MOLECULAR TARGETS OF BOTULINUM TOXIN SEROTYPES A AND C: AN EXPERIMENTAL *IN-VITRO* AND *IN-VIVO* STUDY

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Dedico este trabalho ao meu pai, que sei que estará sempre orgulhoso de mim.

I dedicate this work to my father, who I know will always be proud of me.

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Keywords

Botulinum toxin type A, botulinum toxin type C, ND7/23 cells, DRG cells, urinary bladder, LAL muscle model, co-administration, sprouting.

Abstract

Botulinum neurotoxins (BoNTs), the most potent toxins known, are produced by anaerobic bacteria of the genus *Clostridium* and cause a persistent paralysis of peripheral nerve terminals, which is known as botulism. Seven immunologically distinct serotypes (BoNT/A–G) have been identified. All serotypes produce neuronal paralysis through catalytic inactivation of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which are involved in the exocytosis of synaptic vesicles, preventing neurotransmitters release.

The high toxicity of BoNTs, together with their specificity, includes them in the list A of potential bioterrorism weapons and, at the same time, among the therapeutics of choice for a variety of human syndromes. Recently, BoNT/A has been used for the treatment of several urologic conditions, such as neurogenic detrusor overactivity (DO) and Bladder pain syndrome/interstitial cystitis (BPS/IC). Also, the possibility of this toxin having indirect influences in central mechanisms was exploited, and great enthusiasm has been shown around the possible ability of BoNT/A to undergo retrograde axonal transport. Despite the huge success of BoNT/A, some issues about the biological actions of BoNTs are still unresolved, namely in what concerns their binding, internalization, neuroparalysis and duration of action. Therefore, in this work we aimed to study the molecular targets of serotypes A and C and to evaluate their potency and duration of neuroparalysis in different *in-vitro* and *in-vivo* models.

We showed that a model of small cranial muscles, namely the levator auris longus (LAL) muscle, is a sensitive and reproducible model for the study of BoNTs. The action of BoNT/A was detected with doses as little as 0.1 ng and its thin thickness allows for the application of direct immunofluorescence methods. Additionally, other methods would also be easily applied such as Wester-blotting or gene expression.

Using the LAL model as a model for skeletal muscle and the urinary bladder as a model for smooth muscle, we demonstrated that the different types of muscle react differently to the action of toxins. After 7 days of injection, the skeletal muscle showed an intense sprouting network, contrarily to the bladder, where no signs of sprouting were shown. This may be the reason behind the different durations of action of BoNTs in the two types of muscle, but further evidence is needed. Furthermore, the possibility of toxins having synergistic or cumulative effects when co-administered was showed with the LAL model, demonstrating that the serotype C is a promising candidate for the co-administration with BoNT/A. Co-administration of BoNTs can be a novel and efficient way to treat bladder pain and bladder reflex activity, as the antinociceptive action could be increased with the possible synergistic effects. Also, lower doses of toxins would be necessary. However, further studies are needed and this is just the beginning of a big project that may result in a new and unexplored approach to fight pain. With this project we also hope to answer some of the outstanding questions about the mechanisms of BoNTs, providing new and relevant insights for the possible uses of these toxins.

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List of Abbreviations

AAL – Abductor Auris Longus muscle

ABC – Avidin-Biotin Complex

ABC – HRP - ABC - horseradish peroxidase

AraC – Cytosine Arabinoside

AS – Auricular Superior muscle

BoNT(s) - Botulinum Neurotoxin(s)

BoNT/A (B, C, D, E, F, CD, DC) - BoNT type A (B, C, D, E, F, CD, DC)

BPS/IC - Bladder Pain Syndrome/ Interstitial Cystitis

BSA - Bovine Serum Albumin

CGRP - Calcitonin Gene-Related Peptide

COX2 – Cyclooxigenase-2

cSNAP-25 - cleaved SNAP-25

DAB - 3,3'-diaminobenzidine tetrahydro-chloride

DME/F-12 – DMEM Mixture F-12 Ham

DMEM – Dulbecco's Modified Eagle Medium

DO - Detrusor Overactivity

DRG – Dorsal Root Ganglia

FDA – Food and Drugs Administration

FGFR3 - Fibroblast Growth Factor Receptor 3

GAP-43 – Growth Associated Protein 43

H – Heavy (chain**)**

HC domain - C terminus of the H chain

HCC - C terminal of the HC domain

HCN - N terminal of the HC domain

HN domain - N terminus of the H chain

I.P. - Intraperitoneal

IS – Interscutularis muscle

L – Light (chain)

LAL – Levator Auris Longus muscle

LUT – Low Urinary Tract

NANC - Non-adrenergic, Non-cholinergic Transmitters

NGF – Nerve Growth Factor

NGS - Normal Goat Serum

NO – Nitric Oxide

NTNH – Non-toxic Non-hemagglutinins

PB – Phosphate buffer

PBS -Pphosphate-buffered saline

PBST - PBS containing 0.3% of Triton X-100

PBST 1% - PBS containing 1% of Triton X-100

PFA - Paraformaldehyde

PSG - Polysialoganglioside

SNAP-25 - Synaptosome-Associated Protein of 25 kDa

SNARE - Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor

SV2 -Synaptic Vesicle Transmembrane Protein 2

Syn-I – Synapsin-I

Syt (I, II)– Synaptotagmin (I, II)

TRPV1 – Transient Receptor Potential Cation Channel Subfamily V Member 1

TrxR–Trx - Thioredoxin reductase–Thioredoxin system

tSNAREs – Target Membrane SNAREs

VAChT - Vesicular Acetylcholine Transporter

VAMP - Vesicle-Associated Membrane Protein

vSNAREs – Vesicle-associated SNAREs

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I. Introduction

1. Introduction

Botulinum neurotoxins (BoNTs) are one of the most poisonous biological substances known. These toxins are produced by anaerobic, gram-positive, spore-forming, rod-shaped bacteria of the genus *Clostridium*, commonly found on plants, soil, water and the intestinal tracts of animals [1]. Namely, six phylogenetically distinct clostridial groups (*C. botulinum* groups I–III, *Clostridium argentinense* and some strains of *Clostridium baratii* and *Clostridium butyricum*) produce seven distinct BoNT serotypes, named A to G. Each toxin serotype is then categorized into several subtypes, on the basis of their amino acid sequences [2]. Several dozens of toxin subtypes within each serotype have been discovered in the last few years, and it is predicted that many others will be revealed [3]. Moreover, two mosaic toxins have been discovered: BoNT type CD (BoNT/CD) and BoNT type DC (BoNT/DC) [4].

Four of the BoNT serotypes (A, B, E, and F) can cause human botulism, a neuroparalytic disease which can result in death [5]. Food-borne and infant botulism are the predominant forms of the disease in humans (**Figure 1**). In these cases, the toxins cross the intestinal wall and enter the bloodstream. BoNTs then reach peripheral cholinergic nerve terminals and produce neuronal paralysis through catalytic inactivation of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins involved in the exocytosis of synaptic vesicles [2]. The blockage of vesicles fusion with the presynaptic membrane prevents neurotransmitters release, leading to all symptoms of botulism (such as diplopia, ptosis, difficult swallowing, autonomic dysfunctions, and skeletal muscle paralysis) [6]. The paralysis associated with botulism is called flaccid paralysis, as the blockage of acetylcholine release at the neuromuscular junction prevents the contraction of the target muscle [7].

The high toxicity of BoNTs, together with their absolute neurospecificity and catalytic activity makes them the most powerful known toxins, and includes them in the list A of potential bioterrorism weapons [6]. On the other hand, the same characteristics of BoNTs, enables them to be used as the therapeutic of choice for the treatment of human diseases characterized by the hyperfunction of peripheral cholinergic nerve terminals [2]. Controlled local injection of minute amounts of these toxins counteracts hyperactivity of the nerve terminal, leading to local relaxation lasting many months. It was first applied by Alan Scott, whom pioneered the use of BoNT type A (BoNT/A) to treat several pathological ophthalmic conditions. Nowadays, BoNT/A is used to treat a diversity of conditions, from dystonias, to hyperhidrosis, to gastrointestinal and urinary disorders, as well as for cosmetic purposes [8]. Recently, BoNTs have been evaluated for treatment of other new indications, for example, diabetic neuropathic pain, depression, cancer and wound healing [9].

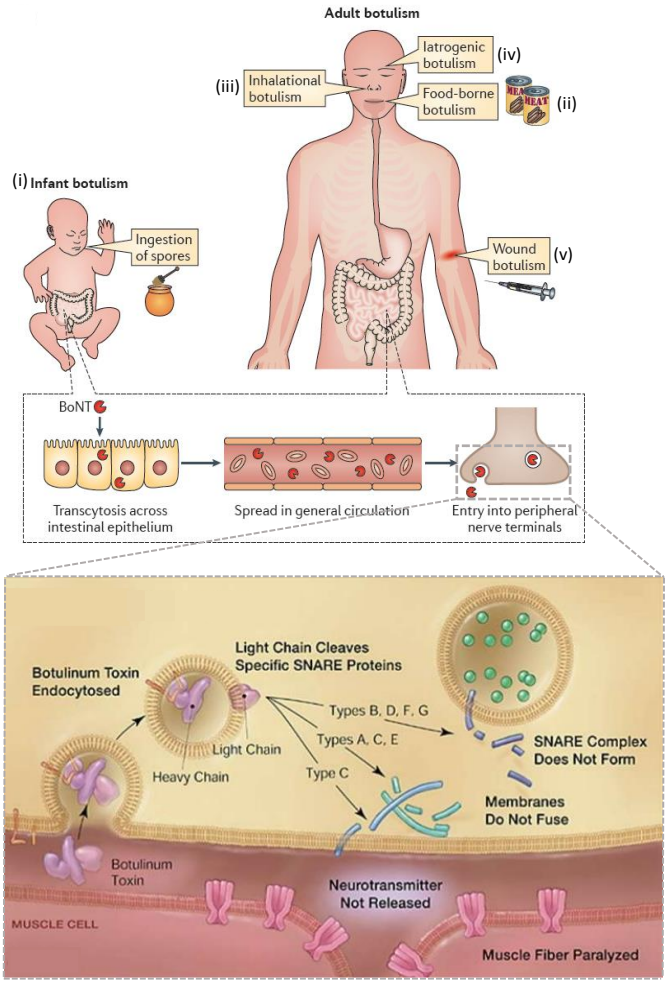

Figure 1. Botulism Causes.

Human botulism may occur in five forms: (i) infant botulism (caused by the ingestion of food contaminated with spores, that germinate and proliferate in the gastrointestinal tract), (ii) food-borne botulism (caused by the ingestion of BoNT-contaminated foods, normally, canned foods), (iii) inhalational botulism (inhalation of aerosols containing BoNTs), (iv) iatrogenic botulism (caused by the excess doses injected in clinical practices) and (v) wound botulism (usually associated with drugs injection). The first two are the most common forms of botulism, in which BoNTs cross the intestinal epithelium and enter the lymphatic system and then into blood circulation. Subsequently, BoNTs bind specifically to peripheral skeletal and autonomic cholinergic nerve terminals, where they are endocytosed and cleave specific SNARE proteins. This results in the prevention of the formation of a stable SNARE complex, leading to the blockage of vesicle fusion with the presynaptic membrane. Consequently, it leads to the blockage of neurotransmitters release, namely acetylcholine, which thereby causes muscle paralysis. Adapted from: [2, 10]

2. Historical Landmarks of Botulinum Toxin

Botulism has probably accompanied mankind since the primeval times, when men started to preserve and store food in conditions that were optimal to the presence and growth of the anaerobic bacteria *Clostridium botulinum*. However, the relationship between the consumption of contaminated food and death by a paralytic disease was not recognized at that time [11]. Only in the late 1700s, during the Napoleonic Wars, the first reports of poisoning by contaminated food emerged in the southwest German region of Württemberg, where many deaths were attributed to the consumption of smoked blood sausages [12]. At first, by 1811, the 'prussic acid' (nowadays known as hydrocyanic acid) was considered the cause of the 'sausage poisoning' by the medical section of the Department of Internal Affairs of the Kingdom of Württemberg [13]. Later, professors of the Medical Faculty of the University of Tübingen suspected a biological poison as the toxic agent in sausages [11].

In 1820 and 1822, Justinus Kerner (1786-1862), a German physician, after analyzing more than 230 case reports, published two monographs containing an accurate description of all symptoms of what we now call botulism, including vomiting, diarrhea, mydriasis, reduction of lacrimal and salivary gland secretion, ptosis, diplopia, respiratory failure and gastrointestinal and bladder paralysis [11, 13]. Furthermore, based on animal experiments and high-risk experiments on himself, Kerner concluded that (1) the 'sausage poison' develops in sour sausages under anaerobic conditions, (2) the toxin acts by interrupting motor signal transmission in the peripheral and autonomic system and, (3) it is lethal even in small doses [12]. Moreover, Kerner proposed prevention methods and possible treatments to the disease and in a visionary way, he hypothesized that small doses of the toxin could have therapeutic applications in conditions with hyperactivity and hyperexcitability of the nervous system [11]. Because of his findings, Kerner is considered the godfather of BoNT research [14].

After Kerner's remarkable job, Müller, another German physician, introduced the term 'botulism' (from the Latin word *botulus*, meaning sausage) to describe the 'sausage poisoning' [15].

The following significant step was the discovery of the botulism-causing bacteria, in 1895, by the microbiologist Emile-Pierre van Ermengem (1851-1922), who worked with Robert Koch, the first investigator to show animal diseases driven by microorganisms, such as tuberculosis and cholera [12, 13]. After a botulism outbreak occurred in the small Belgian village of Ellezelle, van Ermengem correlated for the first time botulism with an anaerobic bacterium that he found in smoked ham and in the postmortem tissue of the victims who had eaten the intoxicated meat [13]. van Ermengem was the pioneer on the isolation of the bacterium that he called *Bacillus botulinus* (later renamed *Clostridium botulinum*), and on the description of its toxin [12].

Comparing the bacterium isolated by van Ermengem and a bacterium isolated from another botulism outbreak in Germany (1904), J. Leuchs found that the strains were different and the toxins they produce were serologically distinct. Later (1919), Georgina Burke named the two different serotypes as type A and B [11]. Since then, the other five serotypes of BoNT were identified (**Table 1**).

Table 1. Timetable for the identification of the different BoNT serotypes [12].

1910	Leuches	Discovery of two different botulinum toxin serotypes
1919	Burke	Designation of the two serotypes as type A and B
1922	Bengston Seddon	Identification of the botulinum toxin serotype C
1928	Meyer Gunnison	Identification of the botulinum toxin serotype D
1936	Bier	Identification of the botulinum toxin serotype E
1960	Moller Scheibel	Identification of the botulinum toxin serotypes F and G

Dr. Herman Sommer was the first to obtain the purified form of BoNT type A as a stable acid precipitate, in the 1920s [14]. However, the first batch of the crystalline form of the toxin was only produced by Dr. Edward J. Schantz, in 1946, during World War II, when the United States government was seeking for potential biological weapons [13].

Later, in the early 1950s, Burgen and Dr. Vernon Brooks discovered the temporary paralysis of hyperactive muscles due to the blocking capacity of BoNT in the release of acetylcholine at neuromuscular junctions [12]. This breakthrough encouraged the research of BoNT as a potential therapeutic agent, namely, by Dr. Alan Scott, an ophthalmologic surgeon who was searching for alternatives to strabismus' conventional surgery [13]. Scott, unsuccessfully injected several substances in monkeys with induced strabismus. Only, in 1968, when he approached Dr. Schantz about the toxin, he found that a few picograms of BoNT/A injected in primates resulted in induced local paralysis without side-effects and with long duration [12, 13]. After experimentations in humans, Scott obtained the FDA approval, in 1989, for the use of BoNT/A to treat strabismus and other ophthalmological conditions, under the brand name of 'Oculinum', which became known as Botox® after its acquisition by the Allergan company [12, 13]. Thereafter, BoNTs have been intensively studied for various medical purposes and also, have been used to unravel some inner aspects of the machinery of synaptic release [8].

3. Characterization of Botulinum Toxins

3.1. Structure of Botulinum Toxins

Despite the high number of different BoNT isoforms, they are all structurally similar [3]. Generally, each BoNT isoform is initially synthesized by *Clostridium* bacteria as an inactive, single polypeptide chain of ~150 kDa, which is then cleaved by proteases into a 50 kDa light (L) chain and a 100 kDa heavy (H) chain, yielding the mature toxin. The L chain and the H chain are kept together by an essential interchain disulfide bond and by the belt, a loop from the H chain that wraps around the L chain (**Figure 2**) [5].

Structurally, the activated mature toxin consists of three primary domains that fulfill different functions during the intoxication process: the HC domain (C terminus of the H chain), responsible for presynaptic binding and endocytosis; the HN domain (N terminus of the H chain), required for translocation of the L chain across the endocytic vesicles membrane into the neuronal cytosol; and the L chain or the catalytic domain (N-terminal), which is a zinc-dependent endopeptidase that specifically cleaves the SNARE proteins, being responsible for the toxic effects (**Figure 2**) [2, 5].

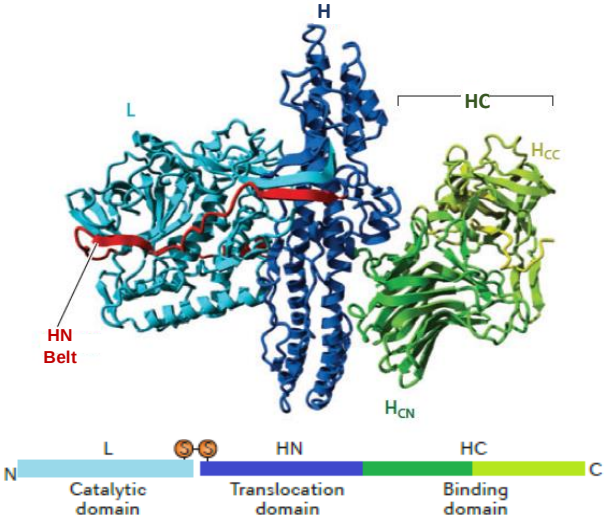

Figure 2. Structure of BoNT molecules. Representation of the crystal structure of BoNT/A (PDB 3BTA), and the organization of the different domains. The HC domain (binding domain) is represented in a green gradient that highlights the two subdomains – H_{CC} (C terminal of the HC domain) and H_{CN} (N terminal of the HC domain) – that have different folding and binding properties. HN domain (translocation domain) is in dark blue, and the L chain or catalytic domain (zinc-dependent endopeptidase or metalloprotease) is in cyan blue. The HN belt is depicted in red and the disulfide bond in orange. Adapted from: [5]

It is important to notice that BoNTs are not released to the surrounding media by *Clostridium* bacteria as homogeneous neurotoxins. Instead, some accessory proteins form heterodimeric complexes with BoNTs between 300 and 900 kDa [16]. The best known accessory proteins are hemagglutinins (HA) or molecules called non-toxin non-hemagglutinins (NTNH) [17]. However, although these proteins may have a role in the entry mechanisms of BoNTs, namely in the protection of the toxins' exposure to damaging agents

in the gastrointestinal tract (like pH denaturation and proteases), they are not involved in the actions of the toxins after they enter the blood circulation. Indeed, some factors contribute to the dissociation of these proteins from the toxins, such as the pH and salt composition of the blood [18]. This way, after absorption, BoNTs proceed as homogenous proteins [17].

3.2. Botulinum Toxins Mechanism of Action

Physiologically, neurotransmitters release occurs due to a stimulation of the nerve fiber that causes a depolarization along the presynaptic membrane, which results in the opening of voltage-dependent Ca^{2+} channels and the subsequent influx of Ca^{2+} into the presynaptic terminal. The increase of Ca^{2+} concentration induces Ca^{2+} binding to Synaptotagmin (Syt) which thereby stimulates the binding of Syt to the SNARE complex (**Figure 3**) [19]. This complex is formed by vesicle associated-SNAREs (v-SNAREs) and target membrane-SNAREs (t-SNAREs) and mediates the fusion of the synaptic vesicles with the pre-synaptic membrane during exocytosis [20]. When the synaptic vesicles approach the plasma membrane, the v-SNARE VAMP (Vesicle-Associated Membrane Protein) interacts with t-SNAREs SNAP-25 (Synaptosome-Associated protein of 25 kDa) and Syntaxin, forming the SNARE complex, in a process that is regulated and assisted by Munc18, complexin and other proteins [19]. Membrane fusion results in transient exposure of the lumen of synaptic vesicles to the outside and neurotransmitters release at the synaptic cleft, a process that takes less than milliseconds [2]. Neurotransmitters then bind to specific receptors in postsynaptic membrane, resulting in muscle fiber contraction [21]. After the transient exposure of the lumen of the synaptic vesicles to the outside and neurotransmitters release, vesicles are internalized into the nerve terminal by endocytosis and recycled [2].

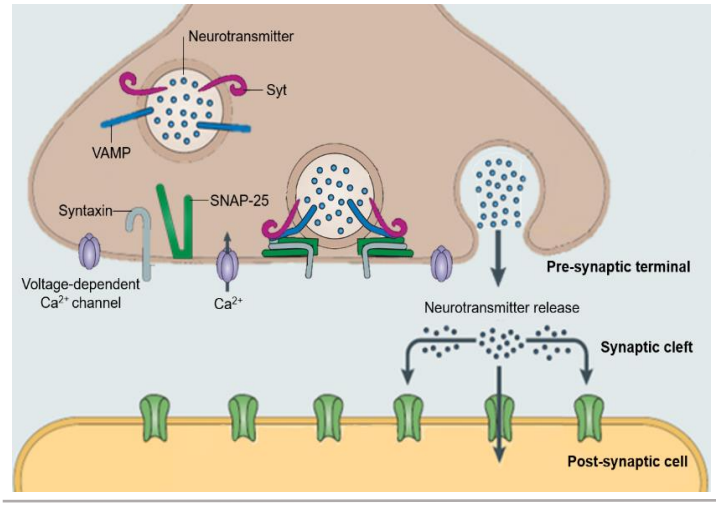

Figure 3. Synaptic neurotransmission in physiological conditions. The increase of intracellular Ca^{2+} leads to its binding to Syt and the subsequent formation of the SNARE complex, comprised by VAMP, Syntaxin and SNAP-25. The SNARE complex mediates membrane fusion, resulting in neurotransmitters release to the synaptic cleft. Adapted from: [2]

BoNTs can interfere with the physiological exocytosis process of neurotransmission, being the model accepted to describe their neurotoxic effect a multistep mechanism, composed by four stages: binding, internalization, membrane translocation and target SNARE cleavage [5].

3.2.1. Binding to target cell

The specificity of BoNTs is absolute, as they only bind to peripheral nerve terminals, particularly those of skeletal and autonomic cholinergic nerves. To selectively target the presynaptic membrane of these peripheral nerve terminals, BoNTs use a dual receptor mechanism, involving a low and a high affinity receptor [5].

The carboxy-terminal of the HC domain (H_{CC}) of BoNTs first binds to polysialoganglioside (PSG) receptors (GD1b or GT1b), that are present at a high density in the presynaptic membrane (**Figure 4.a)**). The PSG binding site of the H_{CC} is a hydrophobic cavity with a highly conserved lactose-binding motif between almost all BoNTs, the SxWY motif [20]. The BoNT-PSG low affinity interactions concentrate BoNTs on the cell surface [2]. Once anchored in the membrane, conformational changes facilitate intermolecular interactions of BoNTs with additional lower density but higher affinity protein receptors [22].

The protein receptors have some serotype specificity, since the receptor for BoNT types A, E, and F is the synaptic vesicle transmembrane protein 2 (SV2) [5, 23], a putative transporter protein with undefined function [24]; and for BoNT types B, and G are the synaptic vesicle proteins Synaptotagmin-I (Syt-I) and Synaptotagmin-II (Syt-II) [2, 25], calcium sensors which are involved in the docking and fusion of synaptic vesicles [24]. The Fibroblast Growth Factor Receptor 3 (FGFR3) was also identified as a high affinity receptor for BoNT/A (**Table 2**) [22]. A coreceptor for BoNT/C and BoNT/D has not yet been identified [24]. Some studies suggest SV2 as a protein receptor for BoNT/D, but the results are controversial [26, 27]. Only Syt-I and Syt-II have been identified as the second receptors for the mosaic toxin BoNT/DC (which comprises the L chain and HN domain of BoNT/D and the HC domain of BoNT/C). However, these receptors are not used by the C or D toxin serotypes [28].

Unlike PSG, these protein receptors are not exposed on the nerve terminal surface, but they become available following the fusion of the synaptic vesicle with the presynaptic membrane, which transiently exposes the synaptic vesicle lumen to the extracellular environment [2].

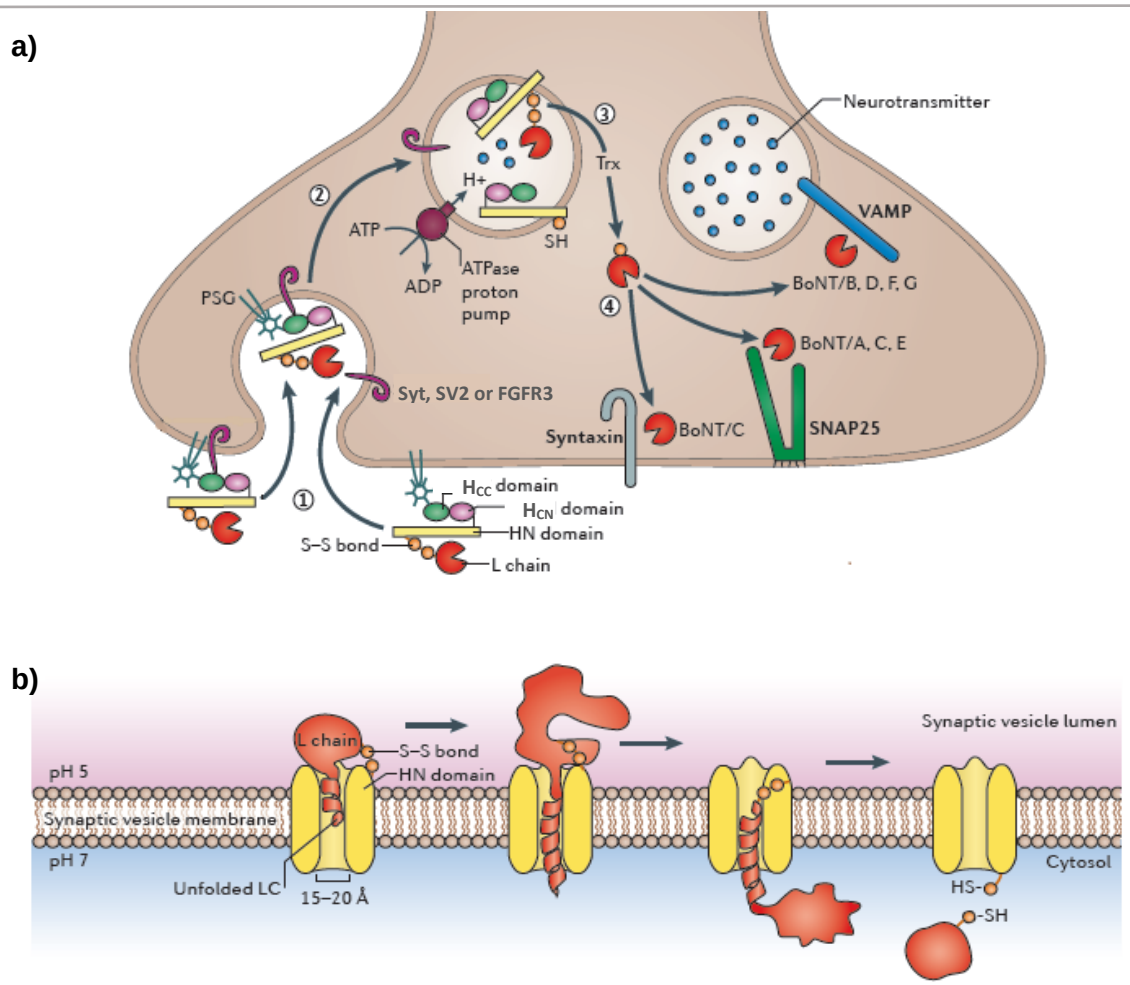

Figure 4. Model for botulinum neurotoxins intoxication mechanism. a) Step 1 – binding of the H_{CC} domain to a PSG in the presynaptic membrane, followed by the binding to a protein receptor (Syt, SV2 or FGFR3); Step 2 – endocytosis of BoNT and acidification of the synaptic vesicle owing to the ATPase proton pump, resulting in the formation of a channel in the membrane by the HN domain; Step 3 – translocation of the L chain across the channel into the cytosol, where it is released after cleavage of the disulfide bond; Step 4 – cleavage of the target SNARE proteins. **b)** molecular events that occur during L chain translocation across the synaptic vesicle membrane. Adapted from: [2]

3.2.2. Internalization

Following the dual binding interactions with PSG and synaptic vesicle receptors, BoNTs enter nerve terminals using the synaptic vesicles as 'Trojan horses' (**Figure 4.a)**) [2].

During physiological activity, the exocytosis process is followed by a compensatory endocytosis to maintain the pool of synaptic vesicles and the size of the presynaptic terminal, being the balance between the two processes critical for maintaining neuronal function [29]. This way, after exposure of the synaptic vesicles lumen to the synaptic cleft, their membranes are recycled to form a new vesicle, engulfing the BoNT that is attached to its receptor. Therefore, the amount of engulfed toxin is higher in nerve terminals with high rates of vesicle recycling, such as hyperactive cholinergic nerves [29].

3.2.3. Translocation of the light chain

Immediately after endocytosis, the synaptic vesicle lumen has a neutral pH. However, owing to the action of the vesicular ATPase proton pump (which is physiologically involved in the accumulation of neurotransmitters and pumps protons into the synaptic vesicles), the pH in the lumen of synaptic vesicles becomes progressively more acidic. This causes a conformational change in the toxin HN domain, increasing its hydrophobicity and therefore, enabling it to penetrate the lipid bilayer, where it forms a channel (**Figure 4.b)**). This results in the translocation of the partially unfolded L chain across the synaptic vesicle membrane into the cytosol, where it is released from the HN domain by the action of the thioredoxin reductase–thioredoxin system (TrxR–Trx), which cleaves the interchain disulfide bond [2, 5]. The reduction of this bond was recently identified as a key event in the intoxication process of BoNTs, since the inhibition of the TrxR-Trx and the consequent inhibition of the disulfide bond reduction, prevents neuroparalysis [30].

3.2.4. Cleavage of the specific SNARE target

Once in the cytosol, the L chain metalloproteases of BoNTs cleave distinct peptidic bonds on different target SNARE proteins (**Figure 4.a)**), that have a crucial role in the docking and fusion of synaptic vesicles [20]. Serotypes A and E cleave SNAP-25, serotypes B, D, F and G cleave VAMP (also known as Synaptobrevin), and the serotype C cleaves both SNAP-25 and Syntaxin (**Table 2**) [5, 31]. Whatever the SNARE protein cleaved, the consequences are the same: prevention of the formation of a stable and functional SNARE complex, leading to the blockage of vesicles fusion with the presynaptic membrane and neurotransmitters release, which thereby causes neuroparalysis [6].

Despite the different SNARE targets cleaved by the different BoNTs, all the serotypes present an extremely conserved sub-region on the L chain, called HEXXH, which is the zinc-binding motif of the endopeptidase [21].

Interestingly, BoNT interaction with the target SNARE protein involves not only the segment containing the peptide bond to be cleaved but also, the L chain has to recognize and bind to several exosites. Therefore, the specificity of each BoNT to its target may be dictated by the diversity and distribution of the exosites [20].

Table 2. Binding receptors, catalytic targets and specific cleavage sites of BoNT serotypes [32].
*NA - non-available information, **Controversial results.

BoNT	Cellular binding receptor	Catalytic SNARE target	Specific peptide bond cleaved (-/-)
A	SV2 and FGRF3	SNAP-25	Glu-Ala-Asn-Gln-/-Arg-Ala-Thr-Lys
B	Syt-I and Syt-II	VAMP	Gly-Ala-Ser-Gln-/-Phe-Glu-Thr-Ser
C	NA*	SNAP-25	Ala-Asn-Gln-Arg-/-Ala-Thr-Lys-Met
C	NA*	Syntaxin	Asp-Thr-Lys-Lys-/-Ala-Val-Lys-Phe
D	SV2**	VAMP	Arg-Asp-Gln-Lys-/-Leu-Ser-Glu-Leu
E	SV2	SNAP-25	Gln-Ile-Asp-Arg-/-Ile-Met-Glu-Lys
F	SV2	VAMP	Glu-Arg-Asp-Gln-/-Lys-Leu-Ser-Glu
G	Syt-I and Syt-II	VAMP	Glu-Thr-Ser-Ala-/-Ala-Lys-Leu-Lys

This is the most accepted mechanism of action of BoNTs and illustrates the effect of these toxins on its most studied target, the neuromuscular junction in striated muscle, where the blockage of neurotransmitters release influences both α and γ motor neurons, which innervate extrafusal and intrafusal muscle fibers, respectively [33].
The same mechanism of action has been suggested to explain the action of these toxins in smooth muscle and other cell types, but many aspects of BoNTs' action are yet to unravel.

3.3. Duration of Action of Botulinum Toxins

Besides its safety, effectiveness and specificity, the effect of BoNT is reversible and lasts only for a few months [14]. After BoNT intoxication, neuromuscular junctions lose their main functionality, since the release of neurotransmitters is blocked. However, a key factor when considering the action of BoNTs is that motor neurons, neuromuscular junctions and muscle fibers remain viable, with no sign of atrophy, and regain function over time [34].
The recovery process occurs in two steps, and involves compensatory sprouting of the affected axon terminal and end-plate remodeling. As the original nerve terminal recovers and exocytosis is restored, sprouts retreat and are eliminated, which translates the synaptic plasticity associated with the neuromuscular junctions [35, 36]. The recovery time varies with the different BoNT serotypes and the local of action.
Actually, the duration of BoNT-induced neuroparalysis is remarkably different between the seven serotypes. Accordingly to the duration of paralysis they cause in human neuromuscular junctions, BoNT types are ordered as follows (from longest to shortest duration): A, C, B, F and E (**Figure 5**) [8].

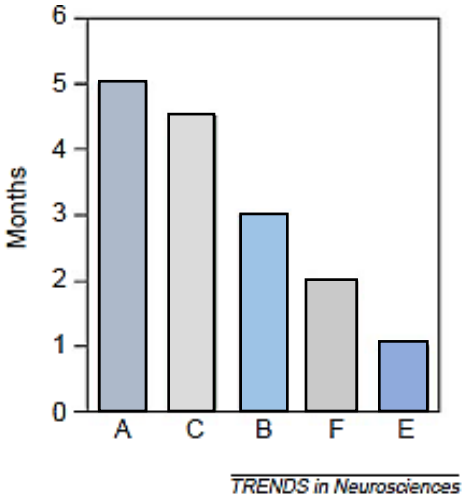

Figure 5. BoNT serotypes duration of action. Neurotoxin serotypes are ordered according to the duration of neuroparalysis they cause in human neuromuscular junctions. In rodents, the order is the same but the duration of neuroparalysis for each BoNT is approximately two times shorter. Adapted from: [8]

It is clearly known that the variable durations of synaptic blockage triggered by BoNTs, are not due to the difference in their molecular targets. Actually, different serotypes with the same SNARE protein target have very different durations of action (like BoNT/A and BoNT/E, with SNAP-25 as the same target) [8]. It is thought that the main factor that contributes to the different durations of paralysis is the longevity of BoNTs L chains within the nerve terminal cytosol. However, the vertebrate species (shorter duration in small size mammals), the activity of the affected muscle and the type of nerve terminal (human skeletal nerve terminals are paralyzed for 3-4 months by BoNT/A whereas human autonomic cholinergic nerve terminals are paralyzed for 12-15 months) and the toxin dose (higher dose equals longer duration), also contribute to the duration of paralysis [2].

The persistence of BoNT activity is the main factor that contributes to their biological action, as it determines the severity of human botulism. Also, the duration of action is important for the therapeutic application of BoNTs, as a toxin that has longer duration of neuroparalysis requires fewer injections and lower doses [2].

4. Overview of Botulinum Toxin Type A Applications

From the seven serotypes of BoNTs that have been identified, BoNT/A and BoNT/B are the most extensively studied and the only serotypes commercially available. Botox® refers to a BoNT/A preparation (OnabotulinumtoxinA), produced by Allergan Inc. (United States). Dysport® (AbobotulinumtoxinA) and Xeomin® (IncobotulinumtoxinA) are also BoNT/A preparations, from different manufacturers: Ipsen (France) and Merz Pharmaceuticals GmbH (Germany), respectively. NeuroBloc (RimabotulinumtoxinB, also called MyoBloc) is the only type B preparation, produced by the Solstice Neurosciences (United States) [37,

38]. Despite the same mechanism of action, the four formulations are significantly different in terms of manufacturing, composition, potency and dosing which in turn influence their therapeutic applications [37].

Because of its long-lasting effects, BoNT/A is the serotype most commonly used to treat several human neuromuscular disorders characterized by hyperfunction of peripheral cholinergic terminals [32]. In 1989, the U.S. Food and Drug Administration (FDA) approved the use of BoNT/A for the treatment of strabismus, blepharospasm and hemificial spasms. Patients with blepharospasm and hemificial spasm treated with BoNT/A revealed 90% and 76% of improvements, respectively [39]. Since then, BoNT/A also received approval for other applications, including cervical dystonia and the popularly recognized cosmetic uses, which are summarized in **Table 3**.

In addition to the therapeutic neuromuscular applications, BoNT/A is also effective in the treatment of hyperhidrosis and hypersalivation (sialorrhea or drooling) and it has been widely explored to treat other ophthalmological, gastrointestinal, orthopedic, dermatological and secretory diseases [20]. Other less common applications for BoNT/A have been suggested, such in vaginism [40], diabetic neuropathy [41] and wound healing [42]. Nevertheless, further studies are needed for application of BoNT/A in these conditions [39].

Table 3. Current uses of botulinum toxins in different therapies. Adapted from: [39]

Status	Indications	Commercial BoNT (Year of approval)	Remarks
FDA approved indications	Strabismus	Oculinum/Botox (1989)	Very effective but repetitive injections are required; more suitable for temporary use
	Blepharospasm	Oculinum/Botox (1989)	Very effective
	Hemificial spasm	Oculinum/Botox (1989)	Very effective
	Cervical distonya	NeuroBloc (2000), Botox (2001), Dysport (2009), Xeomin (2010)	Very effective but larger doses may be needed and so, it may imply immune-resistance
	Cosmetic use	Botox (2000, Canada; 2012, US)	Very effective and safe for long-term use
	Axillary hyperhidrosis	Botox (2001, UK, Canada; 2004, US)	Effective and safe, but painful at the injection sites
	Chronic migraine	Botox (2010)	Safe and effective, but it needs more trials
	Neurogenic detrusor overactivity	Botox (2013)	Remarkable efficacy with minimal side effects
Off-labeled indications	Lower urinary tract disorders	Botox	Remarkable efficacy with minimal side effects
	Gastrointestinal tract disorders	Botox	Short-term effects
	Spasticity	Botox	First-line treatment if used at the early stages
	Spasmodic dystonia	Botox	Effective but more studies are needed
	Sialorrhea	Botox	Effective but more studies are needed
	Temporomandibular disorder	Botox	Effective with right dose and injection technique
	Chronic musculoskeletal pain	Botox	Effective for patients refractory to 1st-line treatments
	Vaginism	Botox	Effective but reports are limited
	Wound healing	Botox	Improvement of wound healing
	Diabetic neuropathy	Botox	Effective and safe but more studies are needed

4.1. Applications of Botulinum Toxin Type A in Urology and Pain

The range of clinical applications of BoNT/A is continuously increasing and recently it has been employed in the field of urology. Injections of BoNT/A in rat prostates shown to cause a decrease in prostate weight in patients with benign prostate hyperplasia (a disease characterized by prostate enlargement and urinary retention) [43]. Also, BoNT/A is under active investigation for the treatment of urinary incontinence in patients with neurogenic detrusor overactivity (DO), characterized by involuntary contractions during the filling phase of micturition [44]. Actually, in 2013 the use of this neurotoxin was approved to treat patients with DO refractory to medical treatment (FDA news release, January 18 2013, available online) [45].

Nevertheless, an emerging field concerning the use of BoNT/A is the control of pain [46]. In recent years, particular interest has been devoted to the use of BoNT/A for treating pain, both in humans [47, 48] and in animal pain models [49, 50]. It was observed that BoNT/A injections can reduce pain in some conditions, like in painful dystonias, trigeminal neuralgia, neuropathic pain, refractory joint pain and low-back pain [51]. Moreover, in 2010, BoNT/A was approved by the FDA for the treatment of chronic migraine [39].

Initially, it was assumed that the mechanism of BoNT/A-induced antinociception was due to disruption of the spasm-pain cycle by inhibiting the release of certain mediators from the primary sensory neurons, such as glutamate, substance P and calcitonin gene-related peptide (CGRP), which are critical for the nociceptive transmission. This way, both pain perception and peripheral sensitization would be prevented [21]. However, recent studies have shown that besides this direct inhibition of pain neurotransmitters release at peripheral injected locations, BoNT/A also has indirect influences on central pain mechanisms [46]. Very recent experimental studies have shown that after intradermal, intramuscular [52], intraocular [53] and intraneural [52] application of BoNT/A, the end product of its catalytic action (the cleaved SNAP-25 protein) could be detected in spinal cord nerve terminals. Moreover, it was recently shown experimentally that BoNT/A (and also BoNT/E) can be internalized by primary spinal cord motor neurons and undergo retrograde axonal transport in these cells to the central nervous system [7, 51, 52]. These results clearly demonstrated that BoNT/A can gain access to the central nervous system after peripheral administration, contradicting the common belief that BoNT/A effects remain localized to synapses near the injection site [52]. This paradigm shift is about to revolutionize the conceptual and practical approaches to the use of BoNT/A in the treatment of pain [7].

Furthermore, it was suggested by some experimental studies that the intrathecal administration of BoNT/A has a central antinociception action in animal models with

neuropathic and somatic pain [54]. This hypothesis was recently demonstrated by Coelho *et al*. in the improvement of Bladder pain syndrome/ interstitial cystitis (BPS/IC) [49].

BPS/IC is a chronic condition characterized by an intense suprapubic pain associated with increased frequency and nocturia that can also be combined with edema and inflammation [49]. Because of its unclear etiology, BPS/IC has no standard effective treatments known, thus being one of the most difficult types of pain to manage.

In a recent study, injections of BoNT/A in the bladder wall proved to be highly effective in improving bladder pain, and daytime and nighttime voiding frequency in patients with BPS/IC refractory to standard treatments [55]. However, the need to repeat BoNT/A administration every 6 to 9 months represented a major throwback of this method, as the repeated injections imply general anesthesia and can cause urinary tract infections that may aggravate the BPS/IC symptoms [49].

An alternative method proposed to overcome these issues was intrathecal administration of BoNT/A in the spinal areas of bladder primary afferents termination (L6 segment). Thus, it was recently demonstrated that intrathecal injections of BoNT/A reduced bladder pain and bladder hyperactivity without somatic or visceral motor impairments observed [49]. However, further data is needed, namely concerning to the optimal doses, long term duration of the effect, and the precise spinal cord structures affected by the toxin.

4.2. Mechanisms of Action of Botulinum Toxin Type A in the Bladder

Urinary bladder is a hollow and elastic organ with a muscular wall composed of smooth muscle bundles that form the detrusor muscle. The bundles of smooth muscle are surrounded by collagen-rich connective tissue, providing the organ great compliance.

The detrusor muscle is then lined by a mucosa layer, lamina propria (suburothelium) and the urothelium in the inside part; and by peritoneal serosa and fascia in the outside [56].

The bladder, together with the bladder neck, the urethra and the urethral sphincter form the low urinary tract (LUT) and the coordination of all these components is essential for a correct process of micturition. These structures are orchestrated by a complex neural control system, receiving innervation of efferent and afferent nerves that transport information from and to specific areas of the brain, spinal cord and peripheral ganglia [57]. This way, the control of the LUT involves complex interactions between the sympathetic, parasympathetic and somatic nervous system [58].

Preganglionic sympathetic innervation arises from T10-L2 segments of the spinal cord and travel through the inferior mesenteric ganglia to the hypogastric plexus. From there, postganglionic sympathetic fibers follow the pelvic nerves until the bladder and urethra,

where they release noradrenaline, which activates β-adrenergic receptors in the detrusor muscle, producing relaxation of the bladder; and activates α-adrenergic receptors, producing contraction of the urethra and bladder neck. Therefore, it is thought that the sympathetic system is responsible for urine storage control [57, 58].

On the other hand, the parasympathetic system appears to be in control of bladder emptying. Parasympathetic fibers are originated in the sacral segments S2-S4 of spinal cord and travel until the bladder through pelvic nerves, passing through the pelvic plexus, where the postganglionic nerves arise. The latter release cholinergic transmitters at the bladder wall, namely acetylcholine, which results in the emptying contraction of the detrusor muscle. This contraction is mediated by muscarinic receptors, especially the M3 receptor, that together with the M2 receptor are the predominant subtypes of muscarinic receptors expressed by the detrusor smooth muscle. Postganglionic parasympathetic fibers also release non-adrenergic, non-cholinergic (NANC) transmitters, which are also responsible for the contractile activation of the bladder. The excitatory transmission of NANC transmitters is mediated by the action of ATP on detrusor purinergic receptors P2X and P2Y. Besides that, NANC transmitters are also involved in the relaxation of the urethral smooth muscle, which is mediated by nitric oxide (NO) [56, 57].

Injections of BoNT/A in the detrusor smooth muscle inhibits the release of noradrenaline from sympathetic nerves, consequently inhibiting α- and β- adrenergic receptors. This could affect bladder compliance, but sympathetic nerves have less expression of SV2 and therefore, a reduced toxin uptake [59]. BoNT/A administration in the urinary bladder also results in obvious blockage of acetylcholine release from both pre- and postganglionic parasympathetic nerves, which leads to a reduced activation of the muscarinic receptors M2 and M3 and the purinergic receptors $P2X_2$ and $P2X_3$, resulting in reduction of the frequency of urgency episodes. This way, detrusor overactivity is reduced through inhibition of motor and sensory pathways of micturition. Moreover, intravesical injections of the toxin also suppresses ATP release, leading to a lack of sensory fibers activation, which thereby results in decreased bladder sensation and amelioration of acute pain [33].

Analgesic and anti-inflammatory effects may also be elicited by BoNT/A through a decrease in afferent sensitization and sprouting by alterations in receptors expression, inhibition of nerve growth factor and blockage of inflammatory mediators' release [59]. In bladder inflammation, as occurs in BPS/IC, the receptor TRPV1 (transient receptor potential cation channel subfamily V member 1), which has a critical role in inflammatory pain perception, is overexpressed in detrusor muscle and urothelium. Injections of BoNT/A might block the exocytosis of TRPV1, thus inhibiting its inflammatory sensitization. It was also shown that intravesical administration of BoNT/A in patients with BPS/IC resulted in analgesic effect by reducing the levels of urinary nerve growth factor (NGF) and brain-derived neurotrophic

factor. Decreased NGF levels are also associated with a reduction in detrusor overactivity, since it leads to a decrease in C-fibers excitability. Moreover, the release of the nociceptive mediators CGRP and substance P is inhibited by BoNT/A, resulting in decreased neurogenic inflammation and bladder excitability [33]. Furthermore, indirect analgesic effects were also observed after BoNT/A bladder injections, for example, with the reduction of the overexpressed COX-2 (Cyclooxigenase-2) in spinal cord in an induced cystitis model [60]. This reflects that BoNT/A is not restricted to the site of injection and that may suffer retrograde transport to the central nervous system after bladder administration (**Figure 6**).

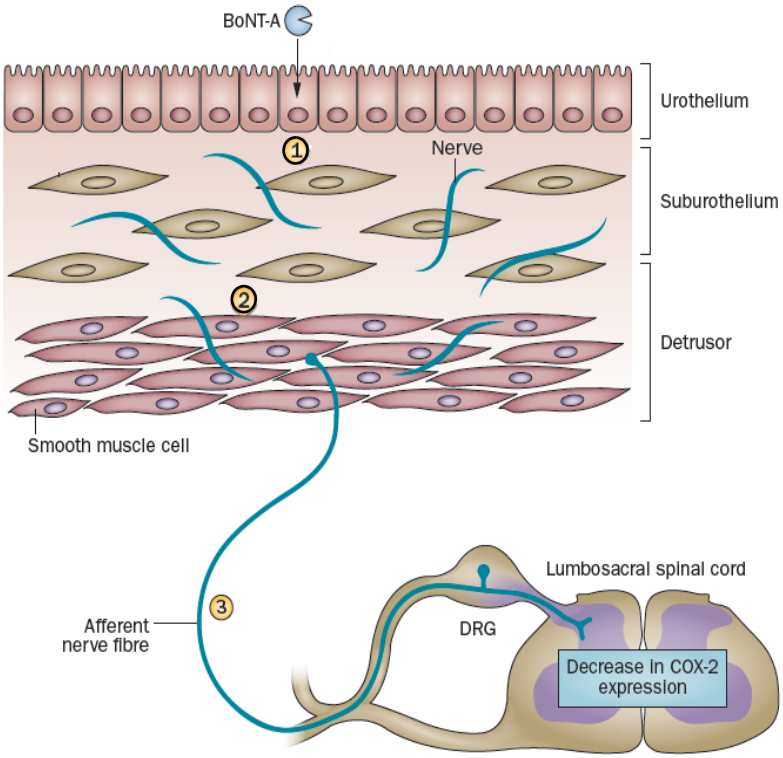

Figure 6. Mechanisms of action of BoNT/A in the urinary bladder. 1) BoNT/A inhibits the release of ATP to the suburothelium space and the expression of TRPV1 in both urothelium and detrusor muscle. 2) It also inhibits acetylcholine release, reducing M2, M3, $P2X_2$ AND $P2X_3$ receptors activation in detrusor muscle. Reduction of NGF, CGRP and substance P from afferent nerves to the detrusor was also verified. 3) Possible retrograde axon transport of BoNT/A to the spinal cord, resulting in decrease of COX-2 expression. All these effects result in amelioration of DO and BPS/IC symptoms. Adapted from: [33].

5. Limitations and Alternatives to BoNT/A

The therapeutic benefits of BoNT/A for treatment of a wide range of conditions are undeniable, and this serotype is by far the most extensively mentioned in literature [8]. Nonetheless, because of its transient effects, repeated injections are needed. In some patients, prolonged treatment could elicit the production of neutralizing antibodies against BoNT/A, thus reducing its beneficial effects or rendering the patient completely unresponsive to further treatment [39]. Moreover, some patients are naturally insensitive to BoNT/A since the first injection [61]. These conditions require the use of a different toxin serotype, and BoNT/B is being used as the substitute of BoNT/A for some applications, such in the cervical dystonia treatment (the only current approved application for BoNT/B) [39]. Yet, BoNT/B induces a shorter neuroparalysis effect (**Figure 5**) and therefore, higher doses are needed for the success of treatments. This could result in a bigger immune response against this serotype, and some studies support the idea that BoNT/B triggers the production of more neutralizing antibodies than BoNT/A [62]. Thus, using BoNT/B as an alternative for BoNT/A-insensitive patients may not be an effective choice.

BoNT/E was also suggested as a substitute for BoNT/A, but its relatively brief synaptic blockage may not be useful in the majority of treatments and very high doses would be necessary [63]. So, why not BoNT/C, the second serotype with the longest duration of action? For unknown reasons this serotype was never produced for therapeuthical uses. Only a few studies suggest BoNT/C as a valid alternative for treatment of BoNT/A-nonresponsive patients. Many years ago, a study revealed that BoNT/C is very effective in paralyzing human neuromuscular junctions when injected in muscles [64]. Another study demonstrated that BoNT/C is effective for the treatment of focal dystonia in humans, presenting a general profile of action similar to that of BoNT/A, and clearly longer than that of BoNT/F [61]. Moreover, BoNT/C has not so far been linked to a single case of human food-borne botulism and only one case of infant human botulism has been reported [8]. This may be a main advantage of this serotype because it would be possible to have the beneficial effects of the toxin, without exposing the patients to the possibility of botulism [64]. Also, a recent study shown the efficacy of BoNT/C in blocking the release of nociceptive peptides (such as CGRP), showing the possible antinociceptive action of this serotype [65]. This may suggest that similarly to BoNT/A, BoNT/C is also effective in the treatment of pain. Accordingly, Meng and co-workers showed an increase in the antinociceptive action when BoNT/A and BoNT/C were simultaneously administered, relatively to BoNT/A alone [65]. These cumulative or synergistic effects of different BoNT serotypes may be advantageous to the management of pain, but more research is needed.

Another approach to surpass some of the limitations presented by these toxins is the engineering of BoNTs. One of the hypothesis to engineering these toxins is to enhance their activity, making more potent toxins. This way, they would present extended duration of clinical effects, avoiding repeated injections [20]. Also, with the relevant progresses in biomolecular technology, the design of hybrid neurotoxins was possible and is a subject of intense research [21]. Recombinant neurotoxins were already synthetized, with the purpose of combine key functional characteristics of different BoNTs, enhancing their affinity and potential [66]. Moreover, due to BoNTs specificity and low immunogenicity, they have already been engineered to be drug delivery vehicles of other cargos different from their L chain [20]. BoNTs engineering will provide a range of therapeutic applications that are not currently addressed by the native toxins, but further studies are needed, namely in what concerns the molecular structure and targets of the different BoNTs [21].

6. Gap-knowledges in Botulinum Toxins Biological Action

Although many studies have already unveiled the molecular basis of BoNTs action, there are still many unresolved issues. The main outstanding questions are related to the mechanistic details of toxin binding to the presynaptic membrane and endocytosis inside synaptic vesicles, and how the translocation of the L chain occurs across the synaptic vesicle membrane and is directed to the cytosol [32]. All these aspects will affect the relative potency and duration of action of BoNTs. Also, there is a major need to establish reliable methods to compare the already identified BoNTs and the BoNTs that may be identified [2]. Another important question is to determine if BoNTs are really retroaxonally transported to the central nervous system and what mechanisms they use [32].

6.1. Diversity of Serotypes and Their Categorization

Until now, about 40 subserotypes of BoNTs have been identified and this number is expected to grow in coming years, once BoNT genes have a high variability and are present in several Clostridium strains [2]. These serotypes have been categorized as subtypes, which are defined as groups of toxins immunogenically related to the parental serotype, but differing in the aminoacid composition from 2.6 to 31.6% [67]. However, this comparison method may be unreliable and many questions have been pointed out, such as: if only the amino acid differences should be included or also the nucleotide changes, if the number of differences justify a new designation, or if the changes affect the function of the toxins [3].

6.2. Binding and Internalization of Botulinum Toxins

As mentioned before, despite all the insights into the binding mechanism of the different serotypes, the coreceptors for BoNT/C and BoNT/D have not yet been identified. Some studies have shown that these two serotypes lack the PSG-binding motif that is conserved in all other BoNTs (the SxWY motif), but contain analogous binding residues in the same position [24]. Other studies have also revealed that these serotypes have a second PSG-binding site in the HC domain, consisting on a WY loop [68]. These WY residues are known to be involved in binding to ganglioside and carbohydrate receptors. Additionally, a sialic acid binding site was also discovered in the HC domain of BoNT/C, indicating other possible binding sites for gangliosides and carbohydrates [24]. Based on these findings, it has been suggested that BoNT/C might not need a protein coreceptor and instead, this serotype utilizes gangliosides as dual host receptors [69]. However, further evidences are needed. Moreover, although the dual-receptor model is the accepted model for the toxins' binding to the presynaptic membrane, other receptors may be involved. Also, it is still unclear if the subserotypes use the same, different, or additional surface receptors than the parental serotypes [24].

Other issue that still needs further studies is the internalization of BoNTs. It has been shown that BoNT/A1 is internalized in the lumen of the synaptic vesicles, but the mechanism that the remaining toxins use has not yet been established [3]. Additionally, it was shown that BoNTs can reach higher structures in the brain, especially when administered in high doses, contradicting the common believe that their effects remain restricted to the peripheral nervous system. It may suggest that other trafficking routes might contribute to toxin entry, namely the retrograde axonal transport [52]. Detailed studies of the effects of the peripherally administered toxins in the central nervous system is mandatory (particularly, the precise site that they affect and mechanism of action) and will provide valuable information for more applications of these toxins [51].

6.3. L Chain Translocation and Neuroparalysis

The mechanism of translocation of the L chain across the synaptic vesicle membrane its only partially elucidated, and the mechanisms by which it reaches the cytosol, where it acts, is still unknown [32]. Only some evidenced have been raised about the temperature and pH gradient dependence for the L chain translocation. It was shown that the time course of translocation varies between serotypes, but it remains in the range of minutes, at 37°C, at a pH range of 4.5-6 [70].

Some aspects regarding the potency of neuroparalysis of the toxins are also unanswered. It has been assumed that once in the cytosol, the L chain of each serotype cleaves specific SNARE proteins, preventing the formation of a stable SNARE complex, leading to the blockage of neurotransmitter release [2]. However, quantitative and topological data of this proteolytic activity are still missing, namely the turnover rate of these metalloproteases and the subcellular localization of their action. Also, the percentage and localization of the cleaved substrate necessary to cause neuroparalysis is still unknown [3]. Moreover, in spite of the identification of the substrates for many of the BoNT serotypes, some subtypes have not already been studied. It is unlikely that different substrates will appear, but there is the possibility for the discovery of new cleavage sites within the SNARE proteins [3].

Furthermore, from a practical point of view, there are still some imprecisions in the measurement of BoNTs potency, and inadequate comparisons between the potency of different BoNT serotypes and brands [71]. The biological potency of BoNTs is usually measured by a LD_{50} assay (the only assay approved by the FDA for this purpose), where the mouse lethal dose is estimated, being the potency expressed in LD_{50} units. These units have to be attributed to each serotype and to each formulation, and conversion ratios are estimated between them [72]. However, these conversion ratios have been questioned and several drawbacks have been pointed to the LD_{50} method, such as large error rates, no standardization, high costs, large number of animals used and nonspecific deaths of the animals. Therefore, there is an increasing need to replace this assay for a more precise and accurate method for the estimation of the relative potency of BoNTs [73].

6.4. Duration of Neuroparalysis

Duratin of action of many subserotypes has not yet been determined and it should be determined in *in vivo* studies [3]. Also, studies to clarify the reasons of such different duration of actions should be performed. The recovery of synaptic function after BoNTs intoxication seems to be related with the replacement of damaged SNARE proteins for new and functional molecules [8]. This way, a deeper knowledge about duration of action of the BoNTs would also provide information about the mechanisms of inactivation and turnover of the cleaved SNARE proteins and the mechanisms of assembly of the SNARE complex [2]. Therefore, these issues are all interconnected and studies in a particular aspect will affect the remaining outstanding questions.

II. Aims of the Work

II. Aims of the Work

There is an evident gap-knowledge about the relative potency and the duration of BoNTs neuroparalysis, characteristics that are intrinsically associated with the molecular targets of these toxins. Therefore, this work had four main goals:

The first goal was to study the molecular targets of newly synthetized botulinum toxins serotypes A and C, and to evaluate their potency and duration of action in cellular and animal models. Immunofluorescence methods were used to evaluate toxins' activity, labeling for the target of both BoNTs type A and C – cleaved SNAP-25 – and for other specific neuronal markers.

The second goal was to investigate if the *in-vivo* co-administration of botulinum toxins types A and C can enhance their potency and duration of action, which would be translated into a potential novel and efficient approach for various applications, especially the management of pain.

Third, we wanted to compare the effects of botulinum toxin type A in skeletal and smooth muscle fibers. For that, we used a model of rat and mouse cranial muscles to represent the skeletal muscle, and the urinary bladder to illustrate smooth muscle.

Lastly, we also intended to optimize reproducible models, where methodological approaches outside the scope of this work may be applied to study botulinum toxins, such as Western-Blotting and gene expression. Four potential models were evaluated: a continuous cell line (the ND7/23 hybrid cell-line), a primary culture of dorsal root ganglia (DRG), the urinary bladder and the model of rat and mouse cranial muscles.

III. Materials and Methods

III. Materials and Methods

1. Animals

Young adult Wistar rats (Charles River, Barcelona, Spain), with 6 to 10 weeks and weighting 80-100 g were used for the *in-vitro* experiments.
In-vivo experiments were performed in adult female Wistar rats (Charles River, Barcelona, Spain) weighting 220-250 g and female C57BL/6N mice (Charles River, Barcelona, Spain) with a weight of 20-30 g.
All animals were housed in our animal facility under controlled conditions of light (12h light/12h dark schedule), temperature (20-24°C) and humidity (55% +/- 10%), with free access to food and water. The procedures were carried out according to the European Communities Council Directive of 22 September 2010 (2010/63/EU). All efforts were made to reduce the stress, suffering, and the number of animals used.

2. Toxins and Antibodies

BoNT/A and BoNT/C (150 kDa purified protein without accessory proteins) were a kind gift of Dr. Thomas Binz (Hannover Medical School, Germany), a world authority in the study of clostridial toxins, who has permission to produce wild type, mutated or reassembled recombinant BoNTs for research purposes. For experiments with cell cultures, toxins were aliquoted in sterile conditions and stored at -80 °C.
For the animal experiments, toxins were diluted in a TRIS 0.1 M pH 8 solution and frozen at -80 °C. Injectable BoNT solutions were freshly made at the time of injection.
Rabbit anti-cSNAP-25 (cleaved SNAP-25), produced against the truncated C-terminal peptide of SNAP-25, was kindly provided by Professor Ornella Rossetto (University of Padova, Italy) and has been previously described by Antonucci *et al* [52]. Characteristics of the other primary antibodies used in this work are summed up in **Table 4**.
Secondary biotinylated swine anti-rabbit was purchased from Palex Medical S.A. (Barcelona, ES) and avidin-biotin complex (ABC - horseradish peroxidase (HRP), Vectastain® Elite Kit) was from Vector Laboratories (Peterborough, UK). Fluorochrome-labeled secondary antibodies were obtained from Molecular Probes Europe, Life Technologies (Paisley, UK) and are described in **Table 4**.

Table 4. Primary and secondary antibodies used in this work.

	Target/ Specification	Dilution	Host	Manufacturer
Primary Antibodies	cSNAP-25	1:2000	Rabbit	Ornella Rossetto
	cSNAP-25	1:2000	Mouse	RDABs (Las Vegas, USA)
	FGFR3	1:1000	Rabbit	Cell signaling Tecnhology (Leiden, NL)
	GAP-43	1:5000	Rabbit	Abcam (Cambridge, UK)
	SNAP-25	1:2000	Mouse	Abcam (Cambridge, UK)
	SV2	1:3000	Mouse	DSHB (Iowa, EUA)
	Synapsin-I	1:2000	Rabbit	Cell signaling Tecnhology (Leiden, NL)
	β3-tubulin	1:10000	Mouse	Abcam (Cambridge, UK)
Secondary Antibodies	Rabbit/ Alexa Fluor 488	1:1000	Goat	Molecular Probes Europe (Paisley, UK)
	Mouse/ Alexa Fluor 568	1:1000	Goat	Molecular Probes Europe (Paisley, UK)

3. *In-vitro* Experiments

3.1. ND7/23 Cell Culture

ND7/23 cells were maintained and grown in DMEM (Dulbecco's Modified Eagle Medium) with high glucose, supplemented with 20% fetal bovine serum, 1% GlutaMAX™ and 1% penicillin and streptomycin, all from Gibco, ThermoFisher Scientific (Waltham, USA). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. After 3 days in culture, cells were plated in 24-well plates covered with poly-L-ornithine (Sigma, Missouri, USA) coated glass coverslips (~30% confluence). The next day, differentiation medium was added to cells, consisting in DMEM with high glucose supplemented with 0,5% fetal bovine serum, 1% GlutaMAX™, 1% penicillin and streptomycin, 0.5 nM of ds-cAMP and 100 ng/mL of NGF. Cells were kept in the differentiation medium for 2 days before use.

3.2. Rat DRG Culture

Young adult Wistar rats were killed with an overdose of sodium pentobarbital (Eutasil 200 mg/Ml; obtained from CEVA, Algés, PT) administered by intraperitoneal injection. After exposure and removal of the spinal cord, DRGs were collected in DMEM mixture F-12 Ham (DME/F-12) (Sigma, Missouri, USA). Connective tissue was digested with DME/F-12 with 0.125% collagenase for 3 hours, at 37 °C. DRGs were then triturated with the help of a fire-polished Pasteur pipette and plated in 24-well plates covered with poly-L-ornithine coated

glass coverslips. Cells were maintained at 37 °C and 5% CO_2 gas phase, in DME/F-12 supplemented with 4% Ultroser G (Pall Life Sciences, USA), 1% GlutaMAX™, and 1% penicillin and streptomycin (complete medium) for 6 days, with an intermediate change of culture medium. 10 µM of cytosine arabinoside (AraC) was also added to DRG cultures, in order to arrest growth of non-neuronal cells.

3.3. Cells' Exposure to Botulinum Toxins

After ND7/23 cells' differentiation and DRG cells incubation periods, various quantities of BoNT type A or C were added to culture medium in a total volume of 500 µL per well, followed by an incubation of approximately 24 hours, at 37 °C and 5% CO_2.

In the case of the depolarizing buffer experiment in DRGs, cells were incubated with a control buffer (140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) or a high-k^+ buffer (same as control buffer but adjusted to 87 mM NaCl and 56 mM of KCl), in the presence of the desired concentration of BoNT/A (10 nM), for 10 minutes, at 37 °C and 5% CO_2 gas phase. After that time, buffers were removed and 500 µL of DRG complete medium with AraC was added to each well. DRG cells were kept in this conditions for 24 hours.

It is important to notice that all experiments were controlled using the same described conditions but without any addition of the toxins.

3.4. Immunocytochemistry

Cells were washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 minutes, and then washed again with PBS. Next, cells were permeabilised for 15 minutes in PBS with 0.3% of Triton X-100 (PBST) and blocked with 10% normal goat serum (NGS) in PBST for 1 hour at room-temperature, prior to 3 overnights incubation with primary antibodies diluted in 2% NGS in PBST, at 4 °C. After incubation time, cells were rinsed 3 times in PBST and incubated with the respective fluorescent secondary antibody diluted in 2% NGS in PBST, for 1 hour at room temperature. After another washing step with PBST (2x) and PBS (1x), cell-containing coverslips were mounted on slides with a glycerol/PBS medium (3:1) or using Fluoroshield Mounting Medium with DAPI (Abcam, Cambridge, UK). Control staining was done to all reactions by omitting the primary antibodies, followed by incubation with secondary antibodies. Immunofluorescence results were observed with a Zeiss Imager Z1 microscope and images were collected using the AxioVision 4.6 software.

4. *In-vivo* Experiments

4.1. Botulinum Toxin Type A Administration in Rat and Mouse Urinary Bladders

Female Wistar rats and C57BL/6N mice were anesthetized with isoflurane anesthesia (IsoFlo; Abbott Laboratories, Maidenhead, UK), delivered in oxygen (5% for induction and 1-3% for maintenance) and their bladders exposed by laparotomy. This method is necessary since in a previous study [74], it was showed that simple instillation of the bladder with BoNT/A did not cross the inner epithelial lining of the organ.

The animals received intramural injections of the indicated doses of BoNT/A (2 ng for rats and 0,2 ng for mice) diluted in a saline 0,5% BSA solution. 6 injections of 10 µL in rats and 6 injections of 1 µL in mice were performed around the entire organ, with a microliter syringe (Hamilton; Missouri, USA). After 2 days of administration, the animals were terminally anesthetized with an overdose of sodium pentobarbital (0.1 mL/ 100 g of body weight) and bladders were collected and fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at 4 °C, and subsequently stored in a 30% sucrose in 0.1 M PB solution. Control non-injected animals were handled the same way.

4.2. Sectioning of Rat and Mouse Bladders

Bladders were embedded in the freezing compound FSC 22 (Leica Microsystems, Wetzlar, Germany) and longitudinal sections with a thickness of 20 µm were cut with a Zeiss Microm® cryostat and collected in Superfrost™ Plus glass slides (Thermo Cientific™, Massachusetts, USA).

4.3. Botulinum Toxins Administration in Rat and Mouse LAL Muscle

Wistar rats and C57BL/6N mice were anesthetized with isoflurane anesthesia, delivered in oxygen (5% for induction and 1-3% for maintenance) and the indicated doses of BoNT/A or BoNT/C were diluted in a saline 0,5% BSA solution and injected subcutaneously in the area of cranial muscles in a total volume of 100 µL for mice and 500 µL for rats. After the indicated times of administration, the animals were killed with an overdose of sodium pentobarbital (0.1 mL/ 100 g of body weight), and the muscles and a representative part of the ears were dissected and fixed in a 4% PFA in 0.1 M PB solution for approximately 2 hours. Posteriorly,

entire muscles were separated from each other and stored in PBS containing 0.01% of sodium azide, at 4 °C. Control non-injected animals were handled the same way.

4.4. Fluorescence Immunohistochemistry

Representative slides of the bladders of the desired animals were washed 3 times in PBS and then permeabilised for 15 minutes in PBST.
Cranial muscles were also washed 3 times in PBS but before PBST permeabilisation, muscles were permeabilised with different ethanol concentrations (50%, 99,6%, 50% v/v) for 30 minutes each, and then rinsed more 3 times with PBS. Permeabilisation was then performed using PBS with 1% of Triton X-100 (PBST 1%) for 15 minutes.
After permeabilisation procedures, slides of the bladders and muscles were blocked with 10% NGS in PBST/PBST 1% (respectively) for 1 hour at room-temperature, followed by 3 overnights incubation with primary antibodies diluted in 2% NGS in PBST/PBST 1%, at 4 °C. After incubation time, bladders and muscles were washed 3 times in PBST and incubated with the respective fluorescent secondary antibody diluted in 2% NGS in PBST/PBST 1%, for 1 hour at room temperature. Finally, bladders and muscles were washed with PBST/PBST 1% (2x) and PBS (1x), mounted with a mounting medium (glycerol 3:1 in PBS), and observed with a Zeiss Imager Z1 microscope. Images were collected using the AxioVision 4.6 software. For LAL muscles and some bladder sections, Z-stack images were collected and originated with the referred software and an example of this technique can be found in **Supplementary Image 1** in attachments.
Importantly, control staining was performed to all reactions by omitting the primary antibodies, followed by incubation with secondary antibodies.

4.5. Avidin-biotin Complex (ABC)-immunohistochemistry

LAL cranial muscles were washed 3 times in PBS, permeabilised with different ethanol concentrations (50%, 99,6%, 50% v/v) for 30 minutes each, and then rinsed 3 times with PBS. Inhibition of endogenous peroxidase activity was performed using a hydrogen peroxide solution at 1% (v/v) in PBS for 30 minutes. Next, muscles were rinsed and permeabilised 3 times with PBST 1% and blocked with 10% NGS in PBST 1% for 1 hour at room-temperature. Then, muscles were incubated with the respective primary antibody diluted in 2% NGS in PBST 1%, for 3 overnight at 4 °C. After washing 3 times with PBST 1%, muscles were incubated overnight with the secondary biotinylated antibody diluted in

2% NGS in PBST 1% (1:200). Subsequently, muscles were rinsed more 3 times with PBST 1% and were incubated with the avidin-biotin complex diluted 1:200 in PBST 1% for 24 hours. ABC complex was prepared 30 minutes prior to use.

After incubation time, muscles were washed 3 times with PBST 1%, 1x with PBS and then washed with a Tris-HCl 0.05 M pH 7.6 buffer for 10 minutes. Immunoreaction revealing was done by incubation with a solution containing 0.05% (m/v) of 3,3'-diaminobenzidine tetrahydro-chloride (DAB) in Tris-HCl 0.05 M pH 7.6 buffer with 0.003% (v/v) of hydrogen peroxide. After revealing of the cromodogenic substrate, muscles were rinsed twice with Tris-HCl 0.05 M pH 7.6 solution and in PBS for 10 minutes.

4.5.1. Muscle mounting and image analysis

After ABC-immunohistochemistry reaction, muscles were dehydrated with an increasing ethanol gradient (50%, 70%, 99,6% and 100% v/v), washed with propylene oxide for one hour and embedded in a mixture of EPON and propylene oxide (1:1) for about 20 minutes at room temperature. After that, muscles were mounted in a glass slice and included with EPON 100% overnight, at 60 °C. Results were observed using a Zeiss Axioskop 40 microscope equipped with a Leica EC3 camera and images were collected using the Leica LAS Core V4.6 software.

For muscles mapping, about 200 images of the entire muscles were taken in a magnification of 5x and then mounted using the Photoshop CS6 Portable software (**Supplementary Image 2**). Drawings of the innervation patterns of the indicated muscles were also done using the referred software.

IV. Results

1. Results of *In-vitro* Experiments

1.1. ND7/23 Neuron Hybrid Cell-line

1.1.1. Morphology of differentiated ND7/23 cells

Neuronal morphology of differentiated ND7/23 cells was evaluated using the antibody against β3- tubulin as a pan-neuronal marker, since this protein is a specific constituent of neurons' microtubules.

Observation of the immunoreaction demonstrated that differentiated ND7/23 cells develop a neuronal phenotype with neurite outgrowth, after 7 days in culture. However, different levels of neurite outgrowth were shown and in some cells, it was absent (**Figure 7**).

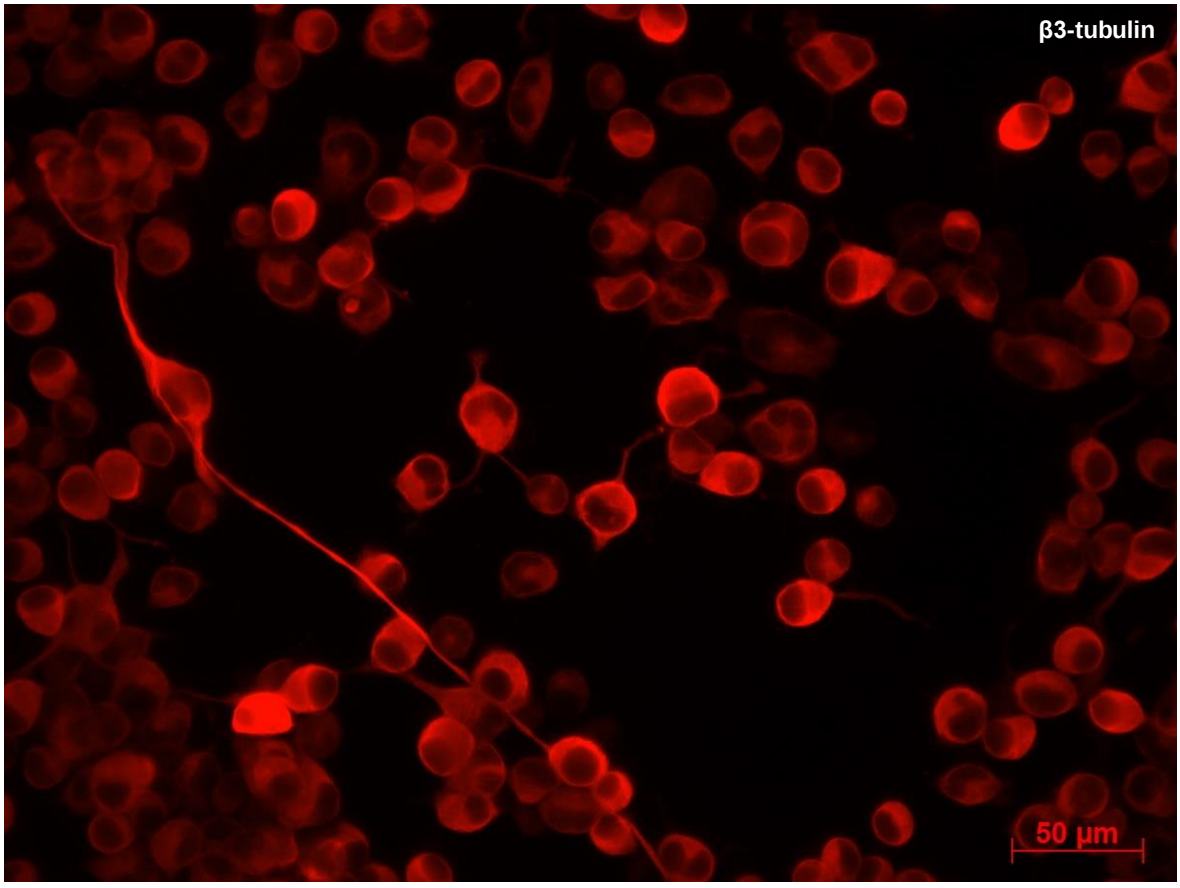

Figure 7. Neuronal phenotype of differentiated ND7/23 cells after 7 days in culture. Differentiated ND7/23 cells immunoreacted against β3-tubulin.

1.1.2. Expression of SV2 and co-localization with cleaved SNAP-25

Once the synaptic vesicle protein SV2 is the specific cellular binding receptor for BoNT/A, its expression was evaluated in differentiated ND7/23 cells by immunochemistry. Also, the co-localization of SV2 and the cleaved form of SNAP-25 was assessed in differentiated ND7/23 cells exposed for ~24 hours to different toxin concentrations. This co-localization was assayed by double staining immunocytochemistry.

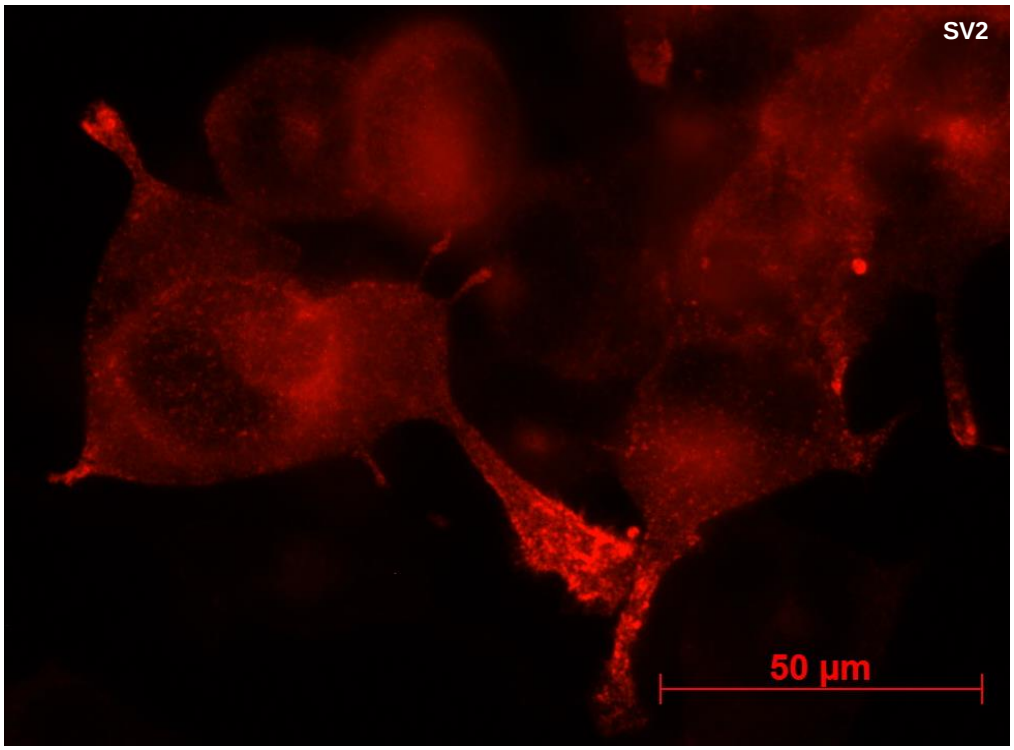

Figure 8. Distribution of SV2 in differentiated ND7/23 cells. ND7/23 cells stained for the protein receptor SV2.

Immunoreaction against the protein SV2 was observed in ND7/23 cells, ensuring that the receptor for BoNT/A is present in these cells. Moreover, SV2 was expressed in synaptic vesicle-like structures, revealing an intense and punctate distribution in the perinuclear region and in the tips of neurites (**Figure 8**).

Although the co-localization of SV2 and cleaved SNAP-25 was not observed in these cells after exposure to BoNT/A, the cleaved form of SNAP-25 appeared in SV2-expressing cells (**Figure 9. c) and f)**). However, independently of the toxin concentration, only a small number of cells revealed the catalytic product of BoNT/A (**Figure 9. b) and e)**). Still, it seems to be more cleaved SNAP-25 in ND7/23 cells exposed to the highest concentration of BoNT/A (10 nM) (**Figure 9. e)**).

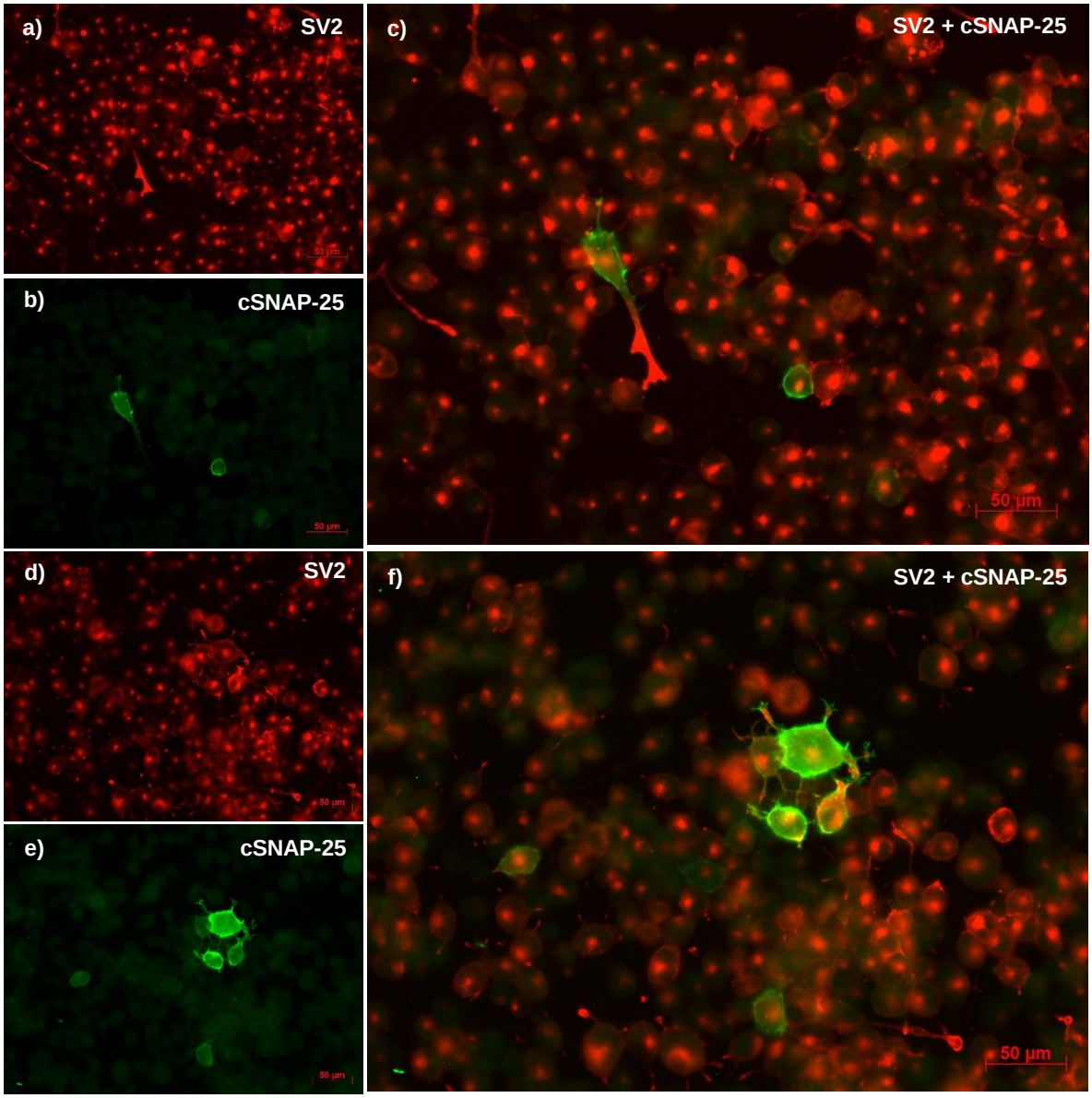

Figure 9. Expression of SV2 and the cleaved form of SNAP-25 in differentiated ND7/23 cells exposed to BoNT/A for 24 hours. a) ND7/23 cells labeled for SV2 after exposure to 5 nM of BoNT/A. **b)** ND7/23 cells labeled for cleaved SNAP-25 after exposure to 5 nM of BoNT/A. **c)** Overlap of a) and b). **d)** ND7/23 cells labeled for SV2 after exposure to 10 nM of BoNT/A. **e)** ND7/23 cells labeled for cleaved SNAP-25 after exposure to 10 nM of BoNT/A. **f)** Overlap of d) and e).

1.1.3. Expression of intact SNAP-25 and co-localization with cleaved SNAP-25 in ND7/23 cells treated with botulinum toxin types A and C

In order to evaluate the expression of the catalytic target of both BoNT type A and C in differentiated ND7/23 cells, and the co-localization with its cleaved form after the exposure of cells to the different toxins, a double staining immunoreaction was performed, using antibodies against intact SNAP-25 and cleaved SNAP-25.

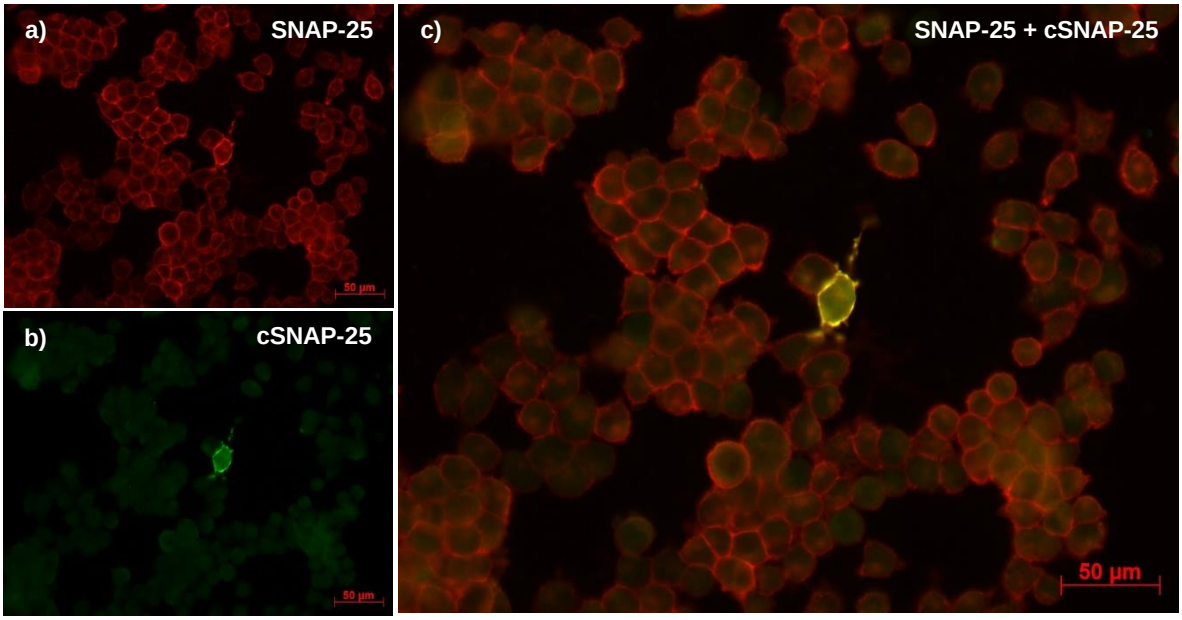

Figure 10. Expression of SNAP-25 and cleaved SNAP-25 in differentiated ND7/23 cells treated with BoNT/A. a) ND7/23 cells labeled for SNAP-25 after exposure to 5 nM of BoNT/A. **b)** ND7/23 cells labeled for cleaved SNAP-25 after exposure to 5 nM of BoNT/A. **c)** Overlap of a) and b). Cells were in culture for a total of 7 days and were exposed to BoNT/A ~24 hours.

The results of immunoreaction showed an intense staining for the intact form of SNAP-25 virtually in all cells, with a denoted expression of this protein in cell membranes (**Figure 10. a)**). On the other hand, the expression of the cleaved form of SNAP-25 after exposure to 5 nM of BoNT/A was only observed in a small number of cells (**Figure 10. b)**). Co-localization of intact and cleaved SNAP-25 was detected in the same conditions (**Figure 10. c)**).

In cells treated with the serotype C, no signs of neurodegeneration were observed and the expression of intact SNAP-25 was similar of that of cells treated with BoNT/A (data not shown). However, the cleaved form of this protein was not observed in ND7/23 cells treated with 5, 10 or even 20 nM of BoNT/C.

1.2. Primary Culture of Rat Dorsal Root Ganglia

1.2.1. Characterization of dorsal root ganglia culture

With the purpose of studying the neurochemical content of DRG cells relevant for this study, immunocytochemical detection was performed against specific neuronal markers.
Once again, a primary antibody against β3-tubulin, a constituent of microtubules, was used as a pan-neuronal marker to evaluate DRG cells phenotype. It was demonstrated that after approximately one week in culture, an extensive neurite network had formed (**Figure 11**). Actually, as long as 24 hours post-plating, cells already revealed some neurite formation. Labelling with DAPI (4′,6-diamidino-2-phenylindole) as a nuclear counterstain, showed that not all $DAPI^+$ cells are β3-$tubulin^+$ cells, indicating that non-neuronal cells are present in the culture.

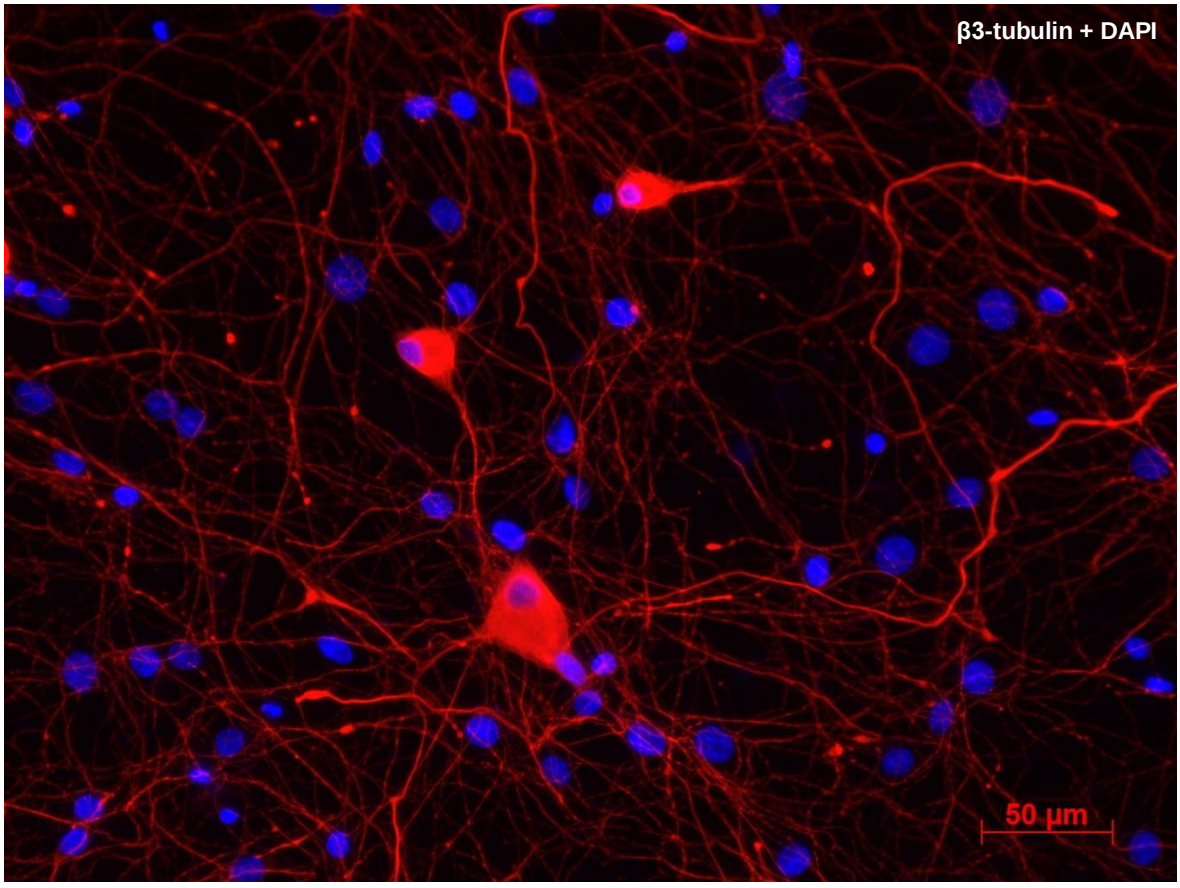

Figure 11. The extensive network of DGR cells after 7 days in culture. Immunoreaction against β3-tubulin (red) and counterstaining with DAPI (blue) of cultured DRG cells.

Distribution of the two known cellular binding receptors for BoNT/A, SV2 and FGFR3, in DRG cells was assessed using specific antibodies against these proteins. Also, the expression of the common catalytic SNARE target of BoNT/A and BoNT/C, the SNAP-25, was studied and its co-localization with the receptor SV2 was evaluated by double staining immunofluorescence, in DRG cells exposed and not exposed to BoNT/A.

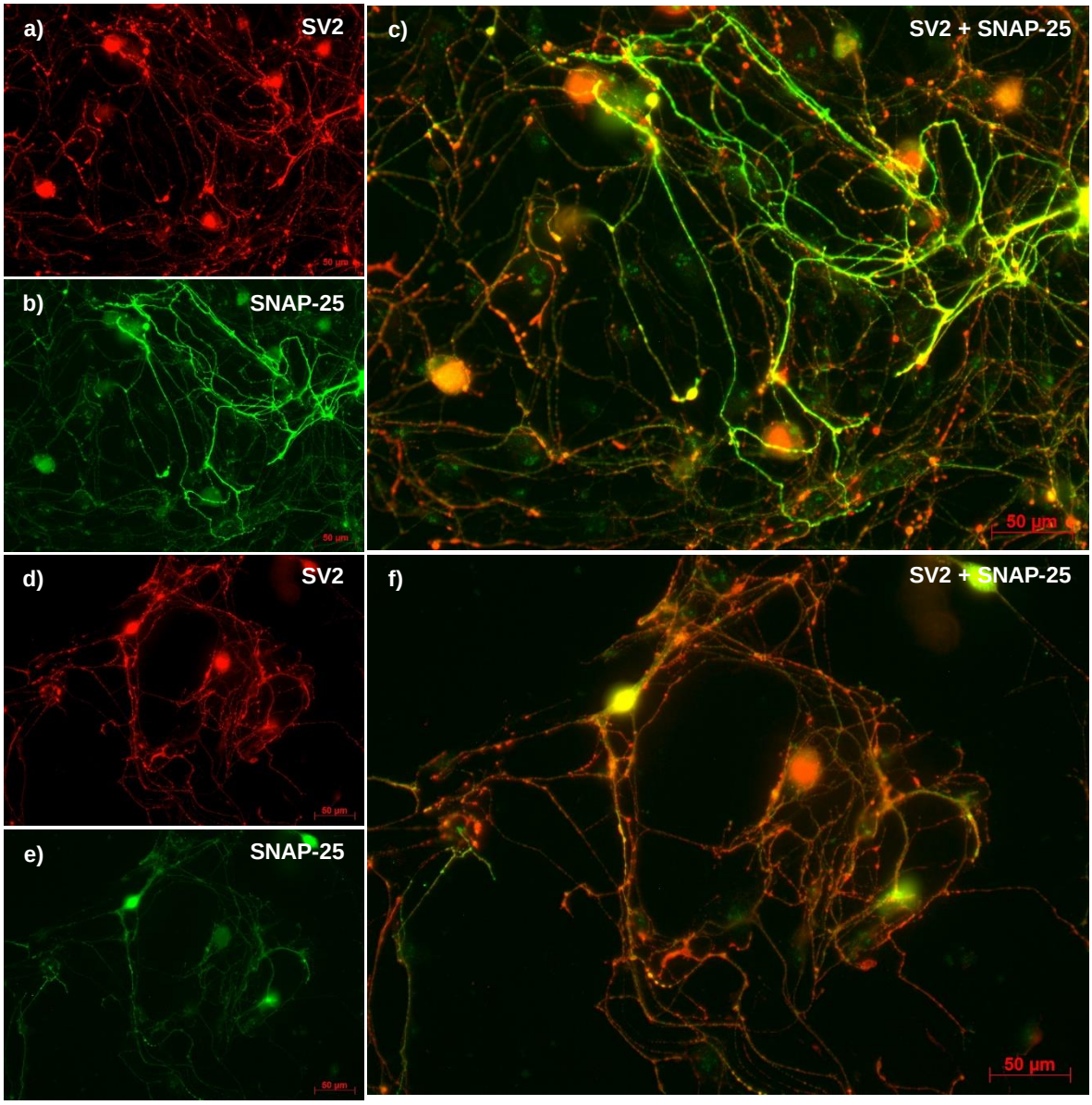

Figure 12. Expression of SV2 and intact SNAP-25 in DRG cells. a) DRG cultured cells labeled for SV2. **b)** DRG cultured cells labeled for intact SNAP-25. **c)** Overlap of a) and b). **d)** DRG cultured cells labeled for SV2 after exposure to 10 nM of BoNT/A. **e)** DRG cultured cells labeled for intact SNAP-25 after exposure to 10 nM of BoNT/A. **f)** Overlap of d) and e). The exposure time to BoNT/A was 24 hours and DRG were in culture for a total of 7 days.

In not exposed cultured neuronal cells, SV2 appeared with a marked expression in the cell body and also distributed through neuronal projections (**Figure 12. a)**). In contrast, SNAP-25 expression was more prominent in neurites than the cell body (**Figure 12. b)**). Co-localization of these two proteins was observed (**Figure 12. c)**), demonstrating that both the protein receptor and the catalytic target of BoNT/A are expressed in DRG cells and thus, the toxin may be able to exert its function.

Some SV2 redistribution was verified in DRG cells after exposure to 10 nM of BoNT/A, appearing with a more homogeneous diffusion (**Figure 12. d)**). A significant decrease in SNAP-25 was detected after exposure to the toxin (**Figure 12. e)**), indicating the possible action of BoNT/A in DRGs. Co-localization of SV2 and SNAP-25 was also shown in treated DRG cells (**Figure 12. f)**).

The negative results for the other specific cellular binding receptor for BoNT/A, the FGFR3, indicated that DRG cells may not express this protein and so, the binding and consequent internalization of the toxin is not dependent of this receptor.

1.2.2. Cleaved SNAP-25 in dorsal root ganglia treated with botulinum toxins

The potency of different concentrations of BoNT types A and C was studied in DRG cells by immunofluorescence detection of cleaved SNAP-25, after approximately 24 hours of exposure to the toxins. Double staining methods were used to co-localize cleaved SNAP-25 with the previously studied neuronal markers: β3-tubulin, SV2 and intact SNAP-25.

Co-localization of the cleaved form of SNAP-25 with β3-tubulin showed that not all cells labeled with cleaved SNAP-25 are of neuronal nature and so, the antibody used against this protein may not work perfectly in this type of cell culture (**Figure 13. c)**). However, β3-tubulin-stained cells (the neuronal cells) showed a more intense staining of cleaved SNAP-25, revealing the action of BoNT/A in DRG neurons. The higher concentration of BoNT/A – 10 nM – resulted in an even more pronounced labeling of cleaved SNAP-25, indicating a higher action of the toxin (**Figure 13. d) and g)**).

Despite the co-localization of cleaved SNAP-25 with the protein receptor SV2 (**Figure 13. f)**) and the intact form of SNAP-25 (**Figure 13. i)**), not all the cells in culture were affected by BoNT/A, whatever the concentration used. The same happened with the DRG cultures treated with BoNT type C. In this case, a very small number of cells showed a weak staining of cleaved SNAP-25 and it only occurred with the highest tested concentration, 30 nM (**Figure 13. j)**). Concentrations below this (10 and 15 nM) resulted in any labeling of cleaved SNAP-25. Also, like in ND7/23 cells, no signs of neurodegeneration were observed.

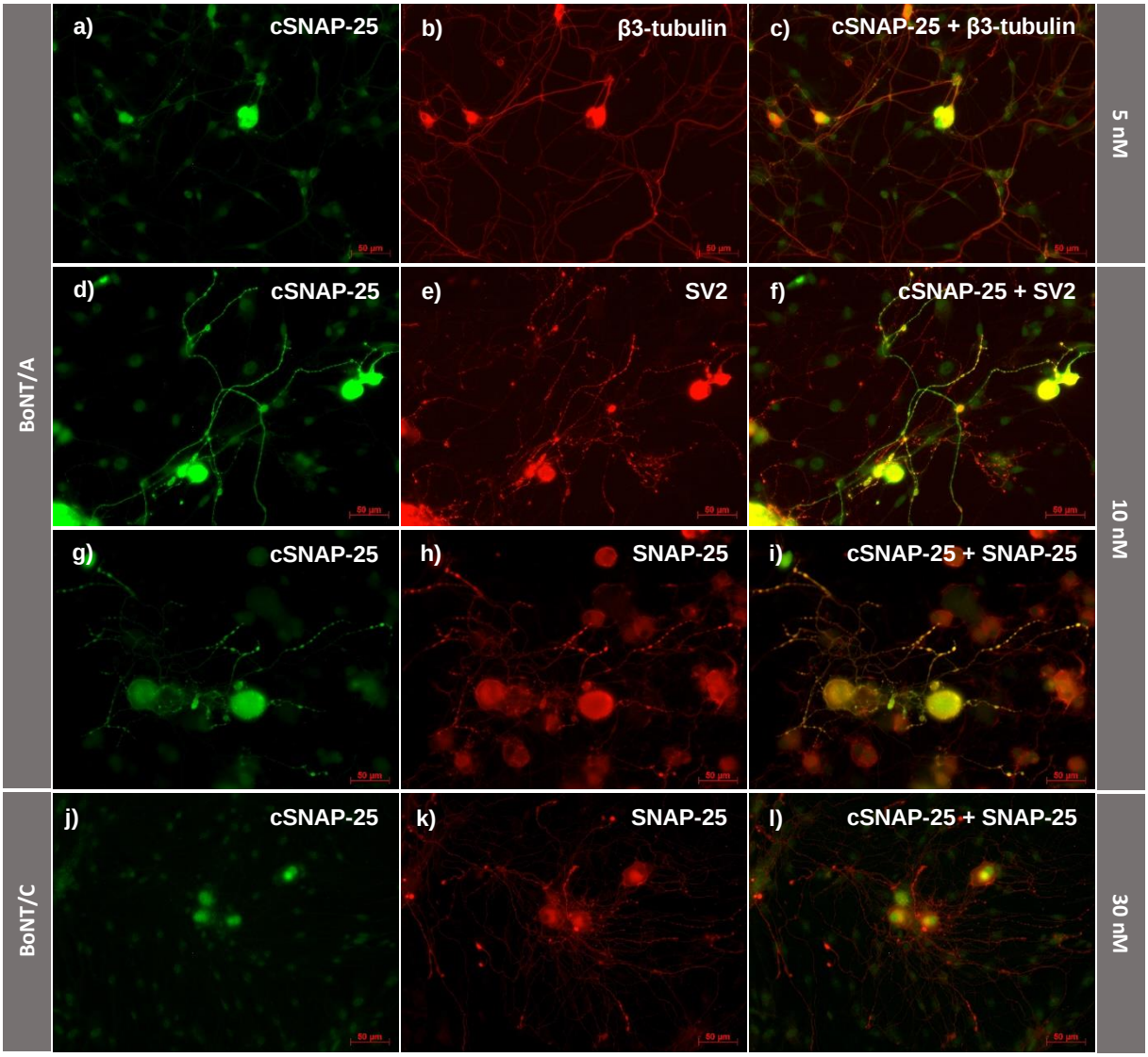

Figure 13. Double staining immunoreactions for cleaved SNAP-25 with various neuronal markers. **a)** DRG cultured cells labeled for cleaved SNAP-25 after exposure to 5 nM of BoNT/A. **b)** DRG cultured cells labeled for β3-tubulin after exposure to 5 nM of BoNT/A. **c)** Overlap of a) and b). **d)** DRG cultured cells labeled for cleaved SNAP-25 after exposure to 10 nM of BoNT/A. **e)** DRG cultured cells labeled for SV2 after exposure to 10 nM of BoNT/A. **f)** Overlap of d) and e). **g)** DRG cultured cells labeled for cleaved SNAP-25 after exposure to 10 nM of BoNT/A. **h)** DRG cultured cells labeled for intact SNAP-25 after exposure to 10 nM of BoNT/A. **i)** Overlap of g) and h). **j)** DRG cultured cells labeled for cleaved SNAP-25 after exposure to 30 nM of BoNT/C. **k)** DRG cultured cells labeled for intact SNAP-25 after exposure to 30 nM of BoNT/C. **l)** Overlap of j) and k). Toxins' treatment time for all the conditions was 24 hours and DRGs were in culture for a total of 7 days.

1.2.3. Cleaved SNAP-25 in dorsal root ganglia stimulated with depolarizing buffer

As not all cells were affected by both BoNT types A and C, we hypothesized that a deficient mechanism of internalization of the toxins was the reason for the low number of cells labeled for cleaved SNAP-25. Consequently, DRG cells were treated with a depolarizing high-k^+ solution for 10 minutes, in the presence or not of 10 nM of BoNT/A. A control buffer was used in the same conditions for results normalization, and the effect of the toxins was evaluated by immunocytochemistry against cleaved SNAP-25.

DRG cells exposed to BoNT/A in high-k^+ buffer showed relatively the same results in comparison with cells exposed to BoNT/A in control buffer and cells treated solely with the toxin (results not shown). This way, depolarization is not a critical factor for the action of the toxins, suggesting that the small number of cells affected might be due to failure of the internal neurotoxic mechanisms.

2. Results of *in-vivo* Experiments

2.1. Injection of Botulinum Toxin Type A in Rat and Mouse Bladders

2.1.1. Expression of GAP-43 in rat and mouse urinary bladder

Immunohistochemistry against growth associated protein 43 (GAP-43), a specific neuronal protein expressed in axons and presynaptic terminals, was used to study rat and mouse bladder innervation patterns, in non-injected animals.

As it is depicted in **Figure 14**, the intense staining revealed a high expression of GAP-43 in both rat and mouse bladder, showing fibers distributed throughout the detrusor muscular layer. Intraepithelial fibers (which are underneath the urothelium) were also detected (**Figure 14. a)**). In mouse bladder, labeling of small granules on the superficial cells of urothelium appeared (**Figure 14. b)**). These granules might be lipofuscin granules, which are part of the normal urothelium structure and present auto-fluorescence.

Ideally, a pan-neuronal marker, like the referred β3-tubulin, should have been used to study fiber distribution, but the antibody against β3-tubulin did not work well in rat and mouse bladder sections. However, as GAP-43 showed a ubiquitous expression, this neuronal marker was considered for the following experiments.

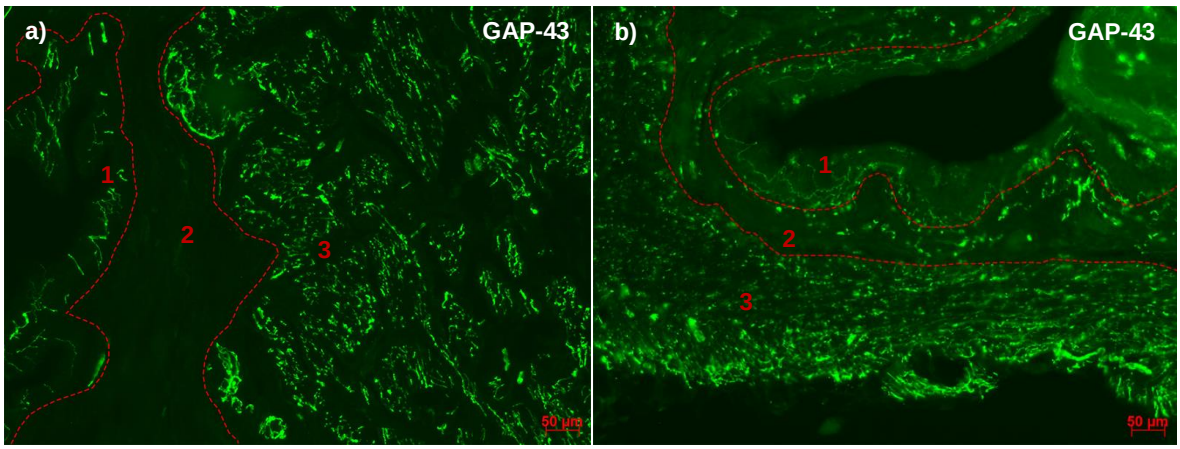

Figure 14. Innervation pattern of rat and mouse bladder. Longitudinal section of **a)** rat bladder and **b)** mouse bladder immunoreacted for GAP-43. **1-** Urothelium; **2-** Suburothelium; **3-** Detrusor smooth muscle.

2.1.2. Detection of cleaved SNAP-25 in rat and mouse bladder after injection of botulinum toxin type A

To evaluate toxin potency, the presence of the product of BoNT type A action, cleaved SNAP-25, was investigated in both rat and mouse urinary bladder, after injections in the bladder wall of 2 and 0,2 ng of the toxin, respectively. For that, immunoreactions against the cleaved form of SNAP-25 were performed. The injection dose used for mice was ten times lower than the one used for rats, once its weight is approximately ten times inferior than the weight of rats.

Also, co-localization of cleaved SNAP-25 with the neuronal marker GAP-43, and the effects of BoNT/A injection in GAP-43 expression were studied by double staining immunofluorescence methods. Moreover, the presence of compensatory sprouting after BoNT/A injection was also evaluated.

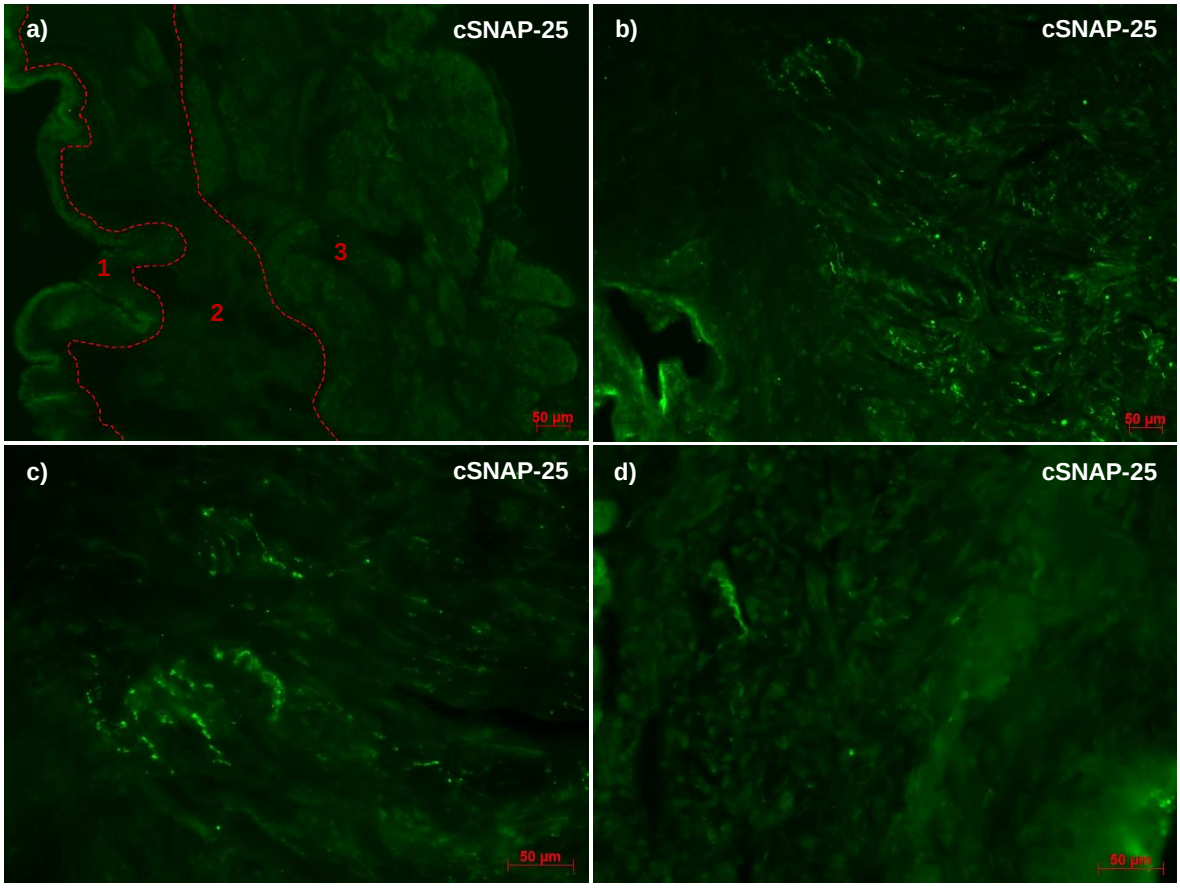

Figure 15. Expression of cleaved SNAP-25 in rat and mouse bladder after BoNT/A administration. **a)** Bladder longitudinal section of a non-injected rat labeled for cSNAP-25, serving as experimental control. **b)** Rat bladder labeled for cSNAP-25 after 2 days of intramuscular injection of 2 ng of BoNT/A. **c)** Z-stack and magnification of the anterior reaction. **d)** Mouse bladder immunoreacted for cSNAP-25 after 2 days of intramuscular injection of 0,2 ng of BoNT/A. **1-** Urothelium; **2-** Suburothelium; **3-** Detrusor smooth muscle.

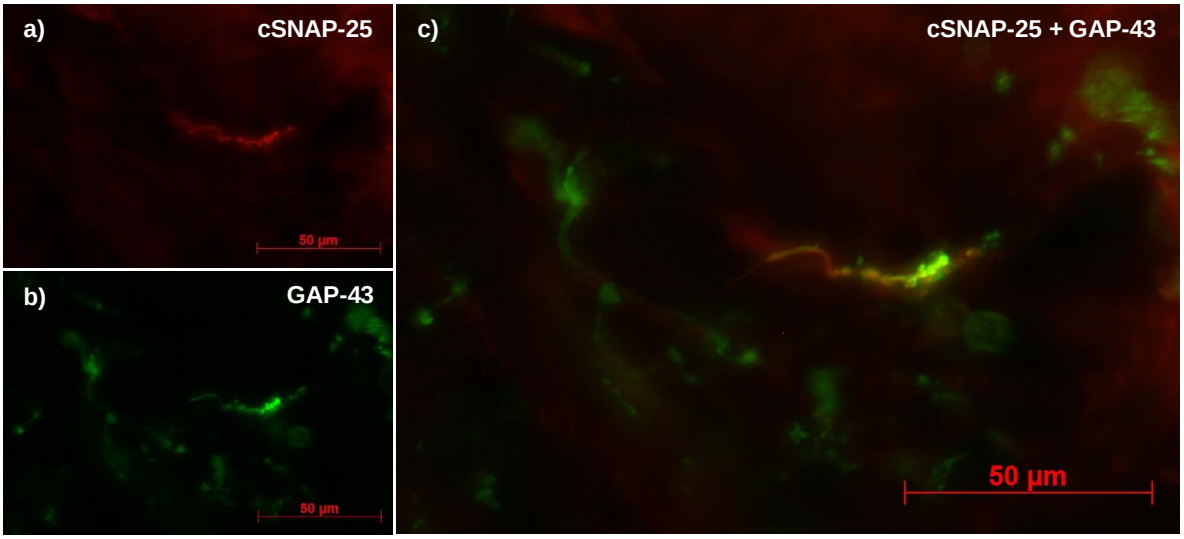

Figure 16. Co-localization of cleaved SNAP-25 and GAP-43 in rat bladder after BoNT/A injection. a) Rat bladder labeled for cSNAP-25 after intramuscular injection of 2 ng of BoNT/A. **b)** Rat bladder labeled for GAP-43 after intramuscular injection of 2 ng of BoNT/A. **c)** Overlap of a) and b).

The reaction against cleaved SNAP-25 showed some auto-fluorescence in the urothelium area (**Figure 15. a)**), which should be due to the high urothelium protein content that has a non-homogeneous distribution. Therefore, control images were always used to compare non-injected to injected animals.

Results demonstrated that after 2 days of injection, only a small population of muscle fibers were affected by the action of BoNT/A, either in rat or mice bladder (**Figure 15. b), c) and d)**). Co-localization of cleaved SNAP-25 with GAP-43 was achieved (**Figure 16. c)**), but the expression of GAP-43 was roughly the same in non-injected and injected animals, indicating that this protein was not subject to up-regulation. Also, the similar innervation patterns showed in non-injected and injected animals by staining with GAP-43, indicate that no nerve degeneration occurred due to BoNT/A injection. Additionally, no signals of cell sprouting were verified after 2 days of injection of BoNT/A in rat or mouse urinary bladder wall.

2.2. Botulinum Toxins A and C in a Model of Rat and Mouse Cranial Muscles

2.2.1. Identification of rat and mouse cranial muscles – the LAL muscle

A group of small cranial muscles was studied in order to optimize a good skeletal muscle model to investigate the mechanism of action, potency, duration of action and plasticity of botulinum toxins. These muscles are responsible for moving the pinna in rats and mice, and are organized in two distinct layers: the superficial layer comprising the levator auris longus (LAL) muscle; and a deeper layer formed by the interscutularis (IS), auricularis superior (AS) and abductor auris longus (AAL) muscles (**Figure 17**).

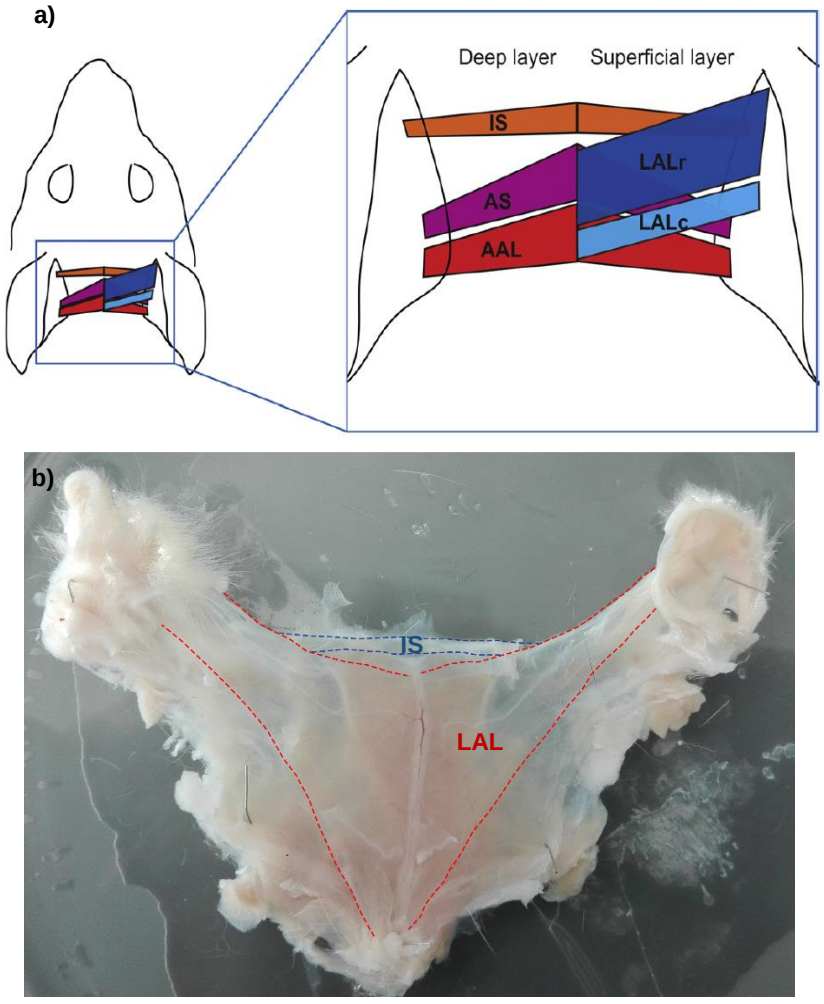

Figure 17. Anatomy of small cranial muscles of rats and mice. a) Schematic representation of cranial muscles responsible for moving the ears in rat and mouse. The right side of midline represents the intact muscle group, after removal of the skin, while the left side represents the deeper layer, after removal of the superficial layer. Adapted from: [75] **b)** Representation of rat muscles after the dissection procedure, which are stretched and pinned out in a Sylgard-covered Petri dish. **LAL** - levator auris longus (r-rostral, c-caudal); **Is** – interscutularis; **AS** - auricularis superior; **AAL** - abductor auris longus.

After successfully reaching an optimized dissection of the small cranial muscles of both rat and mouse, and a correct identification of the constituent muscles according to literature, we evaluated which one of the muscles would be a better model to test the toxins. Being the most superficial muscle and of easier access, we decided to use the LAL muscle for the following experiments, as it should result in a more reproducible model.
It is important to notice that the anatomy of rat and mouse cranial muscles is very similar, only differing in the size and thickness of the muscles.

2.2.2. Innervation pattern of the levator auris longus (LAL) muscle

In order to study the distribution of muscle fibers in the entire LAL muscle, the expression of the pan-neuronal marker Synapsin-I (Syn-I), a phosphoprotein present in possibly all nerve terminals, specially associated with synaptic vesicles, was studied.
For that, immunohistochemistry in mouse LAL muscle and avidin-biotin complex (ABC) immunoreaction methods in rat LAL muscle, using a primary antibody against Synapsin-I, were applied.
In both rat and mouse, an intense staining for Synapsin-I was verified in nerve bundles, end-plates and also, around blood vessels (**Figure 18. b) and c)**).
Figure 18. a) shows the entire mapping of the innervation pattern of rat LAL muscle, where it can be seen that two major groups of end-plates are present in this muscle: one in the middle part of the muscle and other in the most rostral side of the muscle.
Therefore, this cranial muscle model proved to be a method that allows to study in great detail the innervation of a complete muscle in rat and mouse, without bias.

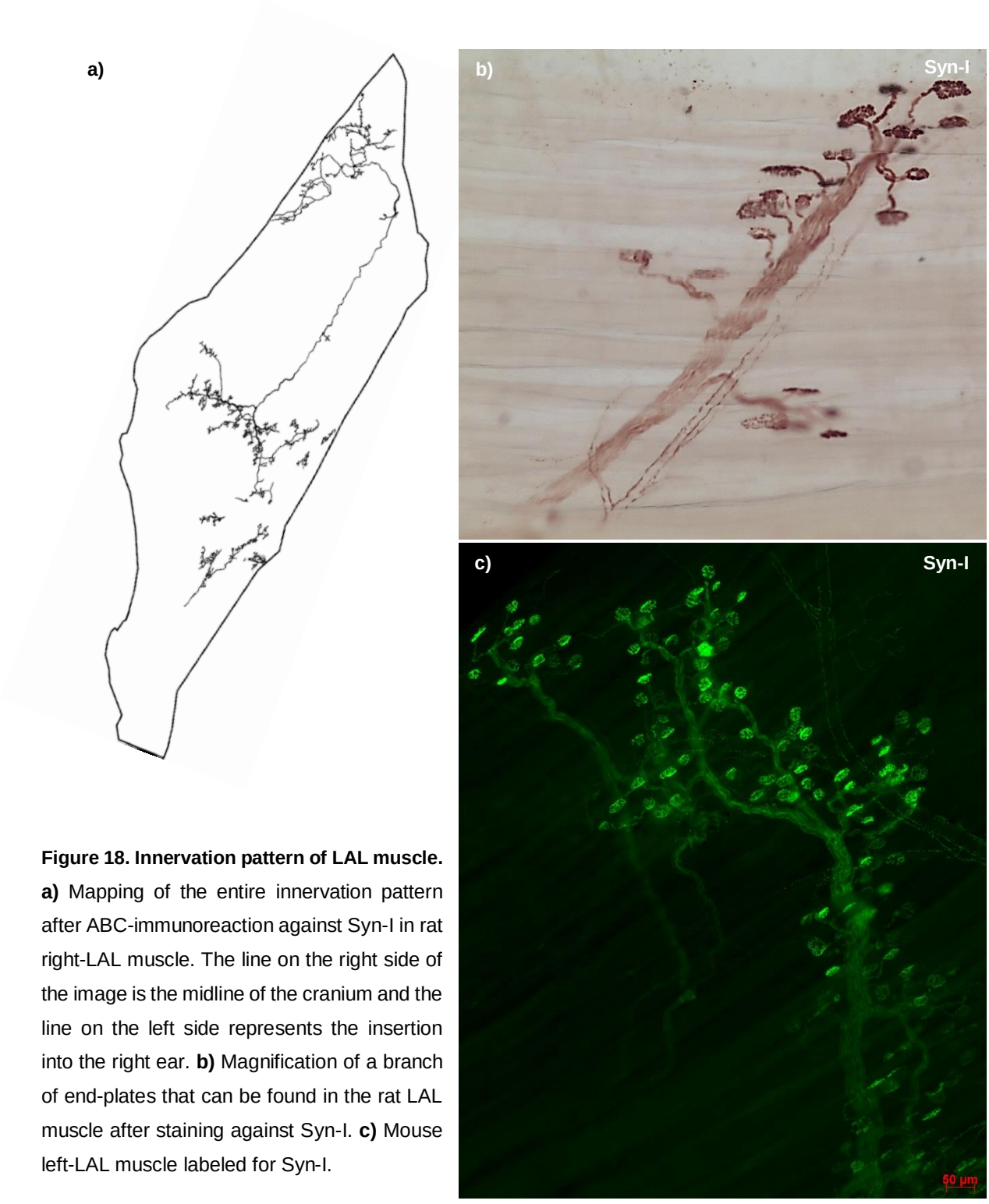

Figure 18. Innervation pattern of LAL muscle. **a)** Mapping of the entire innervation pattern after ABC-immunoreaction against Syn-I in rat right-LAL muscle. The line on the right side of the image is the midline of the cranium and the line on the left side represents the insertion into the right ear. **b)** Magnification of a branch of end-plates that can be found in the rat LAL muscle after staining against Syn-I. **c)** Mouse left-LAL muscle labeled for Syn-I.

2.2.3. Expression and distribution of cleaved SNAP-25 in the LAL muscle after injection of botulinum toxin types A and C

Toxicity of BoNT types A and C was determined by evaluating the expression of cleaved SNAP-25 in mouse and rat LAL muscles, after subcutaneous injections of different doses of the toxins. Immunofluorescence methods were used for this purpose.

Moreover, a mapping of the distribution of the affected motor end-plates in the rat LAL muscle injected with 1 ng of BoNT/A was done by ABC-immunohistochemistry against cleaved SNAP-25.

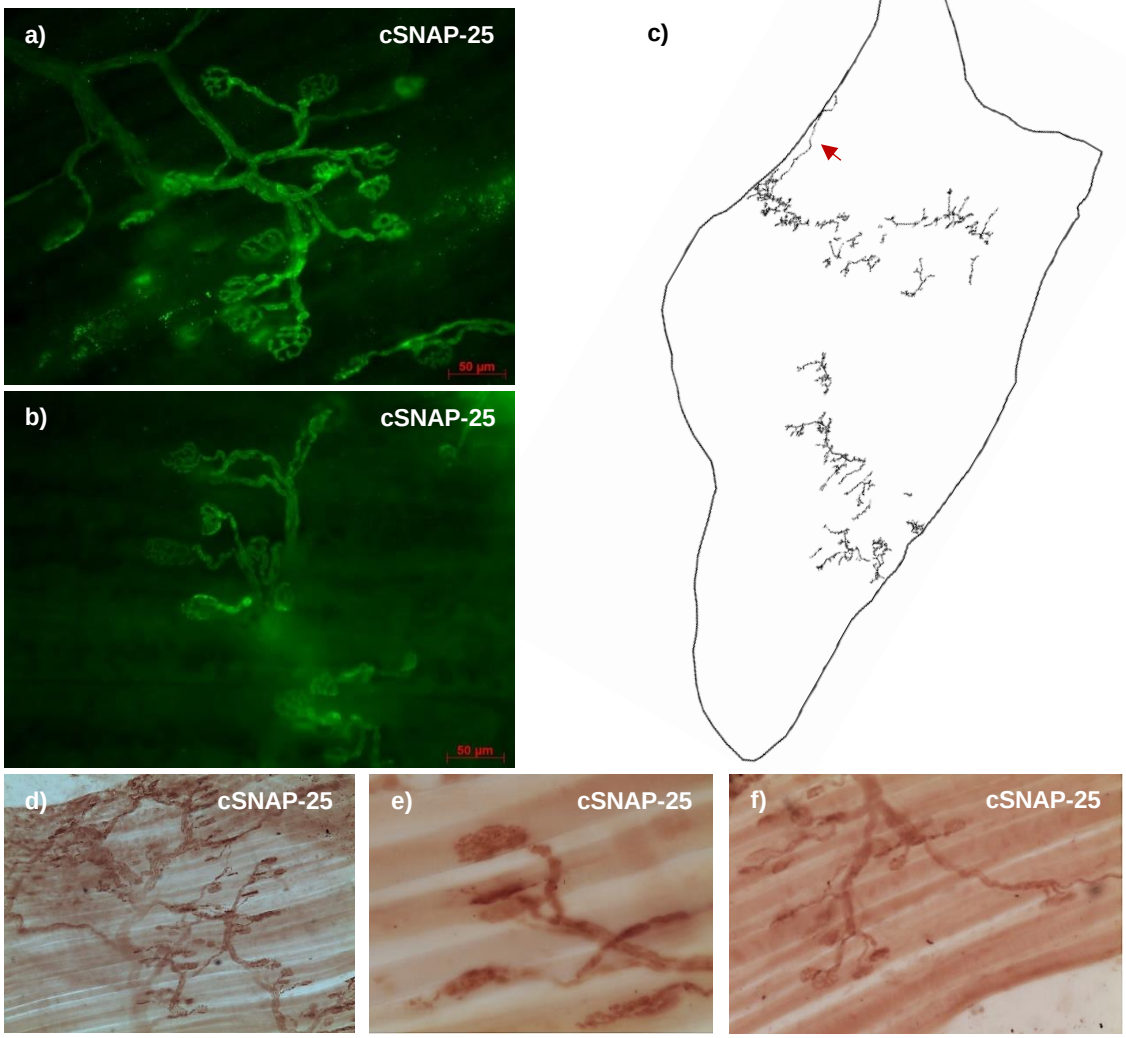

Figure 19. Expression of cleaved SNAP-25 in LAL muscles after BoNT/A injection. a) Mouse right-LAL muscle labeled for cSNAP-25 after 1 day of injection of 0.5 ng of BoNT/A. **b)** Mouse right-LAL muscle labeled for cSNAP-25 after 1 day of injection of 0.1 ng of BoNT/A. **c)** Mapping of the affected end-plates by ABC-immunoreaction against cSNAP-25 in rat right-LAL muscle, after 7 days of injection of 1 ng of BoNT/A. The line on the right side of the image is the midline of the cranium and the line on the left side represents the insertion into the right ear. Red arrow represents a possible nerve sprout. **d)** and **e)** Representations of the type of end-plates that may be found in c). **f)** Mouse left-LAL muscle labeled by ABC-immunohistochemistry against cSNAP-25 after 4 days of injection of 1 ng of BoNT/A.

The catalytic product of BoNT type A, cleaved SNAP-25, was found in the end-plates and nerve bundles in mice injected with 1, 0.5 and 0.1 ng of the toxin, indicating the activity of BoNT/A in the LAL muscle (**Figure 19. a), b) and f)**). Moreover, these injected mice were subjected to BoNT/A treatment for 1 (**Figure 19. a) and b)**) and 4 days (**Figure 19. f)**), indicating the persistence of BoNT/A action over time. Furthermore, comparing the immunofluorescence results, the cleaved form of SNAP-25 showed a more intense staining in the higher injected dose (**Figure 19. a)**).

Mapping of the injected rat LAL muscle (**Figure 19. c)**) showed that a large population of end-plates and nerves was affected by BoNT/A activity, since end-plates of the two groups showed in **Figure 18. a)** appeared labeled for cleaved SNAP-25. Also, a possible sign of sprouting was verified with the mapping of the muscle (red arrow), which was subjected to 7 days of BoNT/A administration.

Unfortunately, the cleaved form of SNAP-25 was not detected in LAL muscles of rats and mice injected subcutaneously in the area of cranial muscles with BoNT type C, indicating the non-activity of the toxin. However, in an experiment where mice were co-administered with a 1 ng dose of both BoNT/A and BoNT/C or administered with the same dose of the separated toxins, one of the animals injected with BoNT/A and C died 4 days after treatment. This raised the possibility of a synergistic effect of the two types of toxins when injected together, but the levels of cleaved SNAP-25 expression did not increase.

2.2.4. Sprouting in LAL muscles after BoNT/A administration

Synaptic plasticity at the neuromuscular junctions of LAL muscles after BoNT/A administration was evaluated by double-staining immunohistochemistry, using antibodies against Synapsin-I and the pan-neuronal marker β3-tubulin.

Co-localization between Synapsin-I and β3-tubulin was verified with the exception of nerve end-plates, which were only stained by Synapsin-I. Non-injected rat LAL muscles showed the presence of a really reduced number of sprouts (**Figure 20. a)-c)**) and no sprouts were shown after 1 day of BoNT/A administration (**Figure 20. d)-f)**). In the other hand, after 7 days of BoNT/A injection in LAL muscle (**Figure 20. g)-i)**), a vast sprouting process was detected, suggesting that a compensatory recovery process starts in the early stages of BoNT/A neurointoxication in the LAL cranial muscle.

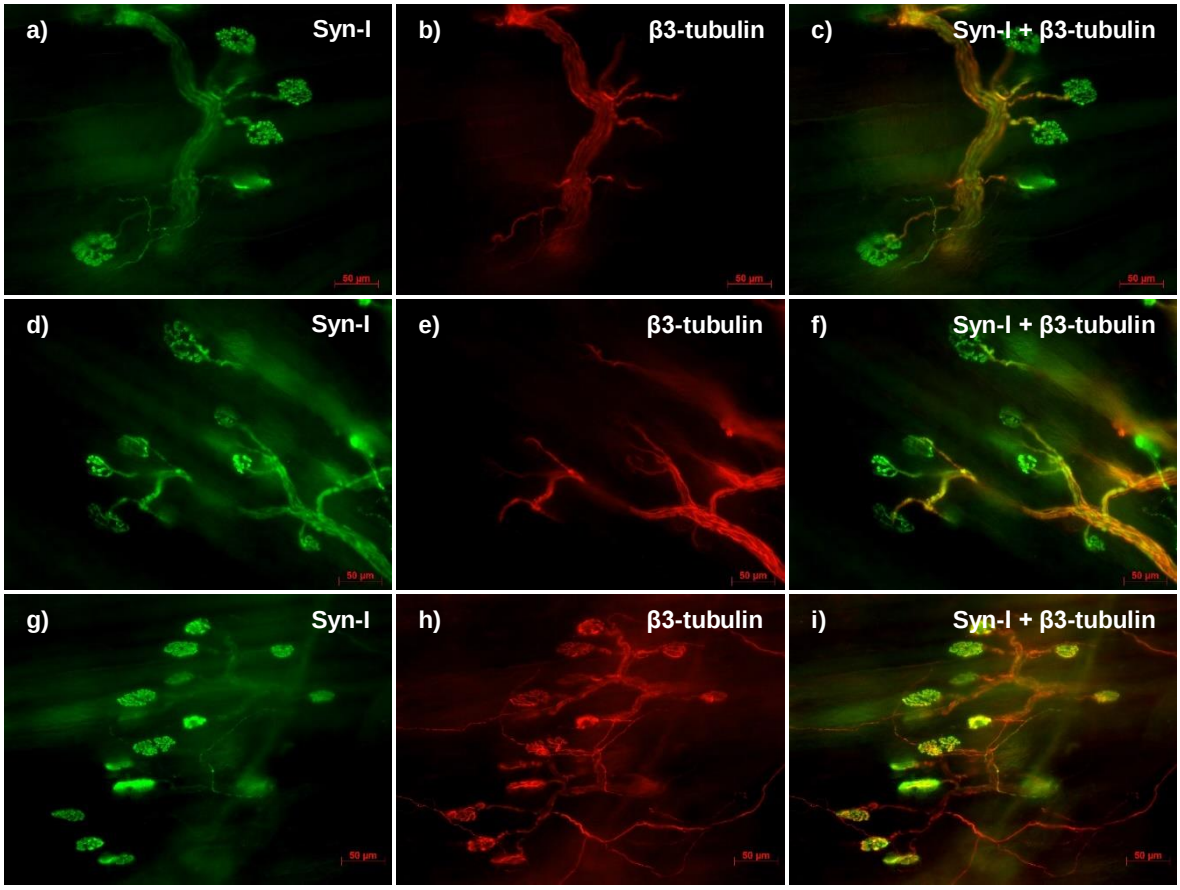

Figure 20. Sprouting in rat LAL muscles after injection of 1ng of BoNT/A. a) Not-injected rat left-LAL muscle labeled for Syn-I. **b)** Not-injected rat left-LAL muscle labeled for β3-tubulin. **c)** Overlap of a) and b). **d)** Rat left-LAL muscle labeled for Syn-I after 1 day of injection of 1ng of BoNT/A. **e)** Rat left-LAL muscle labeled for β3-tubulin after 1 day of injection of 1ng of BoNT/A. **f)** Overlap of d) and e). **g)** Rat left-LAL muscle labeled for Syn-I after 7 days of injection of 1ng of BoNT/A. **h)** Rat left-LAL muscle labeled for β3-tubulin after 7 days of injection of 1ng of BoNT/A. **i)** Overlap of g) and h).

V. Discussion, Final Conclusions and Futures Perspectives

1. Discussion

Previous studies showed the success of BoNT/A in the treatment of a diversity of conditions that affect not only neuromuscular junctions, but also autonomic cholinergic synapses. In particular, BoNT/A has proved its effectiveness in the treatment of some urological conditions, such as benign prostatic hyperplasia [43], bladder hyperactivity and bladder pain [49, 55]. However, several practical issues remain to be solved, namely the need to repeat BoNT/A administration when the therapeutic effect subsides, which can lead to the production of neutralizing antibodies and is painful for patients [39].

New and less invasive administration routes have been successfully showed, such as the intrathecal administration of BoNT/A for the treatment of bladder pain and hyperactivity [49]. Another approach to overcome these issues is the development of ultrapotent toxins that could be administered in lower doses, avoiding immune responses from the patients [9].

Our research group is especially interested in the hypothesis that co-administration of different BoNTs can be a novel and efficient way to treat bladder pain and bladder reflex activity, as the antinociceptive action could be increased with the possible synergistic effects. This way, lower doses of BoNTs would be needed. Being the second serotype with the longest duration of neuroparalysis, BoNT/C is a promising serotype for co-administration with other toxins. However, for unknown reasons it is never used for therapeutical purposes and so, *in-vivo* studies of its effects are needed.

Furthermore, despite the great efforts to understand the molecular basis of BoNT/A and the other serotypes, many questions about their mechanism of action remain unanswered. In the particular case of the A and C serotypes, its mechanism of neurointoxication has raised many doubts. Unlike the remaining serotypes, that cleave large cytosolic segments of their SNARE targets, BoNT/A and C only remove a few residues from SNAP-25, and a stable SNARE complex is formed [2]. Thus, additional studies in their molecular targets are essential.

Moreover, new and accurate models to study the molecular targets, relative potency and duration of action of botulinum toxins are required.

Therefore, in the present work, we studied the molecular targets of botulinum toxins serotypes A and C in different *in-vitro* and *in-vivo* models, evaluating their potency and duration of action. Co-administration of the two serotypes was also tested. It is well established that BoNT types A and C act by cleaving the SNAP-25 protein of the SNARE complex [76]. Thus, the presence of cleaved SNAP-25 was used to evaluate the activity of the toxins. This approach has been used in numerous studies and is considered the most sensitive test for this kind of studies [52, 77].

1.1. *In-vitro* Models

Neuronal cell-based assays have been used to test BoNTs' mechanisms and potency and its sensitivity has exceeding the sensitivity of the mouse LD_{50} bioassay [73]. This type of assay is considered the best *in-vitro* option for testing BoNTs' potency, as it can mimic all the mechanism of intoxication, from binding and translocation, to enzymatic activity. Furthermore, this type of approach reduces dramatically the number of animals used per experiment [78]. In this work, two types of neuronal cell-based assays were evaluated for the application of BoNTs: continuous ND7/23 cell line and primary DRG cell culture.

1.1.1. ND7/23 hybrid cell line

Continuous cells lines are inexpensive, relatively easy to maintain and have already showed good sensitivity to BoNTs detection [78]. Here, the hybrid continuous cell line ND7/23 was used. This line derive from neonatal rat DRGs fused with the mouse neuroblastoma N18Tg2 and exhibit sensory neuron-like properties [79].

In our experiments, differentiated ND7/23 cells showed the expression of the high-affinity receptor for BoNT/A – SV2 – and the specific SNARE catalytic target for BoNT types A and C – SNAP-25, in their expected cellular localization: synaptic vesicles [80] and cell membranes [81], respectively. Moreover, SV2 was found with a more denoted expression in neurite tips, where it is supposed to act. Therefore, these cells gather all the supposed conditions for the action of toxins. However, only a reduced number of cells showed immunostaining against cleaved SNAP-25 when exposed for 24 hours to BoNT/A and no cleaved SNAP-25 detection was obtained in cells exposed to BoNT/C. The limited number of cells affected by BoNTs might be due to the different stages of cell differentiation, what was observed by different levels of neurite outgrowth with immunoreaction against β3-tubulin. It may be a level of differentiation in which the cells are more vulnerable to the action of toxins. Other possibility are the low toxin concentrations and short times of treatment used. Various studies showed that other neuronal continuous cell lines, such as neuro-2a and PC12, are relatively insensitive to BoNTs, necessitating 2 to 3 days of exposure time and large toxin concentrations (in the order of 30 nM) to reveal adequate toxin activity [78]. However, in the case of BoNT/C, we used concentrations in that order and no toxin action was detected. Moreover, although we have used these relatively high concentrations of BoNT/C, no neuronal degeneration was observed, contradicting the results of previous studies [82]. Furthermore, it has been shown that pre-incubation with gangliosides increases cells sensitivity to toxins activity [83]. It may be an interesting procedure to test

for serotype C, as some evidences shown that this serotype uses gangliosides as dual receptors instead of using a specific protein co-receptor [69].

1.1.2. Primary culture of dorsal root ganglia

In-vitro assays utilizing primary neuronal cells usually present higher BoNT sensitivity in comparison with the continuous cell lines, and have been used to unveil some of the biology and mechanisms of BoNTs [73]. Unlike continuous cell lines, primary cultures are tightly controlled systems, yielding very reproducible results, and being cost-effective models [78]. Herein, we used a primary culture from dorsal root ganglia derived from adult animals as it is easily grown in culture, and the sensory neurons derived from DRGs are completely developed neurons that resemble the physiological characteristics of the DRGs *in-vivo* [84]. Moreover, this sensory neuronal cultures allow to evaluate the direct effects of toxins in these type of cells, enabling a closer look of the concentrations of toxins that actually reach the cells and the mechanisms affected [85].

Our results revealed that DRG cells express both SV2 and SNAP-25 and that a co-localization of this proteins exists, ensuring that these cells have both the receptor and catalytic target for BoNT/A. Also, it was demonstrated that an apparent redistribution of SV2 after exposure to BoNT/A happened, indicating its predisposition for toxin binding. Furthermore, a lower expression of intact SNAP-25 was obtained after exposure to the toxin, demonstrating the possible action of BoNT/A in DRG cells. However, despite these encouraging results, a reduced number of cells showed staining for cleaved SNAP-25, indicating that some part of the toxin mechanism of action was failing.

One possibility for these not satisfying results is the lack of expression of the other functional protein receptor for BoNT/A - the FGFR3 receptor - in DRG cells, once it was demonstrated that internalization of BoNT/A is dependent on the levels of FGFR3 expression [22]. However, our results are not in agreement with the literature, that shows that despite only expressed at high levels following injury, DRG cells express FGFR3 [86, 87]. Also, previous studies proved that chemically stimulating the neuronal activity, using buffers with high potassium content, resulted in increased BoNT/A binding to neuromuscular junctions and a consequent increase in SNAP-25 cleavage [23]. Therefore, we tested if exposure to BoNT/A mixed in a high-K^+ solution improved the uptake of the toxin. However, the results with this buffer were equivalent to the results obtained with a control buffer and the results obtained with the normal exposure to the toxin, raising the possibility that the binding and entry mechanism is not the cause for the small number of affected cells. The concentrations used should not also be the key to this problem, since they are in accordance or are even higher

than the doses used in the literature [23, 70, 88]. Also, the culture conditions used (T=37 °C) were shown to be the ideal for the translocation of the L-chain of the toxins into neurons, as it was demonstrated for BoNT/C [70]. However, only the higher concentration of BoNT type C (30 nM) showed little cleaved SNAP-25 in a reduced number of cells. The consistent negative results for this type of toxin may indicate its inactivity or that some extra treatment is needed, like the already referred ganglioside treatment. Moreover, the presence of other important markers for serotype C should be investigated, such as the cleavage of Syntaxin, the other target for this toxin [31]. Furthermore, a more detailed study of the antibody against cleaved SNAP-25 is needed for this serotype. The antibody used is produced against the truncated C-terminal peptide of SNAP-25 and has already been well characterized for the recognition of the cleaved SNAP-25 by BoNT/A [52]. However, no studies were reported for BoNT/C and it is known that this serotype cleaves only 8 residues from SNAP-25, while BoNT/A cleaves 9 [82]. This little difference may influence the recognition of the cleaved form of SNAP-25 by the antibody, but further studies are needed.

1.2. *In-vivo* Models

Obviously, animal models are a more reliable prediction regarding the application of the toxins to the various treatments. In the present work Wistar rats and C57/B6 mice were used as animal models since both the strains are of easy maintenance and handling.

More importantly, these species are well defined and widely used to study urological applications, once the anatomy and physiology of their urinary tract is analogous to humans [89]. Being an *inbred* strain, C57/B6 mice introduce less variability between experiments, being a more reproducible model. Also, because of its lower weight, lower doses of toxins are needed. In the other hand, Wistar rats have larger bladders, facilitating its handling, and a more variety of antibodies may be used with this strain, once the majority of antibodies' host is mice. Therefore, these two strains were used, each one for the most convenient purpose.

We were especially interested in study the action of botulinum toxins in smooth *vs* skeletal muscle. For that, studies in the urinary bladder as a model for smooth muscles and the cranial muscles as a model for skeletal muscle were performed.

1.2.1. Urinary bladder

Urinary bladder was chosen as a model for smooth muscle studies, as it is a self-enclosed organ and therefore, the effects of the toxins can be easily seen in the whole organ, without any external influences. Also, as previously discussed, BoNTs have been used to treat a variety of urological conditions with great success. Moreover, our group is specialized in the urinary bladder, with a vast knowledge about its biology and molecular characteristics, and about the effects of BoNT/A in this organ. Therefore, studies about the expression of the binding receptor SV2, and the intracellular target SNAP-25 in the bladder were not conducted in this work, as previous studies of the group already shown that [90].

Our results demonstrated that after 2 days of intramural injections of 2ng in rats and 0.2 ng in mice of BoNT/A, only a small population of muscular fibers were affected. The cause for these results may be a failure in the distribution of the toxin throughout the bladder, which may be due to the reduced volume of injection used. Volume of injection is in fact an important parameter for the distribution of the toxins, as a recent study described that higher injection volumes of BoNTs cleaved more SNAP-25 and at more distant places than smaller volumes with the same dose [74].

Despite the reduced number of affected fibers, injections of BoNT/A did not result in nerve or muscle cells degeneration, translating the safety of the toxin in the bladder, which is in accordance with other studies [49, 91]. These results were showed by the equal expression of GAP-43 in non-injected and injected animals. GAP-43 is known for being up-regulated after neuronal injury [92, 93] and therefore, the no up-regulation is indicative of no affected neuronal cells.

1.2.2. Cranial muscles

A group of small cranial muscles, that is responsible for moving the pinna in rats and mice, was used as a model for skeletal muscle. Due to its intrinsic characteristics, as the 2-3 fibers thickness, this muscles are easily stained and viewed as wholemount immunofluorescence preparations. Also, its easy accessibility makes this muscles attractive models for a wide variety of studies in the neuromuscular system [75].

Because of the most superficial, subcutaneous location of the LAL muscle, which makes it the most accessible, this muscle was chosen for this experiments. Moreover, the LAL muscle was already used in studies involving injections of BoNT/A [94].

Cleaved SNAP-25 detection was obtained with doses as little as 0.1 ng of BoNT/A, indicating the action of the toxin and a good sensibility of the model. Moreover, the effect of

the toxin was observed in the majority of the end-plates population, as it was demonstrated with the mapping of the entire muscle immunoreacted against cleaved-SNAP-25. This indicates that the LAL model is a good skeletal muscle model because it allows the evaluation of BoNT/A effects throughout the entire muscle, without variables.

On the other hand, and similar to the results obtained for the *in-vitro* models, no cleaved SNAP-25 was detected after injections with BoNT type C. However, there is the possibility of a synergistic effect when BoNT types A and C are injected simultaneously, as an animal injected with both toxins died 4 days after injection, and animals injected with the same dose of the separated toxins did not. Yet, the expression of SNAP-25 in animals co-injected with the two toxins was similar to the expression in animals injected only with the serotype A. This could indicate that BoNT/C is actually working but the detection of its product is failing, suggesting that the antibody is not recognizing the cleaved form of SNAP-25 resultant of the action of BoNT/C, as explained above. Other hypothesis is that BoNT/C action is not involving the cleavage of SNAP-25. Therefore, the presence of the other target of BoNT/C, Syntaxin, or the hypothesis of other catalytic targets for this serotype, should be evaluated.

Contrarily to the results of BoNT/A injections in the bladder wall, where no sprouting was observed, in the LAL muscle, a vast sprouting network was formed after 7days of injection with BoNT/A, translating the recovery process of the neuromuscular junctions after neurointoxication. These results were also shown in previous studies, revealing that skeletal and smooth muscles react differently to the toxin [95]. Furthermore, these findings may be the reason for the longer duration of paralysis in the smooth muscle, that is about 3 times longer than in the skeletal muscle [96]. The delayed axonal outgrowth in the detrusor smooth muscle may lead to a delayed recovery of neuromuscular functions and therefore, to a consequent longer effect of the toxins. However, this possibility has to be tested and more comparative studies of the effect of toxins in smooth and skeletal muscle should be perfomed.

2. Final Conclusions and Future Perspectives

With this work we intended to fill some of the gaps in the mechanism of action of BoNTs, especially in what concerns the molecular targets of the serotypes A and C, and their potency and duration of action, using different *in-vivo* and *in-vitro* models.

With regard to the *in-vitro* models, the primary DRG culture showed more sensitivity to the action of the toxins, being a better model to test the effects of BoNTs than the ND7/23 cells. However, the sensitivity of the *in-vitro* models was not satisfying, as only a reduced number of cells was affected by BoNTs and therefore, they were discarded for the remaining studied. The best model studied in this work was the cranial muscle model, where the LAL muscle proved to be a reproducible model that allows to study in great detail the innervation of a complete muscle in rat and mouse, and the effect of BoNT/A in the entire muscle, without bias. Moreover, it showed a great sensitivity to the toxin, allowing the use of lower doses. Also, its easy accessibility facilitates all the protocols for toxins administration, where there is no need of a surgical procedure, which also reduces animal suffering. In addition, other methodologies should be easily applied to this muscle.

Using the LAL model and the urinary bladder model, we also concluded that after a short period of BoNT/A administration, skeletal muscle suffers a compensatory sprouting process, which translates the beginning of the neuromuscular junctions' recovery, contrarily to the smooth muscle, were no sprouting was detected. This shows that the different types of muscle react differently to the toxin, and therefore these models may be adequate to study the mechanism of action of BoNTs in the different tissues and their versatility in the different clinical applications.

Further studies of the co-administration of BoNT/A and C are imperative, as the possible synergistic or cumulative effects were shown. Also, it may be interesting to study the co-administration effects in the bladder and see if they improve bladder hyperactivity and pain. Furthermore, other catalytic targets for BoNT/C may also be evaluated.

We are extremely confident that upon completion of the project, we will have novel and important results that will be fundamental for the knowledge of the mode of action of BoNTs in the peripheral and central nervous system, and that will open horizons for new clinical applications.

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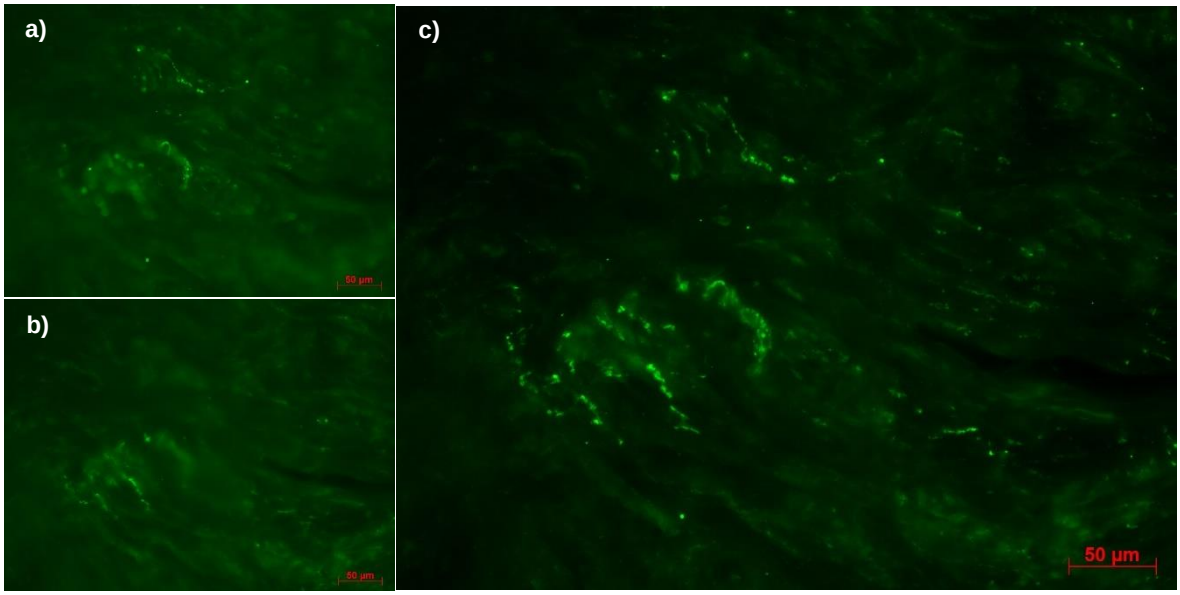
VII. Attachments



Supplementary Image 1. Example of the Z-stack technique used for bladders sections and LAL muscles. Z-stack images were originated with the AxioVision 4.6 software, where **a)** represents the first plan and **b)** the last plan photographed. The Z-stack tool compresses all the images of the plans taken, resulting in the image **c)**.

Supplementary Image 2. Montage of the entire right-LAL muscle labeled for cSNAP-25 after 7 days of injection of 1 ng of BoNT/A. Resultant image montage done in Photoshop CS6 Portable software of the images taken with a magnification of 5x of the referred muscle. Drawings of the innervation pattern were made on this image and resulted in Image 19. c).