urol 187(3): 1121-6

Effect of OnabotulinumtoxinA on Intramural Parasympathetic Ganglia: An Experimental Study in the Guinea Pig Bladder

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Purpose: We investigated whether onabotulinumtoxinA injected in the bladder would affect preganglionic parasympathetic nerve endings in intramural ganglia.
Materials and Methods: Guinea pig bladders were injected with 5 U of botulinum toxin. At 24 hours bladders were collected and processed for immunohistochemistry using tyrosine hydroxylase, and intact and cleaved SNAP-25. To identify the different populations of affected fibers coursing the ganglia we performed double immunoreactions for cleaved SNAP-25 and VAChT, TH or CGRP.
Results: VAChT immunoreactive fibers were identified in axons and varicosities of presynaptic to postganglionic parasympathetic neurons. Those fibers were also immunoreactive to SV2 and SNAP-25. The rare CGRP and TH immunoreactive fibers coursing in the ganglia did not express SV2 or SNAP-25. After onabotulinumtoxinA injection the cleaved form of SNAP-25 was abundantly expressed in parasympathetic fibers.
Conclusions: Botulinum toxin injection in the bladder wall affects preganglionic parasympathetic nerve terminals. This could contribute to the strong effect of botulinum toxin on bladder smooth muscle activity.

Key Words: urinary bladder, overactive; onabotulinumtoxinA; ganglia, parasympathetic; synaptosomal-associated protein 25; guinea pigs

Abbreviations and Acronyms

ACh = acetylcholine
BoNT/A = botulinum toxin type A
CGRP = calcitonin gene-related peptide
CTb = cholera toxin subunit β
IR = immunoreactivity
NF200 = neurofilament 200
OnabotA = onabotulinumtoxinA
PBS = phosphate buffered saline
SNAP-25 = synaptosomal-associated protein of 25 kDa
SV2 = synaptic vesicle protein type 2
TH = tyrosine hydroxylase
VAChT = vesicular acetylcholine transporter

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Study received institutional animal care and use committee approval.
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* Financial interest and/or other relationship with Allergan, Astellas, Rienndati and AMS.

Botulinum toxin type A is under active investigation for the treatment of urinary incontinence caused by idiopathic and neurogenic detrusor overactivity, mainly due to its capacity to decrease bladder smooth muscle contractility.[1,2] The effect of BoNT/A injected in the bladder lasts 6 to 9 months,[1,2] much longer than the effect of the neurotoxin injected in skeletal muscle, which only lasts 3 to 4 months.[1,3] To date no clear explanation has been offered for this difference. Also, there is no concluding justification for the fact that the same dose of BoNT/A induces urinary retention in some patients while others retain the capacity to void.[4,5]

BoNT/A is internalized when synaptic vesicles fuse with the neuronal membrane and the high affinity receptor for the neurotoxin, SV2, is exposed.[6,7] Once inside neurons the toxin cleaves SNAP-25, a key component of the exocytic process, blocking neurotransmitter release.[6,8,9] Inhibition of ACh release from cholinergic neurons is thought to be the pivotal mechanism of decreased bladder contractility.[10] Accordingly recent studies of the human bladder showed that SV2 and SNAP-25 are expressed in almost all parasympathetic fibers and in about half of sympathetic and sensory fibers.[11]

In the bladder the parasympathetic system comprises preganglionic and postganglionic neurons. Pregan-

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glionic cell bodies are located in the ventrolateral part of the intermediolateral gray matter of the sacral spinal cord segments.[12] Their axons end in the parasympathetic ganglia, where postganglionic cell bodies are located. Axons of the latter neurons mainly innervate the detrusor muscle. Axonal endings from preganglionic and postganglionic neurons release ACh, which binds nicotinic receptors on postganglionic neurons and muscarinic receptors in smooth muscle cells.

In humans parasympathetic ganglia are embedded in the bladder wall. Thus, BoNT/A injection in the bladder might reach not only postganglionic nerve fibers but also preganglionic terminals in the ganglia. As a consequence, decreased ACh release from preganglionic neurons, which is expected to occur after botulinum administration, together with inhibited release from postganglionic endings may contribute to the final effect of BoNT/A on the detrusor muscle. Impaired nicotinic cholinergic transmission at the parasympathetic ganglia has profound consequences on bladder function. Ganglion blocking agents such as tetraethylammonium or hexamethonium easily induce urinary retention.[13] In contrast, muscarinic receptor blockade rarely causes urinary retention even when high doses are used.[14]

Demonstrating that preganglionic parasympathetic neurons become inhibited after BoNT/A injection into the bladder wall may be difficult in humans, if not impossible. However, several animal species used in experimental bladder research[15–18] have a parasympathetic organization similar to that of humans. This is the case in the guinea pig, in which the current study was done. To provide evidence of the inhibition of preganglionic parasympathetic neurotransmitter release after BoNT/A injection in the bladder we performed immunodetection of the cleaved fragment of SNAP-25 in the intramural ganglia.

MATERIALS AND METHODS

Animals

A total of 14 adult male Dunkin Hartley Guinea pigs weighing 400 to 450 gm were obtained from Harlan-Ibérica, Barcelona, Spain. All procedures were done in accordance with the European Communities Council Directive of 20 September 2010. All efforts were made to decrease animal stress and suffering, and the number of animals used. The animals were kept at a 12-hour light/12-hour dark schedule with free access to food and water. Well-being was supervised daily.

Reagents and Antibodies

Nonterminal animal handling was performed with the animals under ketamine (6 mg/100 gm body weight) and medetomidine (0.025 mg/100 gm body weight) anesthesia via intraperitoneal injection. For terminal handling animals received an overdose of intraperitoneal sodium pentobarbital. BoNT/A (OnabotA) was obtained from Allergan, Irvine, California. Mouse anti-SV2 antibody[19] was obtained from the Developmental Studies Hybridoma Bank. Rabbit anti-SNAP-25 and sheep anti-CGRP were obtained from Abcam®. Rabbit anti-TH was obtained from Chemicon®. Rabbit anti-VAChT and anti-neurofilament 200 were obtained from Sigma-Aldrich®. Mouse anti-cleaved SNAP-25 was obtained from Research and Diagnostic Antibodies, North Las Vegas, Nevada. Fluorochrome labeled secondary antibodies and Alexa 488 conjugated CTb were obtained from Molecular Probes® Europe.

OnabotA and CTB Administration

Ten animals were anesthetized. An abdominal incision was made and the bladder was exposed. Four animals received 10 injections evenly distributed in the bladder wall for a total volume of 200 μl saline containing 5 U OnabotA. Four controls were injected with saline alone. The other 2 animals were injected in the bladder wall bilaterally near the entry of the ureters with 5 μl 1% CTb diluted in saline.

Tissue Preparation

Three days after OnabotA administration the animals were terminally anesthetized. Bladders were harvested, fixed in 4% buffered paraformaldehyde for 4 hours at 4C and stored in sucrose 30% in phosphate buffer 0.1M. Animals injected with CTb were perfused through the ascending aorta with Tyrode's solution (0.12M NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $NaH_2PO_4 \cdot H_2O$, 5.5 mM glucose and 26.2 mM $NaHCO_3$), followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2. Spinal cord segment S1 was collected, postfixed in the same fixative for 4 hours and stored as described. Transverse sections (20 μM) of the bladder and spinal cord were cut in a cryostat, collected on SuperFrost™ Plus slides and stored at −20C until further processing.

Immunohistochemistry

After thawing, representative sections of each bladder were permeabilized in 50% ethanol for 30 minutes, washed in PBS and blocked with PBS plus 0.3% Triton X-100 and 10% normal horse serum for 1 hour. Incubation with primary antibodies was performed for 48 hours at 4C. Anti-VAChT, anti-TH and anti-CGRP were diluted 1:4,000, anti-SV2, anti-intact SNAP-25 and anti-NF200 were diluted 1:2,000 and anti-cleaved SNAP-25 was diluted 1:500. All antibodies were diluted in PBS plus 0.3% Triton X-100 containing 2% normal horse serum. After several washes immunoreactions were visualized according to species specificity using Alexa Fluor® 488 conjugated donkey anti-rabbit, Alexa Fluor 568 conjugated donkey anti-mouse and Alexa Fluor conjugated donkey anti-sheep, each diluted 1:1,000. Controls were performed by omitting primary antibodies. Representative images were collected in an Axioscope 40 microscope with the appropriate filter sets using the ApoTome system for optical sectioning and AxioVision 4.6 software (Carl Zeiss®).

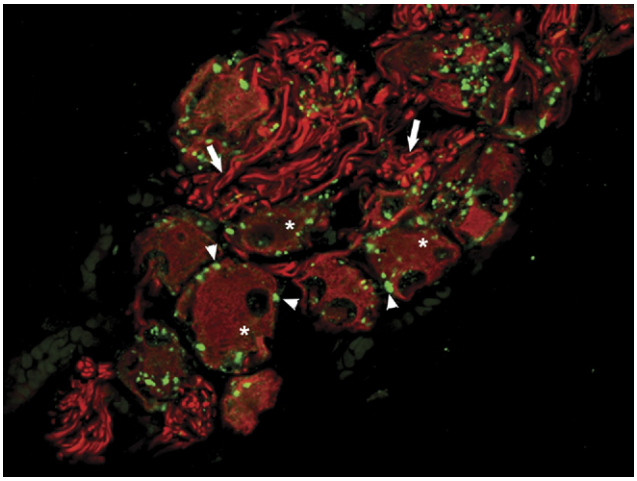

Figure 1. Double staining for NF200 (red areas) and VAChT (green areas) shows NF200 expressed in postganglionic neuronal cell bodies (asterisks) and initial segment of axonal processes (arrows), and VAChT in preganglionic nerve fibers and varicosities wrapping postganglionic perikarya (arrowheads). Reduced from 20 μm.

RESULTS

Intramural ganglia were easily observed in the bladder region near the ureters on the serosal surface or scattered among detrusor smooth muscle bundles. Postganglionic parasympathetic cell bodies and the initial segment of axonal processes were strongly immunoreactive for the neuronal marker NF200 (fig. 1). When simultaneous immunoreaction was done for VAChT, a protein localized in the axonal endings of cholinergic fibers,[20] strong labeling was observed in varicose nerve fibers wrapping postganglionic cell bodies (fig. 1). Abundant VAChT staining was also found in nerve fibers surrounding smooth muscle bundles (data not shown).

Injecting CTb tracer in the bladder produced retrograde labeling in preganglionic neurons of the intermediolateral cell column of the sacral spinal cord and in postganglionic neuronal cell bodies in the bladder wall (fig. 2). Punctate staining, consistent with CTb labeling of nerve terminals, was also observed around postganglionic cell bodies (fig. 2, *B*).

SV2 and intact SNAP-25 IR was found in nerve fibers coursing in the ganglia and in terminals impinging on postganglionic cell bodies, showing extensive colocalization (fig. 3). SV2 and intact SNAP-25 also colocalized abundantly with VAChT IR fibers and terminals (fig. 4). In contrast, no colocalization was detected with TH and CGRP IR fibers (fig. 4). Although these fibers were less abundant than VAChT IR fibers, they were found around ganglia or between perikarya. Ganglionic cells did not express VAChT, CGRP or TH.

In animals treated with OnabotA abundant cleaved SNAP-25 IR was detected in ganglia in nerve endings apposed to neuronal cell bodies (fig. 5). As a control, to show that the antibody against cleaved SNAP-25 antibody did not recognize the intact protein tissue from saline injected animals was also reacted. No signal was observed in these experiments (data not shown). Colocalization analysis between cleaved SNAP-25 and the 3 nerve types revealed that in ganglia only parasympathetic fibers expressed the cleaved form of the protein (fig. 5).

DISCUSSION

Organization of the guinea pig bladder parasympathetic innervation is similar to that of humans since the parasympathetic ganglia of these rodents are also embedded in the bladder wall.[21] In contrast, in the rat, a more commonly used experimental animal, the preganglionic fibers do not reach the bladder wall since the synaptic contacts are located in the major pelvic ganglion and, thus, are outside the bladder. This characteristic of the guinea pig was reported by Gabella in 1990, who described abundant intramural parasympathetic ganglia in the equatorial region of the bladder near the entry of the ureters.[21] These observations were fully confirmed in our study. We further confirmed preganglionic parasympathetic axonal endings in the guinea pig bladder wall.

After injecting CTb in the bladder wall this neuronal tracer was transported retrograde to the spinal cord, where it could fill preganglionic parasympathetic cell bodies of the sacral intermediolateral column.[22] These facts made us consider that the guinea pig was an appropriate experimental animal in which to answer our initial question.

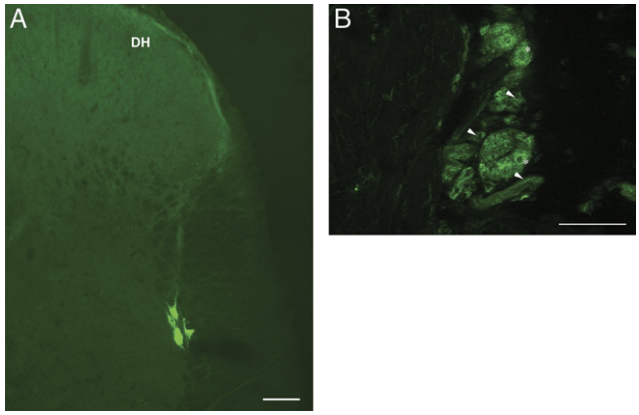

Figure 2. Retrograde labeling with CTb of neuronal cell bodies after tracer injection in bladder wall reveals sacral spinal cord section with labeled motoneurons in intermediolateral cell column (*A*), and bladder section with labeled ganglionic neurons (asterisks) and nerve terminals (arrowheads) (*B*). *DH*, spinal gray matter dorsal horn. Scale bar indicates 50 μm.

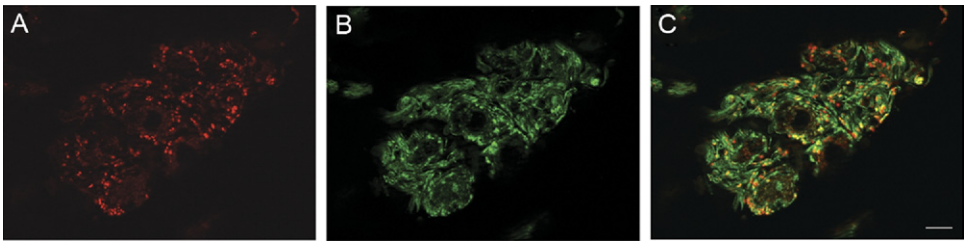

Figure 3. Double staining for SV2 (red areas) (*A*) and SNAP-25 (green areas) (*B*) demonstrates abundant colocalization (yellow/orange areas) (*C*). Scale bar indicates 20 μm.

Preganglionic and postganglionic parasympathetic neurons release ACh from their axonal endings. ACh is synthesized in nerve terminals from choline and acetyl coenzyme A by the cytoplasmic enzyme choline acetyltransferase. The neurotransmitter is thereafter transported into synaptic vesicles, where it is stored until release. This process requires a protein called VAChT. We identified VAChT IR in areas of the central and peripheral nervous system rich in cholinergic nerve endings, including sympathetic and parasympathetic ganglia.[20]

Accordingly in this study abundant nerve endings and varicosities immunoreactive to VAChT were noted in bladder ganglion presynaptic-to-postganglionic neuronal cell bodies and postsynaptically around smooth muscle fibers. These nerve endings were also immunoreactive to SV2, the high affinity binding site for OnabotA, or to intact SNAP-25, the final target of the toxin, making them likely targets of OnabotA action. The same preganglionic nerve endings showed strong labeling for cleaved SNAP-25 in animals injected in the bladder with OnabotA. This indicates that preganglionic parasympathetic terminal axons were also affected by OnabotA delivered in the bladder wall, fully confirming our initial hypothesis.

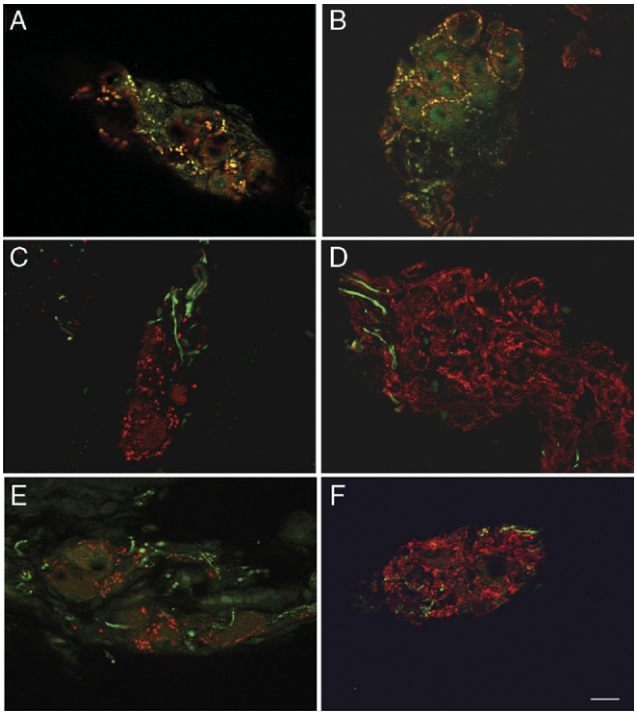

Figure 4. Co-expression of SV2 or intact SNAP-25 (red areas) with VAChT (*A* and *B*), TH (*C* and *D*) and CGRP (*E* and *F*) (green areas). Note extensive colocalization between SV2 or intact SNAP-25 and VAChT (yellow/orange areas). Scale bar indicates 20 μm.

The abundant cleaved SNAP-25 in preganglionic parasympathetic neurons is highly suggestive that ACh release from these nerve endings should be significantly decreased after the toxin injection. The consequence should be decreased cholinergic stimulation of nicotinic receptors in postganglionic parasympathetic neurons. The relative contribution of this finding to the paralyzing effect of OnabotA on the detrusor muscle can only be an object of speculation. However, ganglionic blocking agents easily evoke urinary retention, in contrast to muscarinic receptor antagonists,[23] suggesting that parasympathetic preganglionic neuron intoxication can provide a remarkable contribution to the final effect of OnabotA on the detrusor muscle.

The number of postganglionic axonal endings and respective synapses is much higher than the number of preganglionic ones.[24] Thus, complete upstream blockade of nicotinic transmission seems more likely than full blockade of the muscarinic transmission, provided that parasympathetic ganglia are impaired by the toxin. In clinical practice despite the same number of injections the exact delivery of OnabotA may vary, eventually affecting the number of parasympathetic ganglia reached by the toxin. This could contribute to the variability of effects in humans after OnabotA treatment.[4,5]

The effect of OnabotA on the human detrusor muscle lasts an average of 6 to 9 months, twice the duration of the effect in skeletal muscle.[1,3] In the latter case only 1 motor neuron is affected. Thus, it

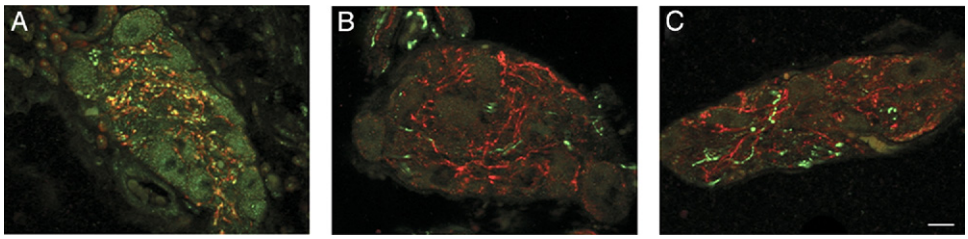

Figure 5. Co-expression of cleaved SNAP-25 (red areas) with VAChT (*A*), TH (*B*) and CGRP (*C*) (green areas) after BoNT/A administration. Note extensive colocalization between cleaved SNAP-25 and VAChT (yellow/orange areas) (*A*). Scale bar indicates 20 μm.

is tempting to link the longer duration of OnabotA in the human bladder with the intoxication of 2 synapses. While the OnabotA effect persists at least in 1 of the 2 neurons (preganglionic or postganglionic), parasympathetic transmission remains impaired. However, this hypothesis is put forward with caution. If preganglionic and postganglionic neurons recover in an equivalent time, the period of decreased neurotransmission cannot be doubled, although the 2 neurons are positioned in sequence. Nevertheless, a BoNT/A effect is expected to occur until the whole parasympathetic chain becomes fully operative. To answer this question comparative studies must be performed of the persistence of cleaved SNAP-25 in preganglionic and postganglionic fibers.

The physiology of parasympathetic ganglia is far from being well elucidated. However, enough evidence has accumulated to state that ganglia are more than a structure in which only nicotinic cholinergic transmission occurs. Parasympathetic transmission can be facilitated or delayed by sympathetic or sensory nerves, further contributing to the final tuning of the parasympathetic outflow.[25] TH IR detected in fibers around and in the ganglia in this study was never noted to our knowledge. Adrenergic receptors in the parasympathetic ganglia had already been observed on the neuronal surface of ganglionic cells,[26] explaining the observation that adrenergic stimulation modulates parasympathetic flow through the ganglia.[25] However, these fibers were not substantially affected by OnabotA since they did not express a detectable amount of cleaved SNAP-25.

Sensory fibers, like CGRP IR fibers observed in ganglia, have already been suggested to be collateral fibers from afferents whose terminals end in the suburothelium, modulating parasympathetic activity.[26] Thus, intoxication of these fibers would probably influence cholinergic transmission. However, like TH positive fibers, CGRP IR fibers were minimally affected by the neurotoxin.

CONCLUSIONS

OnabotA targets and byproducts of OnabotA action are present in guinea pig intramural ganglia, mainly in preganglionic parasympathetic axonal endings. After OnabotA administration SNAP-25 cleavage occurred mainly in these nerve endings. Thus, when injected intramurally, OnabotA action is also exerted on preganglionic nerve terminals, a fact that may contribute to treatment efficacy in clinical practice.

ACKNOWLEDGMENTS

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REFERENCES

1. da Silva CM and Cruz F: Has botulinum toxin therapy come of age: what do we know, what do we need to know, and should we use it? Curr Opin Urol 2009; **19:** 347.
2. Apostolidis A, Dasgupta P, Denys P et al: Recommendations on the use of botulinum toxin in the treatment of lower urinary tract disorders and pelvic floor dysfunctions: a European consensus report. Eur Urol 2009; **55:** 100.
3. Thwaini A, Shergill I, Radhakrishnan S et al: Botox in urology. Int Urogynecol J Pelvic Floor Dysfunct 2006; **17:** 536.
4. Popat R, Apostolidis A, Kalsi V et al: A comparison between the response of patients with idiopathic detrusor overactivity and neurogenic detrusor overactivity to the first intradetrusor injection of botulinum-A toxin. J Urol 2005; **174:** 984.
5. Kuo HC: Urodynamic evidence of effectiveness of botulinum A toxin injection in treatment of detrusor overactivity refractory to anticholinergic agents. Urology 2004; **63:** 868.
6. Dong M, Yeh F, Tepp WH et al: SV2 is the protein receptor for botulinum neurotoxin A. Science 2006; **312:** 592.
7. Mahrhold S, Rummel A, Bigalke H et al: The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. FEBS Lett 2006; **580:** 2011.
8. Schiavo G, Matteoli M and Montecucco C: Neurotoxins affecting neuroexocytosis. Physiol Rev 2000; **80:** 717.
9. Sudhof TC: The synaptic vesicle cycle. Annu Rev Neurosci 2004; **27:** 509.
10. Apostolidis A and Fowler CJ: The use of botulinum neurotoxin type A (BoNTA) in urology. J Neural Transm 2008; **115:** 593.

11. Coelho A, Dinis P, Pinto R et al: Distribution of the high-affinity binding site and intracellular target of botulinum toxin type A in the human bladder. Eur Urol 2010; **57:** 884.

12. Blok BF: Central pathways controlling micturition and urinary continence. Urology 2002; **59:** 13.

13. Aviado DM: Action of ganglion-blocking agents on the cardiovascular system. In: Pharmacology of Ganglionic Transmission. Edited by DA Kharkevich. New York: Springer-Verlag 1980; vol 53, pp 237–249.

14. Chapple CR, Van Kerrebroeck PE, Junemann KP et al: Comparison of fesoterodine and tolterodine in patients with overactive bladder. BJU Int 2008; **102:** 1128.

15. Stolzenburg JU, Schwalenberg T, Do M et al: Is the male dog comparable to human? A histological study of the muscle systems of the lower urinary tract. Anat Histol Embryol 2002; **31:** 198.

16. Dass N, McMurray G, Greenland JE et al: Morphological aspects of the female pig bladder neck and urethra: quantitative analysis using computer assisted 3-dimensional reconstructions. J Urol 2001; **165:** 1294.

17. Ganzer R, Neuhaus J, Dorschner W et al: Muscle systems of the lower urinary tract of the male rhesus monkey (Macaca mulatta): histomorphology and 3-dimensional reconstruction. J Urol 2002; **168:** 1603.

18. Neuhaus J, Dorschner W, Mondry J et al: Comparative anatomy of the male guinea-pig and human lower urinary tract: histomorphology and three-dimensional reconstruction. Anat Histol Embryol 2001; **30:** 185.

19. Buckley K and Kelly RB: Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. J Cell Biol 1985; **100:** 1284.

20. Arvidsson U, Riedl M, Elde R et al: Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. J Comp Neurol 1997; **378:** 454.

21. Gabella G: Intramural neurons in the urinary bladder of the guinea-pig. Cell Tissue Res 1990; **261:** 231.

22. Kuipers R, Izhar Z, Gerrits PO et al: Location of bladder and urethral sphincter motoneurons in the male guinea pig (Cavia porcellus). Neurosci Lett 2004; **362:** 57.

23. Chapple C and Cruz F: Open to debate. The motion: antimuscarinics are the mainstay of therapy for overactive bladder. Eur Urol 2008; **54:** 226.

24. Westfall T: Neurotransmission: the autonomic and somatic motor nervous system. In: Goodman and Gilman's The Pharmacological Basis of Therapeutics, 12th ed. Edited by L Bruton. New York: McGraw Hill 2011; pp 171–218.

25. de Groat WC: A neurologic basis for the overactive bladder. Urology 1997; **50:** 36.

26. Gillespie JI, Markerink-van Ittersum M and de Vente J: Sensory collaterals, intramural ganglia and motor nerves in the guinea-pig bladder: evidence for intramural neural circuits. Cell Tissue Res 2006; **325:** 33.

Publication IV

Coelho A, Oliveira, R, Rosetto O, Cruz F, Cruz CD, Avelino A. (2013) Intrathecal administration of botulinum toxin type A improves urinary bladder function and reduces pain in rats with cystitis. (Submitted)

Intrathecal administration of botulinum toxin type A improves urinary bladder function and reduces pain in rats with cystitis

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Abstract

There is increasing evidence that botulinum toxin A (Onabot/A) has an antinociceptive effect. Indeed, the urinary bladder trigonal injection of the toxin has been shown to reduce visceral pain associated with bladder inflammation. The explanation for this antinociceptive effect has been attributed to an impairment of neuropeptide release from nociceptive nerve terminals. Recent results showed that, in addition, botulinum toxin has an effect on the central nervous system that may contribute to this analgesic effect.

In this work we aimed to study the effect of intrathecal administration of botulinum toxin A in an animal model of bladder pain and hyperactivity induced by cyclophosphamide (CYP).

Female rats were subjected to implantation of an intrathecal (IT) catheter at the L6 segment. Bladder pain was induced by the intraperitoneal (IP) injection of CYP. Four experimental groups were created: (1) Saline IT + IP (2) Onabot/A IT (3) CYP + saline IT (4) CYP and Onabot/A IT 48h after CYP. Mechanical pain threshold was assessed in the abdomen and hindpaws by the von Frey filament test. Motor activity was assessed by measuring the distance travelled by rats in an open field arena. Bladder reflex activity was evaluated by cystometry. At the end, animals were perfused and bladders and spinal cord collected and immunoreacted (IR) against cleaved SNAP-25 (cSNAP-25) and the markers of spinal neuronal activation c-Fos and p-ERKs.

We found that the toxin reduced pain symptoms and the increased bladder reflex activity induced by cyclophosphamide. We also observed a direct effect on the expression of

neuronal markers of activation, typically up-regulated in this inflammatory model. Furthermore we detected the presence of cSNAP-25 not only in nerve fibers throughout the L6 spinal cord segment but also in the urinary bladder wall in animals treated with Onabot/A. No somatic or visceral motor impairments were observed.

Overall, our results suggest that intrathecal administration of botulinum toxin A has a strong analgesic effect in a model of severe bladder pain. This route of administration can be further explored to treat intractable forms of pain.

Introduction

Botulinum toxin (BoNT/A) administration in the bladder wall is a highly effective treatment of neurogenic detrusor overactivity (NDO) (63, 107). Another emerging field in the use of BoNT/A is the control of pain, namely that arising from the bladder. Bladder pain syndrome/Interstitial cystitis (BPS/IC) is a debilitating condition characterized by an intense suprapubic pain associated with increased daytime and nighttime voiding frequency (108). It can also be combined with typical cystoscopic and histological features such as edema and inflammation (109). The etiology of the disease is still unclear and no effective treatment is known. Available treatments are usually focused on the relief of pain but their efficacy is often limited (110).

In a recent study, the injection of toxin in the bladder trigonal area proved to have a beneficial effect in BPS/IC patients, decreasing pain and improving their quality of life (66). However, this method of BoNT/A administration requires repeated injections controlled by cystoscopy every 6-9 months under general anaesthesia. Moreover, injections cause frequent urinary tract infections which aggravate BPS/IC symptoms (61, 63).

Recently, some experimental studies suggested that the intrathecal (IT) delivery of BoNT/A has been shown to have a central antinociceptive effect in animal models of neuropathic and somatic pain (111, 112). Thus, the IT administration of BoNT/A may also be advantageous to treat intractable bladder pain. In the present work we explored this issue by using an animal model of bladder pain to determine the effects of IT administration of BoNT/A. The analysis concerned the visceral and somatic effects, nociceptive behavior, urinary bladder reflex activity and the immunohistochemical observation of cleaved SNAP-25 and spinal markers of neuronal activation.

Methods

Animals: All experiments were performed in female Wistar rats weighing 220-250g (Charles River, France) that were housed under a 12h light/12h dark schedule with free access to food and water prior to experimentation. All procedures were carried out according to the European Communities council Directive (2010/63/EU). All efforts were made to reduce the number of animals used and their suffering.

Reagents and drugs: Surgeries for intrathecal placement of a catheter were performed under deep anaesthesia induced by intraperitoneal injection (IP) of a mixture of ketamine (6 mg/100 g body weight) and medetomidine (0.025 mg/100 g body weight) diluted in saline.
For cystometries and terminal handling animals received a subcutaneous injection of urethane (1,2g/kg). Bladder inflammation was induced by a single IP injection of cyclophosphamide (CYP; 200mg/kg) (Baxter Medico Farmacêutica, Portugal). Botulinum toxin type A (Onabot/A) was purchased from Allergan, Irvine, California. Rabbit anti c-Fos was obtained from Calbiochem, UK. Rabbit anti-phospho ERK1/2 protein was obtained from Neuromics, USA. Biotin-conjugated swine anti-rabbit was purchased from Dakopatt A/5, Denmark. The avidin-biotin complex was purchased from Vector, UK. Rabbit anti-GAP43 was obtained from Abcam, UK. Anti-cleaved SNAP-25 was a kind gift from Professor Ornella Rossetto from University of Padova, Italy. Fluorochrome labeled secondary antibodies were obtained from Molecular Probes Europe.

Catheter implantation: Animals (n=6 per experimental group) were deeply anaesthetized and a silicon catheter was implanted in the lumbar subarachnoid space as described in previous studies (113). A laminectomy was performed between T9 and T10, the catheter was inserted and pushed until the tip reached the L5/L6 spinal cord segment. The other tip of the catheter was externalized at the forehead for posterior drug delivery. Animals were allowed to recover for at least 5 days, during which they were carefully monitored. Data from rats with the catheter in an inappropriate position were excluded from the study.

Drug administration: CYP or its vehicle was administered IP at day 0. Forty-eight hours after CYP administration, 5 Units of Onabot/A diluted in 50 µl of saline or saline alone was administered IT. Animals were divided into four experimental groups: (1) Saline IP + IT, (2) Saline IP + Onabot/A IT, (3) CYP + saline IT, (4) CYP + Onabot/A IT.

Behavioral Tests: All animals were daily handled, prior to behavioral testing, to avoid stress and fear motivated behaviors. To assess cutaneous mechanical sensitivity the Von Frey test was used. Rats were placed in individual observation chambers atop a metal mesh floor and allowed to acclimatize for 15 min. Cutaneous sensitivity was assessed in the lower abdomen and in the hindpaws using von Frey monofilaments (rated at 2, 4, 6, 8, 15, 26, 60 and 100g). Filaments were applied for 5 s perpendicularly to the plantar surface and tested 5 times with an interval of 30 s between filaments. A response was considered positive when the animal reacted to the filament (paw withdrawal or licking) in at least three out of the five filament applications. The test was performed at day 0 before any procedure and 4, 24, 48 and 72 h after CYP administration or its vehicle.

In addition, the open field test was performed to seek for any motor impairment that could have been caused by surgery or toxin administration. Experiments were conducted in a standard open-field maze using a 50x50 cm arena with opaque walls 30 cm high. During the test the animals were allowed to explore the maze environment for 5 minutes. An overhead video tracking system (CinePlex,Plexon Inc., Dallas, TX, USA) was used to provide information about the animal position. Custom MatLab (MathWorks, MatLab release 2009a) and Python (version 2.7) in-house software were used to calculate navigation maps.

Cystometries: Animals were anaesthetized and maintained at 37^{o}C using a heating pad. A low abdominal middle incision was performed to expose the bladder. To measure bladder reflex activity a 21-gauge needle connected to an infusion pump and to a pressure transducer was inserted in the bladder dome. Animals were left untouched for 15-30 min to allow bladder stabilization. After that time, saline was infused at a constant rate of 6 ml/h and intraluminal pressure recorded for 1 hour. The cystometrograms were analyzed using the LabScribe software from World Precision Instruments.

Tissue processing: After cystometry animals were perfused through the ascending aorta with calcium free Tyrode's solution (NaCl 0.12M; KCl 5.4 mM; $MgCl_2.6H_2O$ 1.6 mM; $MgSO_4.7H_2O$ 0.4M; $NaH_2PO_4.H_2O$ 1.2 mM; glucose 5.5 mM; $NaHCO_3$ 26.2 mM) followed by 4% paraformaldehyde in 0.1M phosphate buffer. During tissue collection, the position of the intrathecal catheter was verified. Bladder, spinal cord and submandibular salivary glands were collected, post-fixed for 4 hours and stored overnight in 30% sucrose in phosphate buffer 0.1M. Serial transverse sections were cut in a cryostat (20 µM for bladder and 40 µM for spinal cord) and collected on Superfrost Plus slides or free-floating on cryoprotective solution. Slices were stored at -20^{o}C until further processing. For immunofluorescence, slices were thawed,

washed with phosphate buffer containing 0.3% Triton X-100 (PBST) and blocked with 10% normal horse serum (NHS) in PBST for one hour. Primary antibodies (anti-GAP43 1:5000 and anti-cleaved SNAP-25 1:4000) were diluted in 2% NHS in PBST and incubated for 2 days at 4^{o}C. After several washes they were detected according to species specificity using Alexa Fluor® labeled antibodies. Sections were mounted in Vectashield mounting medium and observed using a Zeiss microscope (Axioimager Z1). Representative images were collected using the AxioVision 4.6 software. A minimum of 8 sections per animal was used and the average intensity of immunofluorescence for GAP43 analyzed in the dorsal horns using the Fiji software (114). Background intensity was subtracted from the average intensity. Detection of c-Fos and p-ERK immunoreactivity was visualized using the ABC method (79). After inhibition of endogenous peroxidase activity, slices were washed in PBS and PBST and incubated in 10% normal swine serum (NSS) in PBST for 1 hour. c-Fos antibody was diluted 1:10000 and p-ERK antibody diluted 1:1000, both in 2% NSS in PBST for 48h. Slices were washed and incubated with swine anti-rabbit biotin conjugated antibody (1:200). Immunoreaction was visualized with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris HCl pH 7.4 plus 0.003% hydrogen peroxide. Sections were mounted, air-dried for 12h, cleared in xylene and cover-slipped. Representative images were collected in a Zeiss Axioscope 40 microscope using the AxioVision 4.6 software. Immunoreactive cells for p-ERK or c-Fos were counted in at least 8 sections per animal and averaged.

Statistical analysis: Statistical analysis of data regarding cystometrogram parameters, open field behavior and immunohistochemistry was performed using the one-way ANOVA with Tukey's multiple comparison test. Data are presented as mean +/- standard deviation and $p \leq 0.05$ was considered to be statistically significant. Von Frey results were analyzed using a two-way ANOVA with Tukey's multiple comparison test. All statistical analysis was performed using the Graph Pad Prism software.

Results

1. **Intrathecal administration of Onabot/A cleaves SNAP-25 in the spinal cord and in the urinary bladder**

The immunohistochemical detection of cleaved SNAP-25 was used to verify the activity of Onabot/A via intrathecal administration. Spinal cord segments from C2 to S1 were analyzed in terms of the cleaved protein in order to observe the toxin diffusion. Cleaved SNAP-25 immunoreactivity (IR) was detected in lumbosacral sections from animals that received IT Onabot/A (Figures 1, B-D), whereas no IR was found in other experimental groups (Figure 1, A). Onabot/A lead to the appearance of a robust cSNAP-25 IR in nerve fibers of the L6 segment where the tip of the intrathecal catheter terminated. Cleaved SNAP-25 IR on the L6 segment was abundant in the dorsal horn, where most of the urinary bladder afferents reach the spinal cord, but also through all the peripheral area of the segment (Figure 1, B). Some labeled fibers penetrated deeper in laminae III and IV with long axons projecting through lamina X. Labeled fibers were also found in the ventral horn around the cell bodies of motor neurons and in the intermediolateral gray matter (ILG) (Figure 1, C). The IR observed in the periphery of the sections was still present until L1 but was occasional or absent in thoracic and cervical segments. In addition, to exclude a systemic effect of the toxin, the salivary glands, which are highly innervated by cholinergic fibers, were also analyzed. No cleaved SNAP-25 fibers were observed in those glands (data not shown).

Finally, as it is known that Onabot/A can have a long-distance effect due to axonal transport of the toxin and since bladder projections were being affected, bladder sections were also immunoreacted. A few IR-fibers for cSNAP-25 were found scattered in the detrusor muscle along the smooth muscle bundles (Figure 1, D). Cleaved SNAP-25 IR was detected 24 hours after Onabot/A administration and maintained with similar levels of expression for at least 30 days.

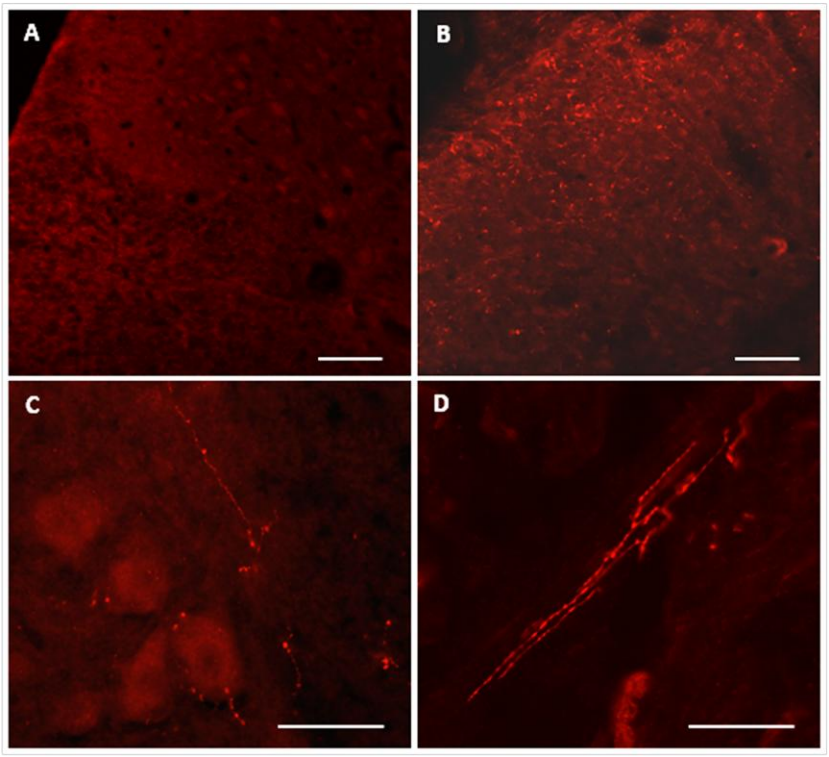

Figure 1: Cleaved SNAP-25 immunoreactivity after IT administration of Onabot/A. Control reaction showing no immunoreactivity for cleaved SNAP-25 in a saline injected animal (A). Nerve fibers labeled for cleaved SNAP-25 were detected in the superficial laminae of the L6 dorsal horn (B), around the motor neuron cell bodies of the ventral horn (C) and around the smooth muscle bundles of the bladder wall (D) after administration of intrathecal Onabot/A. Magnification bars represent 50 µm.

2. Intrathecal administration of Onabot/A reduces pain behavior induced by bladder inflammation

Animals injected with CYP showed altered behavior characterized by rounded back, immobile posture and closed eyes as previously described by other studies (115, 116). Accordingly, inflamed bladders analyzed at the end of the experiment showed evident signs of inflammation including petechial hemorrhage on the bladder serosal surface (115). CYP-induced inflammation is usually associated with an increased cutaneous sensitivity as well as referred visceral pain that can be assessed in the hindpaws and in the lower abdominal area (117). In the present work, the Von Frey test was used to analyze the cutaneous sensitivity and was performed in all experimental groups. Baseline values were obtained 1 week after catheter implantation and prior to any further experimentation. No differences were observed in animals receiving IP saline irrespective of the intrathecal treatment. For this reason, both groups were considered together and are represented in the graph as the non-inflamed controls group (Figure 2, A and B). The withdrawal thresholds in non-inflamed animals were

similar in both hindpaws and abdomen and were maintained almost constant for the overall time course. Immediately after baseline evaluation, animals received a CYP injection or its vehicle and the Von Frey test was performed again at 4, 24 and 48 hours after IT injection of saline or Onabot/A. A significant decrease in the mechanical threshold was detected in the hindpaw and in the abdomen of CYP-injected animals treated with IT saline, starting from 4H after CYP and not being altered until the end of the experiment (Figure 2, A and B). A significant improvement was observed 24H after Onabot/A injection in CYP-inflamed animals. In these animals, mechanical threshold increased to levels similar to control animals. The surgery alone and the IT injection of Onabot/A by itself did not alter the hindpaw and the abdomen sensitivity (Figure 2, A and B).

The open field test showed that control animals (naïve or sham-operated animals) walked similar distances of approximately 3,5m/min while exploring the arena. Animals submitted to intrathecal delivery of Onabot/A exhibited similar behavior, walking similar distances. No limping or any other deficits in the motor coordination of the limbs were observed caused by either surgery of catheter implantation or by the Onabot/A injection (Figure 2, C).

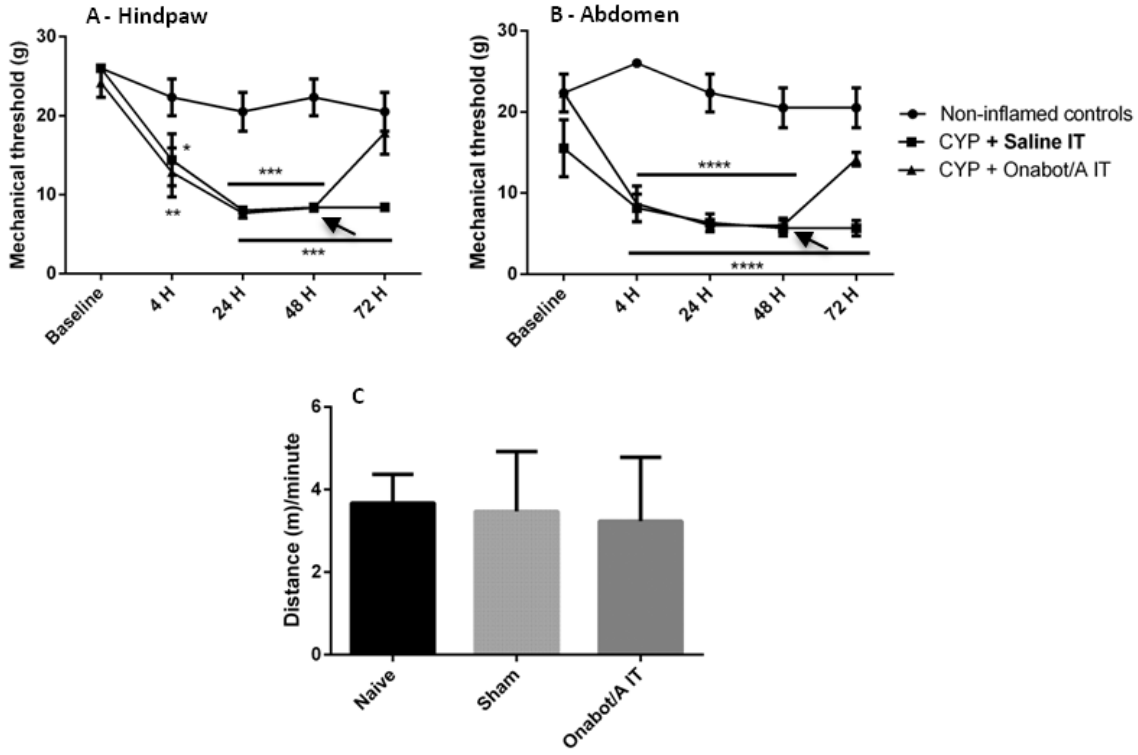

Figure 2: Pain behavior associated with CYP-induced inflammation. Intrathecal administration of saline or Onabot/A in non-inflamed animals did not induce any alteration in the mechanical sensitivity in the abdomen and hindpaw. For this reason and for increased clarity of the graph they were grouped together and are represented as the non-inflamed controls group. Animals receiving CYP injection showed a significant decrease in the mechanical threshold in both areas analyzed. Onabot/A IT

administration (arrows) increased the threshold to levels similar to the non-inflamed controls (Figure 2, A and B). Asterisks indicate significant differences between the CYP-inflamed and non-inflamed animals (*p≤0.05, **p≤0.01, *** p≤0.001 and ****p≤0.0001). The open field test showed no differences in the distance traveled by naïve, sham-operated or Onabot/A injected animals (Figure 2, C). Error bars show the SEM.

3. Intrathecal administration of Onabot/A decreases urinary frequency induced by bladder inflammation

On the last day of experiments, cystometric recordings were obtained from all experimental groups. Intact animals that received saline or Onabot/A showed normal reflex activity (0.35±0.04 and 0.47±0.05 contractions/min, respectively) (Figure 3, A and B). CYP-induced inflammation increased urinary frequency (0.82±0.09 contractions/min) and reduced the intercontraction intervals (Figure 3, C and E). On the other hand, IT Onabot/A administrated to CYP-inflamed animals counteracted the increased urinary frequency associated with the CYP injection (0.41±0.28 contractions/min) (Figure 3, D and E). Macroscopically, no differences were observed in the size of the bladders that could suggest an increased post-void residual volume after toxin administration. Also, no differences were found in other cystometric parameters analyzed (peak pressure and area under the curve) (Figure 3, F and G).

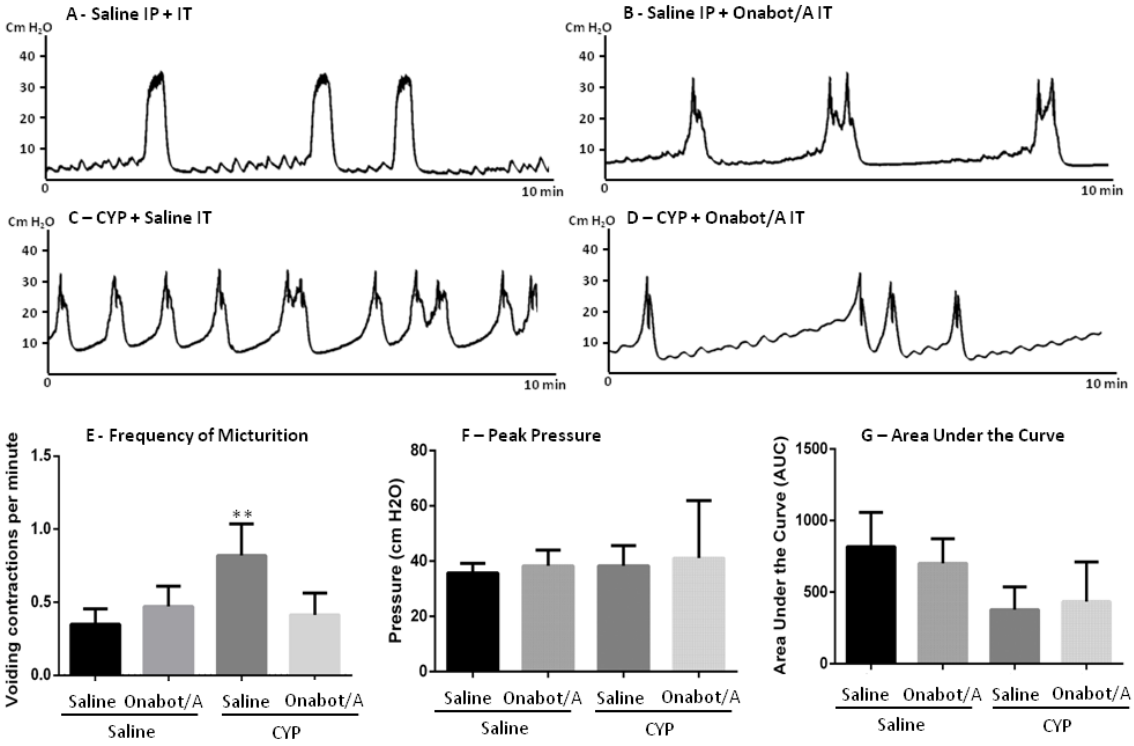

Figure 3: Cystometrograms depicting urinary bladder reflex activity. Non-inflamed animals treated with Saline (A) or Onabot/A (B) exhibited normal reflex activity. Inflamed animals showed an increased urinary frequency (C). Onabot/A IT administration in inflamed animals decreased the urinary frequency to values similar to the non-inflamed animals (D). Urodynamic parameters evaluated during cystometry procedure are represented in the graphs E to G. Evaluation of the micturition frequency showed increased activity in the inflamed group without toxin administration (E). No differences were found in the peak pressure (F) and area under the curve (G) among experimental groups. Data is represented as mean ± SEM. **P < 0.01 was considered statistically significant.

4. Intrathecal administration of Onabot/A reduces c-Fos and p-ERK immunoreactivity induced by bladder inflammation

In normal animals a modest amount of p-ERK IR cells (14.99±1.11) were observed bilaterally in laminae I-II, in the dorsal commisure (DCM, around lamina X) and in the intermediolateral grey matter (ILG) as already showed by previous studies (113) (Figure 4, A and E). IR occurred in both the nucleus and the cytoplasm as well as along the neuronal processes. The injection of Onabot/A by itself did not increase the number of IR cells in any area of the sections (13.69±1.13) (Figure 4, B and E). The basal activity of p-ERK, induced by cistometry, was substantially increased in CYP animals (31.66±4.97) (Figure 4, C and E). In these, IR cells exhibited long axons that crossed the lamina X until the other side of the section. However, the injection of Onabot/A in animals with CYP-induced inflammation showed a decrease of IR cells throughout the L6 segment to levels similar to the controls (19.82±2.14) (Figure 4, D and E).

As described before (118), c-Fos expression was detected in the lumbosacral spinal cord mainly in the lamina I, in the ILG and in the DCM (41.8±3.37) (Figure 5, A, B and E). Onabot/A injection in saline animals did not change the basal number of c-Fos cells (49.17±3.35). CYP-induced inflammation significantly increased the number of IR cells throughout the described areas (100.4±11.97) (Figure 5, C and E). As observed above, the injection of Onabot/A in animals with CYP-induced inflammation showed a decrease of IR cells throughout the L6 segment to levels similar to the controls (58.64±5.18) (Figure 5, D and E).

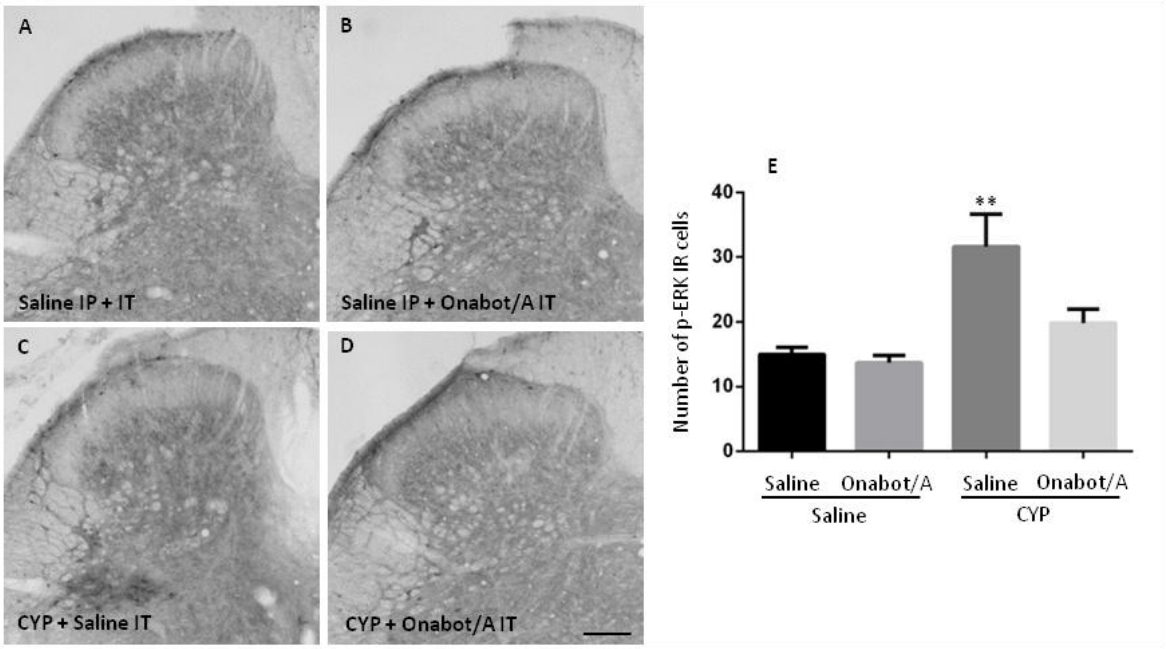

Figure 4: Photomicrographs of the L6 spinal cord segment showing the distribution of p-ERK immunoreactive cells. Animals that received an intrathecal injection of either saline or Onabot/A exhibit similar number of p-ERK IR cells on the spinal cord (A and B). The number of IR cells was significantly increased in animals with CYP-induced inflammation (C). Administration of Onabot/A in inflamed animals showed that the number of p-ERK IR cells was similar to that found in non-inflamed animals (C and D). Graph depicts the average number or p-ERK IR cells found in the L6 spinal cord segment (E). Data is represented as mean ± SEM. **P < 0.01. Magnification bars represent 50uM.

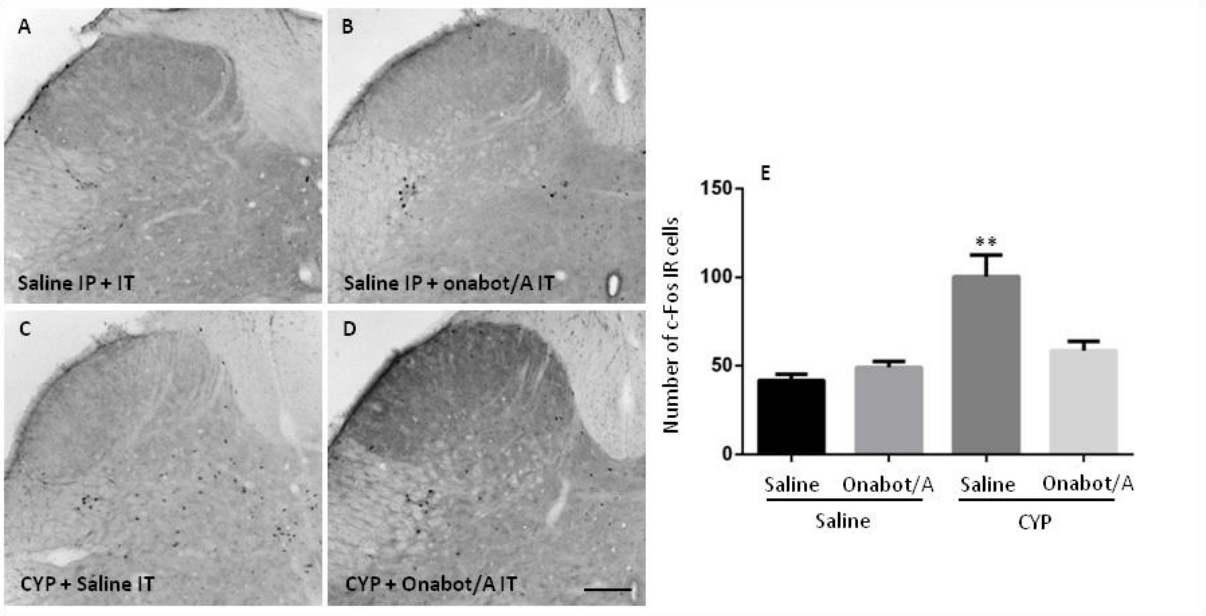

Figure 5: Photomicrographs of the L6 spinal cord segment showing the distribution of p-ERK immunoreactive cells. Animals that received an intrathecal injection of either saline or Onabot/A exhibit similar number of c-fos IR cells on the spinal cord (A and B). The number of IR cells was significantly

increased in animals with CYP-induced inflammation (C). Administration of Onabot/A in inflamed animals showed that the number of c-fos IR cells was similar to that found in non-inflamed animals (C and D). Graph depicts the average number or c-fos IR cells found in the L6 spinal cord segment (E). Data is represented as mean ± SEM. **P < 0.01. Magnification bars represent 50uM.

5. Intrathecal administration of Onabot/A did not alter GAP43 immunoreactivity

In normal animals, the L6 segment of the spinal cord exhibits a discrete IR for GAP43 in nerve fibers of dorsal horn superficial laminae, in the DCM dorsally to the central canal, in the dorsolateral funiculus, in the corticospinal tract and in the ILG (Figure 6, A and E). In CYP-inflamed animals, the intensity of GAP43 IR is clearly increased in the described areas (Figure 6, C and E). IT delivery of Onabot/A, in either saline or CYP-inflamed animals, did not alter the expression of GAP43 (Figure 6, B, D and E).

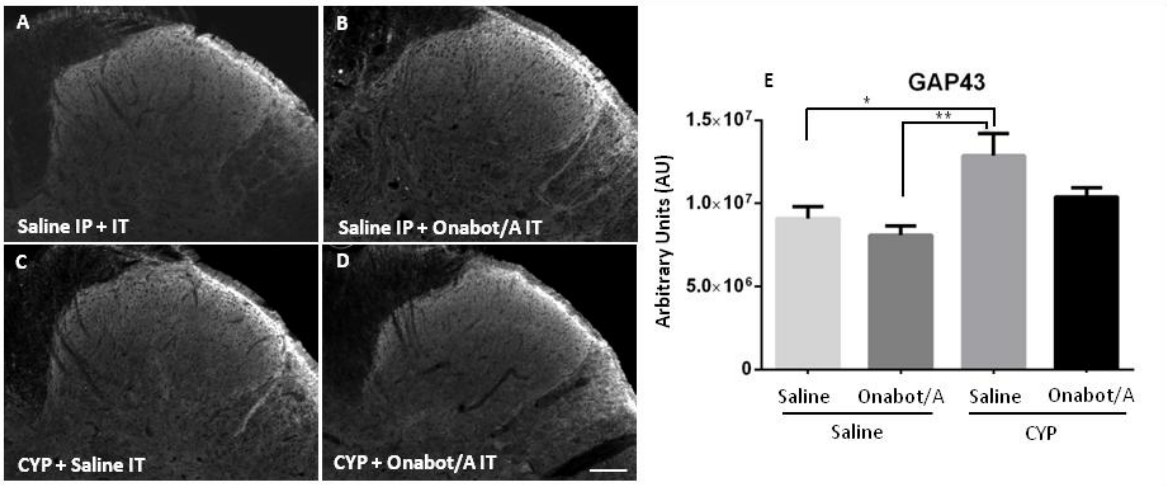

Figure 6: Distribution of GAP43-IR in the L6 segment by fluorescence microscopy. GAP43-IR intensity is increased during CYP-induced inflammation (C) when compared to animals without inflammation (A and B). After IT delivery of Onabot/A the intensity of GAP43-IR was maintained high in levels similar to the CYP-inflamed animals (D). The average intensity of IR is graphically represented for each experimental group (E). Data is represented as mean ± SEM. *p≤0.05, **P < 0.01.

Discussion

In the present study we used the CYP-induced model of bladder inflammation that mimics bladder pain observed in BPS/IC patients. CYP is metabolized in the liver to acrolein, an irritant compound that is excreted in the urine, eliciting an extreme bladder inflammation. CYP-inflamed animals showed clear signs of pain such as rounded back posture, piloerection, closed eyes and lowered mechanical sensitivity (119). Intrathecal delivery of Onabot/A in animals with CYP-induced inflammation rapidly improved pain behavior and cutaneous sensitivity in the abdomen and hindpaws. Our results are, therefore, in line with recent studies that suggested an antinociceptive effect of the toxin after its IT administration. Indeed, the IT administration of Onabot/A reduced the formalin-induced nociceptive responses in mice during 21 days (85, 111, 112). Likewise, it significantly decreased thermal and mechanical hypersensitivity induced by diabetic neuropathy, an effect that started within 24 hours and lasted for almost one month (111). On the other hand, our results also corroborate other studies showing the peripheral administration of Onabot/A exerting an indirect effect on the central nervous system. Cui *et al* showed that Onabot/A administration in the rat hindpaw decreased inflammatory pain induced by formalin, along with a decrease of Fos-like immunoreactive cells in the dorsal horn (85).

Concerning urinary bladder reflex activity, the IT administration of Onabot/A counteracted the increased urinary frequency associated with CYP-induced inflammation without causing urinary retention. At the best of our knowledge, this is the first study that shows an effect of IT delivery of toxin on bladder function.

In addition to these physiological findings, we also found changes in the expression of cleaved SNAP-25 as its immunohistochemical detection has been used as an indicator of Onabot/A activity (120). Nerve fibers labeled with cleaved SNAP-25 were primarily detected in the L6 segment of the spinal cord. Immunoreactivity was found mainly in the periphery of the sections but also in laminae I and II, in some fibers deeper in the dorsal horn and in the ventral horn, in fibers surrounding the cell bodies of motor neurons. IR gradually decreased in spinal cord segments more rostral or caudal than L6 being scarce in the thoracic and sacral segments and inexistent in the cervical segments. This strong IR in the cord periphery could be explained by toxin diffusion, since this area is in contact with the cerebrospinal fluid. A systemic effect was excluded since observation of salivary glands, highly innervated by cholinergic fibers, did not show any cleaved SNAP-25 IR. On the other hand, some labeled fibers were detected in the urinary bladder coursing the smooth muscle bundles, a fact that requires another explanation rather than diffusion. The presence of cleaved SNAP-25 in the urinary bladder

could only be explained if axonal transport occurred. Actually, this hypothesis has been already suggested in other studies, which found cleaved SNAP-25 in areas distant from the delivery site of Onabot/A that was suppressed by colchicines (104, 105, 121-123). However, in the majority of studies, the axonal transport was retrograde from the peripheral to the central nervous system (104, 105, 123) or anterograde but in a small distance from the optic tectum to retinal cells (124). In the present work we showed for the first time that the axonal transport is bi-directional since it also occurs from the CNS to the periphery.

Furthermore, we verified the effect of Onabot/A in the pain-related sensory pathways where ERKs activation and Fos expression occur. The appearance of phosphorylated ERKs occurs in the spinal cord after somatic and visceral noxious stimulation (113). Likewise, the detection of the immediate early gene c-Fos is commonly used to detect noxious sensory input generated in the bladder and conveyed to the spinal cord and its activity correlates with ERK activation (125). We showed that Onabot/A decreased the CYP-induced expression of c-Fos and phosphorylated ERK, suggesting an indirect effect of the toxin. Botulinum toxin affects the anchoring of fusion vesicles with the plasma membrane. It affects channel availability by decreasing the trafficking of at least a well known receptor implicated in pain pathways, the TRPV1 receptor (84). If the insertion of this receptor in the plasma membrane is decreased, it could be speculated that, through this pathway, botulinum toxin decreases neuronal activity in the spinal cord. Thus, our data corroborate previous results showing a pronounced effect of the toxin in central sensory pathways, justifying the beneficial effects observed in pain and urodynamic parameters. Additionally, all these observations are temporally correlated with the appearance of cleaved SNAP-25 in the spinal cord suggesting that the protein cleavage might be the trigger that initiates all other outcomes.

No effects were observed in the GAP43 IR upregulation after Onabot/A administration. GAP43 is a neuronal growth-associated protein that is usually up-regulated during neuronal injury, regeneration and synapse formation (126). An increase of GAP43-IR has been described in the lumbosacral segments of the spinal cord in rat models of bladder inflammation (127). Thus, we confirmed the distribution of this protein and analyzed if Onabot/A could alter GAP43 expression. We found that the expression of this marker was increased in the L6 segment during bladder inflammation possibly due to an attempt of sensory fibers to recover from the injury caused by bladder inflammation (127). However, Onabot/A did not counteract this effect suggesting that the factors responsible for GAP43 upregulation during inflammation are not dependent on exocytosis but rather on non-vesicular pathways.

To discard any negative effect on the somatic motor function caused by the intrathecal placement of the catheter or the toxin administration, the open field test was performed. No

impairments were observed since animals walked similar distances at similar speed while exploring the field. This is in accordance with other studies which performed the rota-rod test to check the neurotoxicity of Onabot/A IT administration, showing no differences when compared to controls (112).

Conclusion:

We verified that Onabot/A IT administration has a powerful antinociceptive effect in an animal model of bladder pain. The toxin acts by direct cleavage of SNAP-25 protein in the CNS but also through an indirect effect on central pain pathways. This intrathecal route of toxin delivery should be further studied due to its potential application in cases of intractable pain.

Acknowledgments:

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Final Considerations

1. Distribution of SV2 and SNAP-25 in the human bladder

The first important outcome of the present thesis was the mapping of SV2 and SNAP-25 expression in the human bladder (Publication I). So far, this was the first systematic and detailed description of both proteins in this organ. The only previous study of SNAP-25 in the urinary bladder was limited to small biopsies of bladder dome from NDO patients (128). In the present work both proteins were shown to be present exclusively in nerve fibers throughout the whole organ suggesting that an entire neuronal net can be potentially targeted by BoNT/A. As the urinary bladder is innervated by three different neuronal populations it would be important to analyze to which population the targeted fibers belonged.

The expression of SV2 and SNAP-25 was observed in parasympathetic, sympathetic and sensory fibers. Ninety-five percent of the parasympathetic fibers population possessed SV2 and SNAP-25 which was not surprising since Onabot/A injections in the human bladder decrease bladder frequency (73). This is in accordance with the BoNT/A effect on the striated muscle where cholinergic terminals are affected and muscular contraction impaired (34). This fact might also indicate that parasympathetic fibers are more susceptible to BoNT/A because its activity is required to initiate the detrusor voiding contractions and maintain the tonic activity. In addition a permanent sympathetic fiber activity might occur to promote detrusor relaxation. This connection between nerve activity and the degree of intoxication has already been addressed in the rat phrenic nerve where a higher nerve fiber activity led to faster nerve intoxication (24). Furthermore, both SV2 and SNAP-25 proteins were found in sympathetic and sensory fibers in considerable amounts, 69% and 58%, respectively. This result corroborates previous studies where the toxin was able to decrease the release of neuropeptides and transmitters typically present in non-cholinergic fibers (90).

Another important aspect of the first work was the absence of SV2 or SNAP-25 expression in the urothelium of human bladders. This is a controversial finding since other authors detected both SNAP-25 and SV2 in urothelial cells (97, 98). In both studies their presence was observed by RT-PCR, a technique considered more sensible than immunohistochemistry, which could explain the different results. However, their expression was very low and did not prove a direct effect of the toxin in these cells. In addition, technical issues could also be the reason for such difference since SNAP-25 was detected in mucosa samples that might include nerve endings and in urothelial cell lines that could differentiate during culture (Birder L, in press).

Hence, the results of publication I, demonstrated for the first time that all types of urinary bladder nerve fibers are potential targets of the toxin action.

2. Distribution of the cleaved form of SNAP-25 after botulinum toxin administration

Although the essential proteins for Onabot/A action were expressed throughout the human bladder there was no direct evidence that all the nerve populations were actually affected if the toxin was applied. Thus, our next step was the histological analysis of urinary bladders previously injected with Onabot/A. For this purpose, guinea pig bladders were used since it was impossible to perform this analysis in human bladders (Publication II).

First, we confirmed if the expression of SV2 and SNAP-25 was in accordance with what had been observed in our previous work on the human bladder. Indeed, both proteins showed a distribution throughout the organ and expression levels in the three neuronal populations similar to that in human bladders.

The effect of Onabot/A on the guinea pig bladder was detected through the analysis of the cleaved form of SNAP-25. It is well established that BoNT/A cleaves SNAP-25 (6) and this cleaved form had been detected before in cultured cells (18) and human bladder biopsies (59) after treatment with BoNT/A. Thus, we considered the detection of cleaved SNAP-25 a reliable and accurate approach to study the activity of Onabot/A.

After Onabot/A administration in guinea pig bladders, a detailed analysis of all bladder structures was carried out. Cleaved SNAP-25 was detected throughout the whole detrusor muscle and on the three neuronal populations studied. Parasympathetic fibers were the most affected as 85% of them expressed cleaved SNAP-25. Sympathetic and sensory fibers also expressed the cleaved form but in lower amounts (42% and 36%, respectively), showing an widespread effect of the toxin on the three neuronal populations.

The effect of the toxin was shown to be very fast since the maximum density of affected fibers was observed within 24 hours and lasted for at least 7 days without changes (Publication II).

These results clearly support recent basic and clinical evidences that suggest an effect of BoNT/A on all urinary neuronal pathways. BoNT/A is known to target efferent pathways, blocking ACh release and consequently decreasing smooth muscle contractility. This effect can be very pronounced as it causes urinary retention in some NDO patients (63, 64). BoNT/A effect on sensory pathways is already well supported since it improves urgency symptoms in OAB patients (61-64) as well as bladder pain in BPS/IC patients (66). Finally, its effect on the sympathetic pathways is still a matter of debate. However, recent studies already showed a decrease on the urinary noradrenaline levels in BPS/IC patients treated with Onabot/A (129).

3. Diffusion of botulinum toxin through the urinary bladder

During the last years, many clinical trials have been performed concerning the beneficial effects of Onabot/A in LUT disorders. However, different protocols were used without a systematic study concerning site of injections, number of injections, dilutions or toxin doses leaving several to be answered (67). As an example of how important injections can be, recently, Kuo *et al* compared the therapeutic effects of different intramural injections. His patients reported the same outcome after detrusor, submucosal or trigonal injections regarding detrusor overactivity. However, injections on the trigone rather than the dome relieved urgency symptoms without affecting bladder capacity (130).

In publication II we aimed to clarify some of these questions by addressing two important issues that were ambiguous. We started to address the effect of Onabot/A administered through two different approaches already tested in clinical trials: the intramural injection and the intravesical instillation. Animals who received an intramural injection of toxin showed fibers labeled with cleaved SNAP-25 throughout the whole bladder, as described in the section above. On the other hand, animals that underwent intravesical instillation of the toxin showed no labeled fibers, neither in the urothelium, nor in the detrusor. This finding could be controversial since many studies reported beneficial effects of Onabot/A after intravesical instillation (68, 131, 132). However, it should be noted that in those studies the urothelial barrier was disrupted by either protamine sulfate (131) or cyclophosphamide (68), eventually allowing the contact of toxin with the bladder interstitium. Since we never observed SV2 or SNAP-25 in the urothelium and considering that Onabot/A has a high molecular weight we could speculate that urothelial disruption would be the only mechanism allowing the contact of the toxin with bladder nerve terminals.

The second issue we addressed was the effect of the injected volume in the activity and diffusion of the toxin. Thus, we evaluated the diffusion of a single Onabot/A injection using two different volumes. We found that using the same dose in a higher volume more fibers containing cleaved SNAP-25 could be detected. Additionally, it was also observed a higher spread throughout the organ. These results show that the volume is a key factor to be considered when performing Onabot/A injections which is in accordance with similar studies performed in striated muscle where bigger volumes of injection induced more relaxation of the muscle due to a higher diffusion of toxin (133, 134).

Publication II showed relevant findings for both basic and clinical research fields. The finding that Onabot/A cleaves SNAP-25 predominantly in cholinergic nerves but also affects

the other neuronal populations added more information about the mechanism of action of the toxin. From the clinical point of view, the finding that the injection of larger volumes of the same toxin dose increased Onabot/A diffusion and efficacy is of extreme importance for the design of future clinical trials.

4. Effect of Onabot/A on the bladder intramural ganglia

The advantage of using the guinea pig as a model instead of the commonly used rat is related with its LUT anatomy. In the guinea pig, as in humans, the parasympathetic ganglia that receive efferent information from supraspinal areas are embedded in the bladder wall (135). Here, preganglionic axonal terminals establish synapses with the postganglionic cell bodies that innervate the detrusor. Both nerve terminals release ACh and are located within the bladder wall. Sympathetic and sensory innervation also surrounds the ganglia, exerting inhibitory or excitatory effects that might modulate its activity (136). Therefore, it would be expected that the intramural administration of botulinum toxin would affect more than one cholinergic synapse, a double-action that would contribute to the final effect of the toxin. Actually, the effect of ganglioplegic agents that block nicotinic transmission in the human bladder has strong consequences on bladder function. These agents easily cause urinary retention, a consequence rarely seen if only the postganglionic muscarinic transmission is impaired (137, 138).

In Publication III we started to identify the location of the three neuronal populations around the ganglia. Parasympathetic fibers were found coursing the ganglia and impinging to the ganglionic cell bodies. Sympathetic and sensory fibers were scarce and found mainly around the ganglia. Further, SV2 and SNAP-25 was observed in the in nerve fibers coursing the ganglia with a total co-localization. In contrast to what was observed in the nerve fiber network throughout the organ, after Onabot/A administration, cleaved SNAP-25 was detected exclusively in parasympathetic fibers.

The results obtained showed that Onabot/A in the urinary bladder affects preganglionic and postganglionic terminals at the same time. Since both nicotinic and muscarinic transmission might be impaired, it is expected that the toxin action, as it happens with the ganglioplegic agents, exerts a stronger effect in the detrusor. Based on these results, we could speculate that this double inhibition could explain the longer duration of effect of Onabot/A in the smooth muscle when compared to the striated muscle. However, this hypothesis should be taken with caution since a direct correlation between both facts was not performed.

It is well known that sympathetic and sensory fibers found inside parasympathetic ganglia modulate nicotinic parasympathetic transmission. Botulinum toxin might be expected to impair sympathetic and sensory fibers, however, the paucity of their number makes a definitive statement of their function after toxin application impossible.

5. Effect of Onabot/A during bladder inflammation

The antinociceptive effect of botulinum toxin has been a major topic of pain research in the last years. Recent studies showed that peripheral administration of Onabot/A in the rat hindpaw was able to reduce symptoms of both inflammatory and neuropathic pain (85, 123). Concerning the urinary bladder, intramural injection of Onabot/A reduced pain symptoms and improved urodynamic parameters along with a reduction in the urinary levels of NGF and BDNF in refractory BPS patients (66).

Recently, intrathecal (IT) administration of botulinum toxin type A was shown to be effective in the treatment of peripheral pain. Actually, Onabot/A administered intrathecally in mice, attenuated inflammatory pain induced by formalin for 21 days (112). In addition, it significantly decreased thermal and mechanical hypersensitivity induced by diabetic neuropathy, an effect that started within 24 hours and lasted for almost one month (111). These improvements in peripheral pain were attributed to the decreased spinal cord expression of CGRP, p-ERK and p-CaMK (112) thus suggesting a central antinociceptive effect of the toxin.

In the present study we investigated the effect of IT Onabot/A in a well established animal model of bladder inflammatory pain. In these animals, nociceptive behaviour and mechanical sensitivity are increased, as we confirmed with the Von Frey test. In addition, bladder reflex activity of inflamed animals is significantly altered, with an increased frequency and decreased maximum bladder capacity. This phenomenon is attributed to an increased activity of nociceptive primary afferents (139). The IT administration of Onabot/A counteracted the effect of bladder inflammation, decreasing the mechanical sensitivity of the hindpaw and abdomen as well as the urinary frequency, to levels similar to the controls.

Furthermore, we investigated the presence of cleaved SNAP-25 in the L6 segment of the spinal cord, the main termination area of bladder primary afferent neurons. After IT delivery of toxin, cleaved SNAP-25 was observed throughout the L6 segment mainly in the periphery and in the dorsal horn but also around cholinergic neurons of the ILG.

The presence of cleaved SNAP-25 in the spinal cord has already been shown in previously. In these studies, toxin was administered in peripheral somatic locations such as the hindpaw, the gastrocnemius muscle and the sciatic nerve. Afterwards, the cleaved protein was observed along the L3 to L5 segments of the spinal cord in small cholinergic fibers with long neuronal processes and in the dorsal horn. Thus, the results suggested an axonal transport of the toxin from the periphery to the central nervous system (106). In our study, besides the appearance of cleaved SNAP-25 in the spinal cord after IT delivery of toxin, the cleaved protein was also detected in the urinary bladder. Thus, to the best of our knowledge, our studies were the first to report the axonal transport of the toxin from the CNS to the peripheral nervous system.

In our work, we also observed other effects of Onabot/A on the spinal cord. Several studies described that CYP-induced inflammation is responsible for an increased expression of CGRP, GAP43, c-fos and p-ERKs at the lumbosacral level (113, 127, 140). Thus, in addition to the previous goals, in Publication IV we also described the effect of IT Onabot/A in the expression of these markers. We first observed that the intrathecal placement of the catheter or the Onabot/A injection by themselves did not induce any alteration in the basal levels of these markers. We also confirmed a significant increase of their expression in the spinal cord of inflamed animals. Finally, we confirmed our hypothesis that locally applied Onabot/A would decrease the expression of CGRP, c-fos and p-ERKs by impairing sensory neurotransmission in animals with bladder inflammation indicating a strong analgesic effect.

In the course of our studies no effects were observed in the expression of the GAP43 protein after IT Onabot/A in inflamed bladders. This increase is in accordance with previous studies (127). GAP-43 protein is associated with functional recovery of nerve terminals from the neuromuscular junction after botulinum toxin A treatment (141) so, it could be expected that IT administration of Onabot/A would also increase its expression. However, in contrast with what was obtained in the skeletal muscle, where Onabot/A induces a strong sprouting of neuronal terminal endings, we did not detect changes in the GAP-43 IR in the spinal cord Onabot/A treated animals.

Thus, IT Onabot/A clearly has an effect on the modulation of pain pathways in both the peripheral and central levels.

Conclusions

From the results obtained in the present work, the following conclusions were withdrawn:

1. The receptor and the intracellular targets of botulinum toxin A are expressed in the three types of nerve fibers present in the human and guinea pig bladder.

2. The cleaved form of SNAP-25 could be detected in the three types of bladder nerves analyzed after intramural injection of botulinum toxin A in the guinea-pig bladder.

3. The volume is a key factor in the efficacy of botulinum toxin injections that should be considered in the improvement of clinical protocols.

4. Botulinum toxin applied intramurally in the urinary bladder affects pre and post ganglionic parasympathetic synapses. This fact might be the reason for the longer duration of the toxin effect observed in the smooth muscle.

5. Intrathecal administration of botulinum toxin improves bladder function and pain associated with chemical-induced bladder inflammation. The intrathecal delivery of botulinum toxin could be considered an alternative and effective treatment for bladder pain syndrome.

References

1. Erbguth FJ. Historical notes on botulism, Clostridium botulinum, botulinum toxin, and the idea of the therapeutic use of the toxin. Movement disorders : official journal of the Movement Disorder Society. 2004;19 Suppl 8:S2-6.
2. Erbguth FJ. From poison to remedy: the chequered history of botulinum toxin. Journal of neural transmission. 2008;115(4):559-65.
3. Hanchanale VS, Rao AR, Martin FL, Matanhelia SS. The unusual history and the urological applications of botulinum neurotoxin. Urologia internationalis. 2010;85(2):125-30.
4. Kerner J. 1822.
5. van Ermengem EP. A new anaerobic bacillus and its relation to botulism. Rev Infect Dis. 1979;1:701-19.
6. Dickerson TJ, Janda KD. The use of small molecules to investigate molecular mechanisms and therapeutic targets for treatment of botulinum neurotoxin A intoxication. ACS chemical biology. 2006;1(6):359-69.
7. Simpson LL, Schmidt JJ, Middlebrook JL. Isolation and characterization of the Botulinum neurotoxins. Methods in enzymology. 1988;165:76-85.
8. Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nature structural biology. 1998;5(10):898-902.
9. Dolly JO, Aoki KR. The structure and mode of action of different botulinum toxins. European journal of neurology : the official journal of the European Federation of Neurological Societies. 2006;13 Suppl 4:1-9.
10. Montecucco C, Schiavo G, Pantano S. SNARE complexes and neuroexocytosis: how many, how close? Trends in biochemical sciences. 2005;30(7):367-72.
11. Rossetto O, Seveso M, Caccin P, Schiavo G, Montecucco C. Tetanus and botulinum neurotoxins: turning bad guys into good by research. Toxicon : official journal of the International Society on Toxinology. 2001;39(1):27-41.
12. Gu S, Jin R. Assembly and function of the botulinum neurotoxin progenitor complex. Current topics in microbiology and immunology. 2013;364:21-44.
13. Simpson LL. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacological reviews. 1981;33(3):155-88.
14. Rummel A. Double receptor anchorage of botulinum neurotoxins accounts for their exquisite neurospecificity. Current topics in microbiology and immunology. 2013;364:61-90.
15. Montecucco C, Schiavo G, Gao Z, Bauerlein E, Boquet P, DasGupta BR. Interaction of botulinum and tetanus toxins with the lipid bilayer surface. The Biochemical journal. 1988;251(2):379-83.
16. Simpson LL, Rapport MM. The binding of botulinum toxin to membrane lipids: phospholipids and proteolipid. Journal of neurochemistry. 1971;18(9):1761-7.
17. Rummel A, Mahrhold S, Bigalke H, Binz T. The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. Molecular microbiology. 2004;51(3):631-43.
18. Dong M, Yeh F, Tepp WH, et al. SV2 is the protein receptor for botulinum neurotoxin A. Science. 2006;312(5773):592-6.

19. Mahrhold S, Rummel A, Bigalke H, Davletov B, Binz T. The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. FEBS letters. 2006;580(8):2011-4.
20. Buckley K, Kelly RB. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. The Journal of cell biology. 1985;100(4):1284-94.
21. Ahnert-Hilger G, Munster-Wandowski A, Holtje M. Synaptic vesicle proteins: targets and routes for botulinum neurotoxins. Current topics in microbiology and immunology. 2013;364:159-77.
22. Berntsson RP, Peng L, Dong M, Stenmark P. Structure of dual receptor binding to botulinum neurotoxin B. Nature communications. 2013;4:2058.
23. Matteoli M, Takei K, Perin MS, Sudhof TC, De Camilli P. Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. The Journal of cell biology. 1992;117(4):849-61.
24. Carpenter FG. Motor responses of the urinary bladder and skeletal muscle in botulinum intoxicated rats. The Journal of physiology. 1967;188(1):1-11.
25. Simpson LL. Identification of the major steps in botulinum toxin action. Annual review of pharmacology and toxicology. 2004;44:167-93.
26. Koriazova LK, Montal M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nature structural biology. 2003;10(1):13-8.
27. Rizo J, Rosenmund C. Synaptic vesicle fusion. Nature structural & molecular biology. 2008;15(7):665-74.
28. Dolly O. Synaptic transmission: inhibition of neurotransmitter release by botulinum toxins. Headache. 2003;43 Suppl 1:S16-24.
29. Kalandakanond S, Coffield JA. Cleavage of SNAP-25 by botulinum toxin type A requires receptor-mediated endocytosis, pH-dependent translocation, and zinc. The Journal of pharmacology and experimental therapeutics. 2001;296(3):980-6.
30. Keller JE, Neale EA. The role of the synaptic protein snap-25 in the potency of botulinum neurotoxin type A. The Journal of biological chemistry. 2001;276(16):13476-82.
31. Meunier FA, Schiavo G, Molgo J. Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. Journal of physiology, Paris. 2002;96(1-2):105-13.
32. Blasi J, Chapman ER, Link E, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature. 1993;365(6442):160-3.
33. Humeau Y, Doussau F, Grant NJ, Poulain B. How botulinum and tetanus neurotoxins block neurotransmitter release. Biochimie. 2000;82(5):427-46.
34. Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. Physiological reviews. 2000;80(2):717-66.
35. Filippi GM, Errico P, Santarelli R, Bagolini B, Manni E. Botulinum A toxin effects on rat jaw muscle spindles. Acta oto-laryngologica. 1993;113(3):400-4.
36. Rosales RL, Arimura K, Takenaga S, Osame M. Extrafusal and intrafusal muscle effects in experimental botulinum toxin-A injection. Muscle & nerve. 1996;19(4):488-96.
37. de Paiva A, Meunier FA, Molgo J, Aoki KR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(6):3200-5.

38. Dressler D, Benecke R. Pharmacology of therapeutic botulinum toxin preparations. Disability and rehabilitation. 2007;29(23):1761-8.
39. Mangera A, Andersson KE, Apostolidis A, et al. Contemporary management of lower urinary tract disease with botulinum toxin A: a systematic review of botox (onabotulinumtoxinA) and dysport (abobotulinumtoxinA). European urology. 2011;60(4):784-95.
40. Behr-Roussel D, Oger S, Pignol B, et al. Minimal effective dose of dysport and botox in a rat model of neurogenic detrusor overactivity. European urology. 2012;61(5):1054-61.
41. Davletov B, Bajohrs M, Binz T. Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. Trends in neurosciences. 2005;28(8):446-52.
42. Scott AB. Botulinum toxin injection of eye muscles to correct strabismus. Transactions of the American Ophthalmological Society. 1981;79:734-70.
43. Scott AB. Botulinum toxin injection into extraocular muscles as an alternative to strabismus surgery. Journal of pediatric ophthalmology and strabismus. 1980;17(1):21-5.
44. Ting PT, Freiman A. The story of Clostridium botulinum: from food poisoning to Botox. Clinical medicine. 2004;4(3):258-61.
45. Naumann M, Flachenecker P, Brocker EB, Toyka KV, Reiners K. Botulinum toxin for palmar hyperhidrosis. Lancet. 1997;349(9047):252.
46. Annese V, D'Onofrio V, Andriulli A. Botulinum toxin in long-term therapy for achalasia. Annals of internal medicine. 1998;128(8):696.
47. Griffiths DJ, Fowler CJ. The micturition switch and its forebrain influences. Acta physiologica. 2013;207(1):93-109.
48. Chu FM, Dmochowski R. Pathophysiology of overactive bladder. The American journal of medicine. 2006;119(3 Suppl 1):3-8.
49. Abrams P, Andersson KE. Muscarinic receptor antagonists for overactive bladder. BJU international. 2007;100(5):987-1006.
50. Chancellor MB, Yoshimura N. Neurophysiology of stress urinary incontinence. Reviews in urology. 2004;6 Suppl 3:S19-28.
51. de Groat WC, Yoshimura N. Afferent nerve regulation of bladder function in health and disease. Handbook of experimental pharmacology. 2009(194):91-138.
52. Kanai A, Andersson KE. Bladder afferent signaling: recent findings. The Journal of urology. 2010;183(4):1288-95.
53. Birder L, Andersson KE. Urothelial signaling. Physiological reviews. 2013;93(2):653-80.
54. Ouslander JG. Management of overactive bladder. The New England journal of medicine. 2004;350(8):786-99.
55. Dykstra DD, Sidi AA, Scott AB, Pagel JM, Goldish GD. Effects of botulinum A toxin on detrusor-sphincter dyssynergia in spinal cord injury patients. The Journal of urology. 1988;139(5):919-22.
56. Schurch B, Stohrer M, Kramer G, Schmid DM, Gaul G, Hauri D. Botulinum-A toxin for treating detrusor hyperreflexia in spinal cord injured patients: a new alternative to anticholinergic drugs? Preliminary results. The Journal of urology. 2000;164(3 Pt 1):692-7.
57. Kuo HC. Effect of botulinum a toxin in the treatment of voiding dysfunction due to detrusor underactivity. Urology. 2003;61(3):550-4.
58. Smith CP, Chancellor MB. Emerging role of botulinum toxin in the management of voiding dysfunction. The Journal of urology. 2004;171(6 Pt 1):2128-37.

59. Schulte-Baukloh H, Michael T, Sturzebecher B, Knispel HH. Botulinum-a toxin detrusor injection as a novel approach in the treatment of bladder spasticity in children with neurogenic bladder. European urology. 2003;44(1):139-43.
60. Apostolidis A, Fowler CJ. The use of botulinum neurotoxin type A (BoNTA) in urology. Journal of neural transmission. 2008;115(4):593-605.
61. Chapple C, Sievert KD, MacDiarmid S, et al. OnabotulinumtoxinA 100 U significantly improves all idiopathic overactive bladder symptoms and quality of life in patients with overactive bladder and urinary incontinence: a randomised, double-blind, placebo-controlled trial. European urology. 2013;64(2):249-56.
62. Nitti VW, Dmochowski R, Herschorn S, et al. OnabotulinumtoxinA for the Treatment of Patients with Overactive Bladder and Urinary Incontinence: Results of a Phase 3, Randomized, Placebo Controlled Trial. The Journal of urology. 2012.
63. Cruz F, Herschorn S, Aliotta P, et al. Efficacy and safety of onabotulinumtoxinA in patients with urinary incontinence due to neurogenic detrusor overactivity: a randomised, double-blind, placebo-controlled trial. European urology. 2011;60(4):742-50.
64. Ginsberg D, Gousse A, Keppenne V, et al. Phase 3 efficacy and tolerability study of onabotulinumtoxinA for urinary incontinence from neurogenic detrusor overactivity. The Journal of urology. 2012;187(6):2131-9.
65. Andersson KE. New developments in the management of overactive bladder: focus on mirabegron and onabotulinumtoxinA. Therapeutics and clinical risk management. 2013;9:161-70.
66. Pinto R, Lopes T, Frias B, et al. Trigonal injection of botulinum toxin A in patients with refractory bladder pain syndrome/interstitial cystitis. European urology. 2010;58(3):360-5.
67. Rapp DE, Lucioni A, Bales GT. Botulinum toxin injection: a review of injection principles and protocols. International braz j urol : official journal of the Brazilian Society of Urology. 2007;33(2):132-41.
68. Vemulakonda VM, Somogyi GT, Kiss S, Salas NA, Boone TB, Smith CP. Inhibitory effect of intravesically applied botulinum toxin A in chronic bladder inflammation. The Journal of urology. 2005;173(2):621-4.
69. Kessler TM, Khan S, Panicker JN, et al. In the human urothelium and suburothelium, intradetrusor botulinum neurotoxin type A does not induce apoptosis: preliminary results. European urology. 2010;57(5):879-83.
70. Haferkamp A, Schurch B, Reitz A, et al. Lack of ultrastructural detrusor changes following endoscopic injection of botulinum toxin type a in overactive neurogenic bladder. European urology. 2004;46(6):784-91.
71. Roosen A, Datta SN, Chowdhury RA, et al. Suburothelial myofibroblasts in the human overactive bladder and the effect of botulinum neurotoxin type A treatment. European urology. 2009;55(6):1440-8.
72. Apostolidis A, Jacques TS, Freeman A, et al. Histological changes in the urothelium and suburothelium of human overactive bladder following intradetrusor injections of botulinum neurotoxin type A for the treatment of neurogenic or idiopathic detrusor overactivity. European urology. 2008;53(6):1245-53.
73. Chancellor MB, Fowler CJ, Apostolidis A, et al. Drug Insight: biological effects of botulinum toxin A in the lower urinary tract. Nature clinical practice Urology. 2008;5(6):319-28.

74. Smith CP, Boone TB, de Groat WC, Chancellor MB, Somogyi GT. Effect of stimulation intensity and botulinum toxin isoform on rat bladder strip contractions. Brain research bulletin. 2003;61(2):165-71.
75. Smith CP, Franks ME, McNeil BK, et al. Effect of botulinum toxin A on the autonomic nervous system of the rat lower urinary tract. The Journal of urology. 2003;169(5):1896-900.
76. MacKenzie I, Burnstock G, Dolly JO. The effects of purified botulinum neurotoxin type A on cholinergic, adrenergic and non-adrenergic, atropine-resistant autonomic neuromuscular transmission. Neuroscience. 1982;7(4):997-1006.
77. Ikeda Y, Zabbarova IV, Birder LA, et al. Botulinum neurotoxin serotype A suppresses neurotransmitter release from afferent as well as efferent nerves in the urinary bladder. European urology. 2012;62(6):1157-64.
78. Aoki KR. Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A. Neurotoxicology. 2005;26(5):785-93.
79. Avelino A, Cruz C, Nagy I, Cruz F. Vanilloid receptor 1 expression in the rat urinary tract. Neuroscience. 2002;109(4):787-98.
80. Zhong Y, Banning AS, Cockayne DA, Ford AP, Burnstock G, McMahon SB. Bladder and cutaneous sensory neurons of the rat express different functional P2X receptors. Neuroscience. 2003;120(3):667-75.
81. Burnstock G. Purinergic signalling in the lower urinary tract. Acta physiologica. 2013;207(1):40-52.
82. Avelino A, Cruz F. TRPV1 (vanilloid receptor) in the urinary tract: expression, function and clinical applications. Naunyn-Schmiedeberg's archives of pharmacology. 2006;373(4):287-99.
83. Apostolidis A, Popat R, Yiangou Y, et al. Decreased sensory receptors P2X3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. The Journal of urology. 2005;174(3):977-82; discussion 82-3.
84. Morenilla-Palao C, Planells-Cases R, Garcia-Sanz N, Ferrer-Montiel A. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. The Journal of biological chemistry. 2004;279(24):25665-72.
85. Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. Pain. 2004;107(1-2):125-33.
86. Mika J, Rojewska E, Makuch W, et al. The effect of botulinum neurotoxin A on sciatic nerve injury-induced neuroimmunological changes in rat dorsal root ganglia and spinal cord. Neuroscience. 2011;175:358-66.
87. Radziszewski P, Crayton R, Zaborski J, et al. Multiple sclerosis produces significant changes in urinary bladder innervation which are partially reflected in the lower urinary tract functional status-sensory nerve fibers role in detrusor overactivity. Multiple sclerosis. 2009;15(7):860-8.
88. Smet PJ, Moore KH, Jonavicius J. Distribution and colocalization of calcitonin gene-related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder. Laboratory investigation; a journal of technical methods and pathology. 1997;77(1):37-49.
89. Chuang YC, Yoshimura N, Huang CC, Chiang PH, Chancellor MB. Intravesical botulinum toxin a administration produces analgesia against acetic acid induced bladder pain responses in rats. The Journal of urology. 2004;172(4 Pt 1):1529-32.

90. Rapp DE, Turk KW, Bales GT, Cook SP. Botulinum toxin type a inhibits calcitonin gene-related peptide release from isolated rat bladder. The Journal of urology. 2006;175(3 Pt 1):1138-42.
91. Lucioni A, Bales GT, Lotan TL, McGehee DS, Cook SP, Rapp DE. Botulinum toxin type A inhibits sensory neuropeptide release in rat bladder models of acute injury and chronic inflammation. BJU international. 2008;101(3):366-70.
92. Liu HT, Tyagi P, Chancellor MB, Kuo HC. Urinary nerve growth factor level is increased in patients with interstitial cystitis/bladder pain syndrome and decreased in responders to treatment. BJU international. 2009;104(10):1476-81.
93. Liu HT, Chancellor MB, Kuo HC. Urinary nerve growth factor levels are elevated in patients with detrusor overactivity and decreased in responders to detrusor botulinum toxin-A injection. European urology. 2009;56(4):700-6.
94. Smith CP, Gangitano DA, Munoz A, et al. Botulinum toxin type A normalizes alterations in urothelial ATP and NO release induced by chronic spinal cord injury. Neurochemistry international. 2008;52(6):1068-75.
95. Khera M, Somogyi GT, Kiss S, Boone TB, Smith CP. Botulinum toxin A inhibits ATP release from bladder urothelium after chronic spinal cord injury. Neurochemistry international. 2004;45(7):987-93.
96. Persson K, Igawa Y, Mattiasson A, Andersson KE. Effects of inhibition of the L-arginine/nitric oxide pathway in the rat lower urinary tract in vivo and in vitro. British journal of pharmacology. 1992;107(1):178-84.
97. Birder LA, de Groat WC. Mechanisms of disease: involvement of the urothelium in bladder dysfunction. Nature clinical practice Urology. 2007;4(1):46-54.
98. Giannantoni A, Amantini C, Proietti S, et al. Normal human urothelial cell lines express onabotulinumtoxinA SV2 high affinity receptors. European Urology Supplements. 2012;11(1):E465-U16.
99. Ravichandran V, Chawla A, Roche PA. Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. The Journal of biological chemistry. 1996;271(23):13300-3.
100. Banerjee A, Li G, Alexander EA, Schwartz JH. Role of SNAP-23 in trafficking of H+-ATPase in cultured inner medullary collecting duct cells. American journal of physiology Cell physiology. 2001;280(4):C775-81.
101. Cruz F. Targets for botulinum toxin in the lower urinary tract. Neurourology and urodynamics. 2013.
102. Wiegand H, Erdmann G, Wellhoner HH. 125I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. Naunyn-Schmiedeberg's archives of pharmacology. 1976;292(2):161-5.
103. Caleo M, Schiavo G. Central effects of tetanus and botulinum neurotoxins. Toxicon : official journal of the International Society on Toxinology. 2009;54(5):593-9.
104. Matak I, Bach-Rojecky L, Filipovic B, Lackovic Z. Behavioral and immunohistochemical evidence for central antinociceptive activity of botulinum toxin A. Neuroscience. 2011;186:201-7.
105. Restani L, Novelli E, Bottari D, et al. Botulinum neurotoxin A impairs neurotransmission following retrograde transynaptic transport. Traffic. 2012;13(8):1083-9.

106. Matak I, Riederer P, Lackovic Z. Botulinum toxin's axonal transport from periphery to the spinal cord. Neurochemistry international. 2012;61(2):236-9.
107. Santos-Silva A, da Silva CM, Cruz F. Botulinum toxin treatment for bladder dysfunction. International journal of urology : official journal of the Japanese Urological Association. 2013.
108. Abrams P, Cardozo L, Fall M, et al. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. Neurourology and urodynamics. 2002;21(2):167-78.
109. van de Merwe JP, Nordling J, Bouchelouche P, et al. Diagnostic criteria, classification, and nomenclature for painful bladder syndrome/interstitial cystitis: an ESSIC proposal. European urology. 2008;53(1):60-7.
110. Giannantoni A, Bini V, Dmochowski R, et al. Contemporary management of the painful bladder: a systematic review. European urology. 2012;61(1):29-53.
111. Bach-Rojecky L, Salkovic-Petrisic M, Lackovic Z. Botulinum toxin type A reduces pain supersensitivity in experimental diabetic neuropathy: bilateral effect after unilateral injection. European journal of pharmacology. 2010;633(1-3):10-4.
112. Lee WH, Shin TJ, Kim HJ, et al. Intrathecal administration of botulinum neurotoxin type A attenuates formalin-induced nociceptive responses in mice. Anesthesia and analgesia. 2011;112(1):228-35.
113. Cruz CD, Avelino A, McMahon SB, Cruz F. Increased spinal cord phosphorylation of extracellular signal-regulated kinases mediates micturition overactivity in rats with chronic bladder inflammation. The European journal of neuroscience. 2005;21(3):773-81.
114. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nature methods. 2012;9(7):676-82.
115. Boucher M, Meen M, Codron JP, Coudore F, Kemeny JL, Eschalier A. Cyclophosphamide-induced cystitis in freely-moving conscious rats: behavioral approach to a new model of visceral pain. The Journal of urology. 2000;164(1):203-8.
116. Auge C, Chene G, Dubourdeau M, et al. Relevance of the cyclophosphamide-induced cystitis model for pharmacological studies targeting inflammation and pain of the bladder. European journal of pharmacology. 2013;707(1-3):32-40.
117. Frias B, Allen S, Dawbarn D, Charrua A, Cruz F, Cruz CD. Brain-derived neurotrophic factor, acting at the spinal cord level, participates in bladder hyperactivity and referred pain during chronic bladder inflammation. Neuroscience. 2013;234:88-102.
118. Avelino A, Cruz F, Coimbra A. Intravesical resiniferatoxin desensitizes rat bladder sensory fibres without causing intense noxious excitation. A c-fos study. European journal of pharmacology. 1999;378(1):17-22.
119. Saitoh C, Yokoyama H, Chancellor MB, de Groat WC, Yoshimura N. Comparison of voiding function and nociceptive behavior in two rat models of cystitis induced by cyclophosphamide or acetone. Neurourology and urodynamics. 2010;29(3):501-5.
120. Coelho A, Cruz F, Cruz CD, Avelino A. Spread of onabotulinumtoxinA after bladder injection. Experimental study using the distribution of cleaved SNAP-25 as the marker of the toxin action. European urology. 2012;61(6):1178-84.
121. Bach-Rojecky L, Lackovic Z. Central origin of the antinociceptive action of botulinum toxin type A. Pharmacology, biochemistry, and behavior. 2009;94(2):234-8.

122. Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M. Long-distance retrograde effects of botulinum neurotoxin A. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28(14):3689-96.
123. Marinelli S, Vacca V, Ricordy R, et al. The analgesic effect on neuropathic pain of retrogradely transported botulinum neurotoxin A involves Schwann cells and astrocytes. PloS one. 2012;7(10):e47977.
124. Restani L, Antonucci F, Gianfranceschi L, Rossi C, Rossetto O, Caleo M. Evidence for anterograde transport and transcytosis of botulinum neurotoxin A (BoNT/A). The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(44):15650-9.
125. Cruz CD, McMahon SB, Cruz F. Spinal ERK activation contributes to the regulation of bladder function in spinal cord injured rats. Experimental neurology. 2006;200(1):66-73.
126. Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends in neurosciences. 1997;20(2):84-91.
127. Vizzard MA, Boyle MM. Increased expression of growth-associated protein (GAP-43) in lower urinary tract pathways following cyclophosphamide (CYP)-induced cystitis. Brain research. 1999;844(1-2):174-87.
128. Schulte-Baukloh H, Zurawski TH, Knispel HH, Miller K, Haferkamp A, Dolly JO. Persistence of the synaptosomal-associated protein-25 cleavage product after intradetrusor botulinum toxin A injections in patients with myelomeningocele showing an inadequate response to treatment. BJU international. 2007;100(5):1075-80.
129. Charrua A, Pinto R, Taylor AM, et al. AUTONOMIC SYMPATHETIC NERVOUS SYSTEM ACTIVITY IS ENHANCED DURING CHRONIC INFLAMMATION AND CONTRIBUTES TO BLADDER HYPERACTIVITY AND PAIN. European Urology Supplements. 2011;10(2):304-.
130. Kuo HC. Comparison of effectiveness of detrusor, suburothelial and bladder base injections of botulinum toxin a for idiopathic detrusor overactivity. The Journal of urology. 2007;178(4 Pt 1):1359-63.
131. Khera M, Somogyi GT, Salas NA, Kiss S, Boone TB, Smith CP. In vivo effects of botulinum toxin A on visceral sensory function in chronic spinal cord-injured rats. Urology. 2005;66(1):208-12.
132. Krhut J, Zvara P. Intravesical instillation of botulinum toxin A: an in vivo murine study and pilot clinical trial. International urology and nephrology. 2011;43(2):337-43.
133. Shaari CM, Sanders I. Quantifying how location and dose of botulinum toxin injections affect muscle paralysis. Muscle & nerve. 1993;16(9):964-9.
134. Kim HS, Hwang JH, Jeong ST, et al. Effect of muscle activity and botulinum toxin dilution volume on muscle paralysis. Developmental medicine and child neurology. 2003;45(3):200-6.
135. Gabella G. Intramural neurons in the urinary bladder of the guinea-pig. Cell and tissue research. 1990;261(2):231-7.
136. Beckel JM, Holstege G. Neuroanatomy of the lower urinary tract. Handbook of experimental pharmacology. 2011(202):99-116.
137. Chapple CR, Van Kerrebroeck PE, Junemann KP, Wang JT, Brodsky M. Comparison of fesoterodine and tolterodine in patients with overactive bladder. BJU international. 2008;102(9):1128-32.

138. Aviado DM. Action of ganglion-blocking agents on the cardiovascular system. In: Kharkevich DA, editor. Pharmacology of Ganglionic Transmission. 1 ed. New York: Springer-Verlag; 1980. p. 237-49.
139. Yoshimura N, de Groat WC. Increased excitability of afferent neurons innervating rat urinary bladder after chronic bladder inflammation. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1999;19(11):4644-53.
140. Vizzard MA. Alterations in neuropeptide expression in lumbosacral bladder pathways following chronic cystitis. Journal of chemical neuroanatomy. 2001;21(2):125-38.
141. Hassan SM, Jennekens FG, Veldman H, Oestreicher BA. GAP-43 and p75NGFR immunoreactivity in presynaptic cells following neuromuscular blockade by botulinum toxin in rat. Journal of neurocytology. 1994;23(6):354-63.