Modulation of Urinary Bladder Innervation: TRPV1 and Botulinum Toxin A

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Contents

Abstract The persisting interest around neurotoxins such as vanilloids and botulinum toxin (BoNT) derives from their marked effect on detrusor overactivity refractory to conventional antimuscarinic treatments. In addition, both are administered by intravesical route. This offers three potential advantages. First, intravesical therapy is an easy way to provide high concentrations of pharmacological agents in the

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bladder tissue without causing unsuitable levels in other organs. Second, drugs effective on the bladder, but inappropriate for systemic administration, can be safely used as it is the case of vanilloids and BoNT. Third, the effects of one single treatment might be extremely longlasting, contributing to render these therapies highly attractive to patients despite the fact that the reasons to the prolonged effect are still incompletely understood. Attractive as it may be, intravesical pharmacological therapy should still be considered as a second-line treatment in patients refractory to conventional oral antimuscarinic therapy or who do not tolerate its systemic side effects. However, the increasing off-label use of these neurotoxins justifies a reappraisal of their pharmacological properties.

Keywords Bladder · BoNT/A · Pain · Prostate · TRPV1b · TRPV1

1 General Characteristics of the TRPV1 Receptor

The TRPV1 receptor is a protein with six transmembrane domains that forms a pore between the fifth and the sixth domain (Caterina et al. 1997). This protein allows the passage of cations, especially calcium, when activated by vanilloids, noxious heat, and low pH (Caterina et al. 1997). Although the TRPV1 heat threshold was established around 43°C, it is known that in acidosis, the receptor is activated at body temperature (Tominaga et al. 1998; Caterina et al. 2000). Several studies have shown that TRPV1 agonists act on different domains of the protein (Welch et al. 2000a). For instance, vanilloids and endovanilloids, such as anandamide, bind to the intracellular region of the third domain (Jordt and Julius 2002), while protons and other ions act on the extracellular loop that forms the pore (Jordt et al. 2000; Ahern 2005).

1.1 Localization of TRPV1 Protein in Lower Urinary Tract

The TRPV1 receptor was first described in the perikarya and in the central and peripheral processes of a subset of small-diameter sensory neurons (Caterina et al. 1997). Later, the presence of the receptor was described in other neuronal structures, such as the trigeminal ganglia (Guo et al. 1999; Ichikawa and Sugimoto 2001) and the vagus nerves (Holzer 1991), among others (Ichikawa and Sugimoto 2001; Michael and Priestley 1999; Kadowaki et al. 2004; Koike et al. 2004). The receptor was also observed in other areas of the nervous system. Several studies reported the presence of TRPV1 in some areas of the brain (Mezey et al. 2000; Tóth et al. 2005). TRPV1 receptor expression has also been shown in cells outside the nervous system, a subject that has generated some controversy. The expression of the TRPV1 receptor in smooth muscle cells is still a matter of debate

(Ost et al. 2002; Yang et al. 2008; Kark et al. 2008; Wang et al. 2008a; Ito et al. 2008). Nevertheless, the expression of this receptor in mast cells and keratinocytes is now widely accepted (Birder et al. 2007; Turner et al. 2003; Ständer et al. 2004; Bodó et al. 2005). It has been shown that TRPV1 activation in those cells promotes the release of proinflammatory molecules (Birder et al. 2007; Southall et al. 2003).

In the urinary bladder and ureter, TRPV1 is expressed in nerve fibers (Avelino et al. 2002) and in urothelial cells (Birder et al. 2001; Lazzeri et al. 2004a; Charrua et al. 2009a). Dense varicose nerve fibers immunoreactive for TRPV1 occur among detrusor smooth muscle fibers and underneath the epithelium (Avelino et al. 2002). In the prostate, TRPV1-expressing fibers were found in the prostatic urethral mucosa, verumontanum, ejaculatory ducts, and periurethral prostatic acini (Dinis et al. 2005).

A recent work published by Everaerts et al. (2009) revealed that antibodies against TRPV1 may have a positive reaction in TRPV1 KO mice, indicating an absolute lack of specificity. The consequence of these observations is the need to confirm any positive TRPV1 immunostainning with a negative control using TRPV1 KO tissues. In addition, the combination of TRPV1 immunoreaction with other methodologies to detect the presence of the receptor, such as reverse transcriptase polymerase chain reaction, blotting analysis, in situ hybridization, among others, is highly recommended.

1.2 The Role of TRPV1 on Lower Urinary Tract Function and Dysfunction

The role of TRPV1 in normal urinary bladder is still controversial. In cystometries performed in awake TRPV1 knockout (KO) mice, these animals have more small volume voids than their wild-type (WT) controls (Birder et al. 2002). However, in anesthetized animals, the findings are conflicting, some groups reporting that the animals have nonvoiding contractions (Birder et al. 2002; Wang et al. 2008b), whereas others found totally normal cystometric traces (Charrua et al. 2007).

TRPV1 activation outside lower urinary tract (LUT) is essential for the development of somatic thermal hyperalgesia during inflammation (Caterina et al. 2000; Davis et al. 2000). TRPV1 receptor activation elicits the release of proinflammatory molecules, which consequently leads to the development of neurogenic inflammation in visceral organs (Veronesi et al. 2000; McVey and Vigna 2001). In LUT, Charrua et al. (2007) have shown that TRPV1 is essential for the development of hyperreflexia and pain associated with cystitis. While inflamed WT mice exhibit bladder hyperactivity and intense spinal Fos expression, TRPV1 KO mice do not (Charrua et al. 2007). In agreement, TRPV1 antagonists were able to reduce the bladder hyperactivity and noxious input associated with cystitis (Charrua et al. 2009b).

1.3 TRPV1 Regulatory Mechanisms During LUT Inflammation

1.3.1 TRPV1 Expression During Inflammatory Conditions

Most of the information about TRPV1 expression in inflammation comes from somatic models. During somatic inflammation, there is an increased number of dorsal root ganglia (DRG) neurons expressing TRPV1 (Carlton and Coggeshall 2001; Amaya et al. 2003). Moreover, the quantification of TRPV1 protein in DRG cells by Western blotting analysis was consistent with these findings (Ji et al. 2002). The same was observed in visceral inflammation. Similarly, Avelino and Cruz (2010) observed an increase in the number of DRG neurons expressing TRPV1 during cystitis. In agreement with these experimental findings, patients with bladder pain syndrome exhibit a higher number of TRPV1-IR fibers in the bladder mucosa (Mukerji et al. 2006).

Interestingly, the increase in the protein levels in DRG cells does not seem to be accompanied by an increase in TRPV1 mRNA, indicating that the process is regulated at a posttranslation level. This observation was made in both somatic (Ji et al. 2002; Tohda et al. 2001; Voilley et al. 2001) and bladder inflammation models (Charrua et al. 2008). However, the receptor translation does increase in urothelial cells upon inflammation (Charrua et al. 2009b). These observations may indicate diverse forms of regulation in different cells.

1.3.2 Endovanilloid Release During Inflammation

TRPV1 can be directly activated by proinflammatory molecules such as *N*-arachidonoyl-ethanolamine, also known as anandamide (Zygmunt et al. 1999), *N*-arachidonoyl-dopamine (Huang et al. 2002), *N*-oleoyl-dopamine (Chu et al. 2003), eicosanoid acids, and leucotrienes (Hwang et al. 2000; Shin et al. 2002). In the urinary bladder, anandamide levels increase during cystitis (Dinis et al. 2004a) and contribute to a TRPV1-dependent bladder overactivity and increased noxious input (Dinis et al. 2004a). Nevertheless, endovanilloids are low-potency TRPV1 activators in the urinary bladder when compared to capsaicin and resiniferatoxin (Dinis et al. 2004a). Therefore, one might speculate that TRPV1 sensitization might be a prerequisite for a robust TRPV1 activation by endovanilloids (Lee et al. 2005).

Anandamide is also a cannabinoid receptor 1 (CB1) agonist (de Haro et al. 2003). This is curious, since CB1 activation promotes analgesia (Stein et al. 1996) and decreases detrusor contractility (Tyagi et al. 2009). However, it is now well demonstrated that CB1 can either enhance or diminish TRPV1 response to agonists, depending on whether or not cyclic adenosine monophosphate (cAMP)-mediated signaling pathway has been concomitantly activated (Hermann et al. 2003). Therefore, anandamide can activate TRPV1 receptor also through CB1 activation. This receptor was shown to induce TRPV1 phosphorylation, through a phosphoinositide

phospholipase C (PLC) pathway, and eliminate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)-mediated TRPV1 inhibition (Hermann et al. 2003). This can explain why the blockade of CB1 receptor in the urinary bladder significantly enhanced anandamide-induced bladder reflex activity in naive but not in cyclophosphamide-injected animals (Dinis et al. 2004a).

1.3.3 TRPV1 Sensitization

TRPV1 phosphorylation can lead to the receptor sensitization (Fig. 1). It is known that TRPV1 needs to be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase to be activated by vanilloids (Hermann et al. 2003). Phosphorylation of all putative sites by protein kinase A (PKA) or protein kinase C (PKC) also leads to TRPV1 sensitization (Jung et al. 2004). Protease-activated receptor 2 (Amadesi et al. 2006) or 5-hydroxytryptamine 7 receptor (Ohta et al. 2006) is known to be involved in PKA-induced TRPV1 sensitization. Conversely, activation of group II metabotropic glutamate receptors (Carlton et al. 2009) or μ-opioid receptor (Vetter et al. 2006, 2008) inhibits TRPV1 activation by modulation of cAMP/PKA pathway. Receptors, such as bradykinin receptor (Premkumar and Ahern 2000; Vellani et al. 2001; Sugiura et al. 2002; Carr et al. 2003; Tang et al. 2004; Mizumura et al. 2005), purinergic receptors (Tominaga et al. 2001, 2003; Moriyama et al. 2003; Lakshmi and Joshi 2005), tyrosine kinase receptor A (trk A) (Bonnington and McNaughton 2003; Zhuang et al. 2004; Zhu and Oxford 2007), among others, when activated, are known to sensitize TRPV1 receptor through a PKC-dependent mechanism. Furthermore, activation of bradykinin receptor can induce PLC and phospholipase A_2 activation, which leads to the production of arachidonic acid

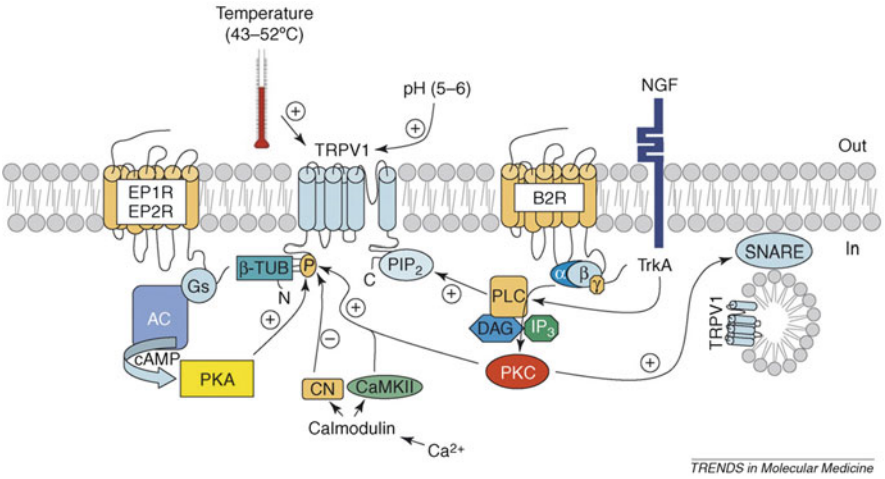

Fig. 1 Schematic model of TRPV1 activation and sensitization [in Szallasi et al. (2006); with Elsevier permission]

metabolites that can activate the TRPV1 channel (Shin et al. 2002; Tang et al. 2004; Ferreira et al. 2004).

Another mechanism of TRPV1 sensitization results from the receptor interaction with phosphoinositides. It has been shown that PtdIns(4,5)P2 sensitizes TRPV1 in the presence of high concentration of some agonists (Prescott and Julius 2003; Liu et al. 2005), but inhibits the receptor at low concentration of agonists (Chuang et al. 2001; Rohacs et al. 2008). Although functional analysis performed in patches from acutely dissociated dorsal root neurons provided evidence that PtdIns(4,5)P2 is in fact a TRPV1 sensitizer (Stein et al. 2006), the mechanisms that lead to such an antagonic effect are still unveiled (Voets and Nilius 2007). TRPV1's direct interaction with ATP molecules is another mechanism of the receptor sensitization. ATP has a binding domain within the ankyrin domain present in the N-terminal of TRPV1 molecule (Lishko et al. 2007). When ATP binds to the receptor, it increases the response of the receptor to further stimulus (Lishko et al. 2007).

1.3.4 TRPV1 Trafficking to the Membrane

Another mechanism that might increase TRPV1-mediated neuronal activity is related with an increase in TRPV1 trafficking to the plasma membrane. In fact, it is known that PKC activation promotes a rapid vesicular TRPV1 transport to the membrane in a soluble NSF attachment protein receptor (SNARE)-dependent mechanism (Morenilla-Palao et al. 2004). Furthermore, TRPV1 phosphorylation by PKA seems to be crucial for TRPV1 cytoplasmic membrane expression, since functional analysis associated with point mutation of putative sites of PKA phosphorylation almost abolished TRPV1 cytoplasmic membrane expression (Bhave et al. 2002). Besides promoting TRPV1 phosphorylation, receptor tyrosine kinase, such as trk A and insulin receptor, promotes TRPV1 trafficking to the membrane through a phosphoinositide 3-kinase (PI3K)-dependent pathway (Stein et al. 2006; Van Buren et al. 2005).

1.3.5 Expression of TRPV1 Splice Variants

The most recently discovered mechanism of TRPV1 modulation results from the receptor interaction with its own splice variants (Wang et al. 2004; Vos et al. 2006; Eilers et al. 2007). The splice variant TRPV1b differs from TRPV1 protein by completely missing exon 7 (Lu et al. 2005). This splice variant, the only one found to be expressed in humans, is known to have a dominant-negative effect over the TRPV1 receptor (Vos et al. 2006). That is, whenever TRPV1 is coexpressed with TRPV1b, the receptor became unresponsive to vanilloids, protons, and heat (Vos et al. 2006). Recently, it was shown that, during urinary bladder inflammation, there is a decrease in the expression of TRPV1b mRNA (Charrua et al. 2008). One possible consequence is the reduction in the amount of TRPV1b available to form heterotetrameric, inactive TRPV1 channels (Wang et al. 2004;

Eilers et al. 2007; Lu et al. 2005). This effect was restricted to neurons that innervate the inflamed urinary bladder, since, during LUT inflammation, TRPV1 and TRPV1b levels were maintained in the perikarya of neurons present in DRG that do not receive bladder afferent fibers (Charrua et al. 2008). It might be interesting to recall at this point that cystitis did not increase the expression of TRPV1 (Charrua et al. 2008).

1.4 Mechanisms of TRPV1 Desensitization

Vanilloid-induced desensitization of urinary bladder type C sensory afferents has been used as a therapeutic approach to ameliorate pain and hyperreflexia associated with some urinary bladder diseases (Fowler et al. 1992, 1994; Geirsson et al. 1995; Chandiramani et al. 1996; Das et al. 1996; Lazzeri et al. 1996, 1997, 1998, 2004b; Cruz et al. 1997a, b; de Ridder et al. 1997; de Séze et al. 1998, 1999, 2004, 2006; Wiart et al. 1998; Chancellor and de Groat 1999; Fagerli et al. 1999; Silva et al. 2000; Giannantoni et al. 2002; Igawa et al. 2003; Kim et al. 2003). However, the mechanisms that lead to TRPV1 desensitization are complex and not fully understood. It is known that desensitization depends on the agonist or agonists that are acting on the receptor (Novakova-Tousova et al. 2007; Vyklický et al. 2008), the phosphorylation state of the receptor (Dinis et al. 2004a; Hermann et al. 2003), the membrane potential (Piper et al. 1999), and the interaction between the receptor and molecules such as ATP, calmodulin, and PtdIns(4,5)P2 (Lishko et al. 2007; Koplas et al. 1997; Mohapatra and Nau 2005). TRPV1 desensitization might be achieved either by applications of high doses of vanilloids onto the bladder, which induces a transient burning sensation followed by a prolonged decrease in pain sensation and urinary bladder frequency (Das et al. 1996; Cruz et al. 1997a, b; de Ridder et al. 1997; Giannantoni et al. 2002; Igawa et al. 2003), or by successive applications of low doses of vanilloids, which improved pain and bladder micturition frequency in each consecutive application (Lazzeri et al. 1996). TRPV1 desensitization by vanilloids occurs after the opening of the phosphorylated channel and it is intrinsically connected to the influx of calcium ions (de Sèze et al. 1999; Docherty et al. 1996). The increase in $[Ca^{2+}]i$ leads to a series of events, which include activation of protein phosphatase 2B (PP2B, calcineurin), which dephosphorylates TRPV1 receptor (Mohapatra and Nau 2005; Docherty et al. 1996) and changes the allosteric coupling between TRPV1 and PtdIns(4,5)P2 (Vyklický et al. 2008). Through the activation of PLC, PtdIns(4,5)P2 is cleaved into diacyl glycerol and inositol 1,4,5-triphosphate, concomitantly with ATP displacement, allowing Ca^{2+}–calmodulin binding to both ankyrin repeat domain and C terminus, promoting the channel desensitization (Lishko et al. 2007; Vyklický et al. 2008). Nevertheless, the use of vanilloids as intravesical therapeutic agents has been hampered by the pungency of capsaicin solutions (Cruz et al. 1997a) and the lack of stability of the resiniferatoxin solutions (Brady et al. 2004b).

1.5 TRPV1 Antagonists

The importance of finding a reliable molecule that blocks bladder TRPV1 is based on the observations that TRPV1 KO mice do not develop bladder hyperreflexia and noxious input during cystitis (Charrua et al. 2007). Therefore, TRPV1 antagonists could offer bladder analgesia, which is difficult to obtain with current analgesics and, in addition, to contribute to control bladder reflex activity.

The first molecule to be used as a competitive TRPV1 antagonist was capsazepine (Urban and Dray 1991; Dickenson and Dray 1991; Perkins and Campbell 1992; Bevan et al. 1992; Szallasi et al. 1993; Walpole et al. 1994). Although in some studies this molecule proved to be extremely useful for TRPV1 investigation, others demonstrated that it is not specific enough and effective as a TRPV1 antagonist (Dinis et al. 2004a; Kuenzi and Dale 1996; Liu et al. 2008; Docherty et al. 1997; Oh et al. 2001; Mahmmoud 2008). Furthermore, capsazepine is not able to counteract all TRPV1 modes of activation (McIntyre et al. 2001; Walker et al. 2003). Although several other TRPV1 antagonists have been produced and commercialized, there is still a lack of information concerning specificity and efficacy in most of them (Szallasi et al. 2007; Wong and Gavva 2008). The most recently synthesized TRPV1 antagonist molecules, which already reached clinical trials, are delivered via oral route (Gunthorpe and Chizh 2009). Japan Tobacco is testing, in Japan, one of those molecules, JTS-653, to treat urinary bladder overactivity, although there is still no data available from that study (Gunthorpe and Chizh 2009; Khairatkar-Joshi and Szallasi 2009). Using an animal model, Charrua et al. (2009b) have shown that oral administration of GRC-6211, a specific TRPV1 antagonist that is already in phase II for neuropathic pain treatment, abolishes both urinary bladder hyperreflexia and hyperalgesia associated with LUT inflammation in rodents and bladder hyperreflexia in animal models of spinal cord transection (Silva et al. 2008a). One undesirable side effect of TRPV1 antagonists is that, by acting on visceral TRPV1 (Jones et al. 2005), they can cause hyperthermia (Steiner et al. 2007; Swanson et al. 2005) after promoting vasoconstriction and increasing thermogenesis (Jones et al. 2005; Gavva et al. 2007). However, recent data have shown that hyperthermia is attenuated after repeated application of TRPV1 antagonist, which renews the interest in already synthesized molecules (Wong and Gavva 2008). Moreover, some compounds, like GRC-6211, did not show this side effect (Charrua et al. 2009b; Silva et al. 2008a).

One curious aspect of TRPV1 antagonist on bladder function is a capacity to decrease bladder reflex activity (Charrua et al. 2009b). In high doses, GRC-6211 was able to transiently block bladder contractions in rats and WT mice. However, the same dose did not produce any effect on bladder activity in TRPV1 KO mice, suggesting that TRPV1 may have mechanoreceptor proprieties, either directly or indirectly. It is tempting to speculate that the formation of TRPV1/TRPV4 dimers may contribute to this unexpected function of TRPV1 receptor in the urinary bladder (Charrua et al. 2009b) and other viscera (Gavva et al. 2008).

1.6 Clinical Perspective of TRPV1 Targeting

1.6.1 TRPV1 Desensitization

Pathologies such as spinal cord injury (De Groat 1997), chronic bladder outlet obstruction (Chai et al. 1998), and in patients with idiopathic detrusor overactivity (IDO) (Silva et al. 2002) have in common the overactivity of a spinal C-fiber initiated micturition reflex. This reflex is ultimately the origin of detrusor overactivity as indirectly shown by the suppression of nonvoluntary detrusor contractions after C-fiber desensitization induced by intravesical application of vanilloids such as capsaicin or resiniferatoxin (Fowler et al. 1994; Lazzeri et al. 1997, 1998; Cruz et al. 1997a, b; de Ridder et al. 1997; de Séze et al. 1998; Silva et al. 2002; Liu and Kuo 2007b).

The effect of vanilloids on bladder C-fibers was clearly demonstrated in patients with neurogenic detrusor overactivity (NDO) of spinal origin. Intravesical resiniferatoxin resulted in a decreased expression of TRPV1 in bladder sensory fibers (Brady et al. 2004b; Silva et al. 2002, 2005) and in urothelial cells (Apostolidis et al. 2005a), which correlated with an increase in bladder capacity and a decrease in urgency (Silva et al. 2007). Intravesical resiniferatoxin was administered either as a single high-dose instillation or as multiple low-dose instillations, in both cases with very positive results (Lazzeri et al. 2000, 2004a; Silva et al. 2007; Dinis et al. 2004b; Kuo 2003, 2005a; Kuo et al. 2006; Chen et al. 2005; Apostolidis et al. 2006a; Peng et al. 2007; Payne et al. 2005). Placebo control trials showed a clear benefit on the resiniferatoxin arm (Silva et al. 2005; Kuo et al. 2006). However, resiniferatoxin instillation did not gain worldwide popularity despite its low pungency (Cruz et al. 1997b) due to technical difficulties in obtaining stable solutions.

1.6.2 TRPV1 Antagonists

Initial experiments with TRPV1 antagonists on rodent models of bladder inflammation or detrusor overactivity induced by spinal cord injury suggest that this new class of drugs will have a relevant future for the treatment of bladder pain, urinary frequency, and urinary incontinence (Charrua et al. 2009b; Silva et al. 2008a). From a theoretical point of view, TRPV1 antagonists will be of special interest in bladder disorders such as interstitial cystitis, a chronic inflammatory condition of the bladder of unknown etiology where TRPV1 expression is increased (Mukerji et al. 2006).

For the same reasons, TRPV1 antagonists might be relevant in detrusor overactivity. In the particular case of NDO, the capacity of TRPV1 antagonists to cause detrusor paralysis makes this class of drugs very appealing (Brady et al. 2004a).

2 General Characteristics of Botulinum Toxin

Botulinum toxin (BoNT) is a neurotoxic protein produced by a great variety of Gram-positive spore-forming bacteria that form *Clostridium botulinum* (Mohanty et al. 2001). Among these bacteria, some produce only one serotype and others produce more than one of the seven known BoNT serotypes A, B, C, D, E, F, and G (Fujinaga et al. 1995; Santos-Buelga et al. 1998; Hill et al. 2007). *Clostridium butyricum* and *Clostridium baratii* also produce type E (Meng et al. 1997) and type F of BoNT (McCroskey et al. 1991), respectively. All BoNT serotypes genes differ in nucleotide sequence and are preceded by the nontoxic nonhemagglutinin gene and other genes that encode toxin-associated proteins (Hill et al. 2007; Popoff and Marvaud 1999). BoNT/A presents four subtypes: BoNT/A1, BoNT/A2, BoNT/A3, and BoNT/A4 that can have up to 15% amino acid differences (Hill et al. 2007). Nevertheless, all BoNT/A genes are translated into a single polypeptide chain (≈150 kDa), afterward being cleaved into a heavy subunit (≈100 kDa) and a light subunit (≈50 kDa) (Simpson 1981; Lacy and Stevens 1999; Binz et al. 2002). The resulting subunits stay connected by disulfide bridges and by noncovalent interactions (Binz et al. 2002; Strauss and Keller 2008). After being produced, the toxin forms a complex with other proteins, becoming more resistant to proteolysis and denaturation (Wagman 1954). The light chain (LC) of BoNT has an amino acid sequence with a motif His-Glu-x-x-His in their intermedial portion (Schiavo et al. 1994; Humeau et al. 2000). This motif is characteristic of the catalytic domain of all Zn-dependent endopeptidases (Binz et al. 2002; Humeau et al. 2000; Li et al. 2000; Rigoni et al. 2001). The heavy chain (HC) of BoNT is subdivided into three portions: a α-helical domain of 50 kDa (H_N), responsible for the LC translocation into the neuronal cytoplasm, and another 50 kDa fragment subdivided into two domains of 25 kDa (H_{CN} and H_{CC}) (Binz et al. 2002; Lalli et al. 1999). The H_N fragment is integrated in the membrane, due to the acidic compartment of the endosome, and forms a selective cationic channel (Blaustein et al. 1987; Kalandakanond and Coffield 2001). It is through this channel that the LC can pass, after conformation rearrangement, possibly helped by the belt region, which has chaperone characteristics (Koriazova and Montal 2003; Fischer and Montal 2007a, b; Galloux et al. 2008; Montal 2009). The function of H_{CN}, the structure of which resembles a lectin, is still unveiled (Binz et al. 2002). The H_{CC} function is associated with both the recognition of neuronal specific areas (nonmyelinated, with complex polysialogangliosides, especially the terminal NAcGalβ3-1Galβ of GT1b and GD1a and glicosfingolipids), and the toxin internalization (Rummel et al. 2004).

2.1 BoNT Mechanism of Action

The BoNT mechanisms of action comprise three steps: the connection of the toxin to the neuronal membrane, its internalization, and finally its intracellular effects (Fig. 2).

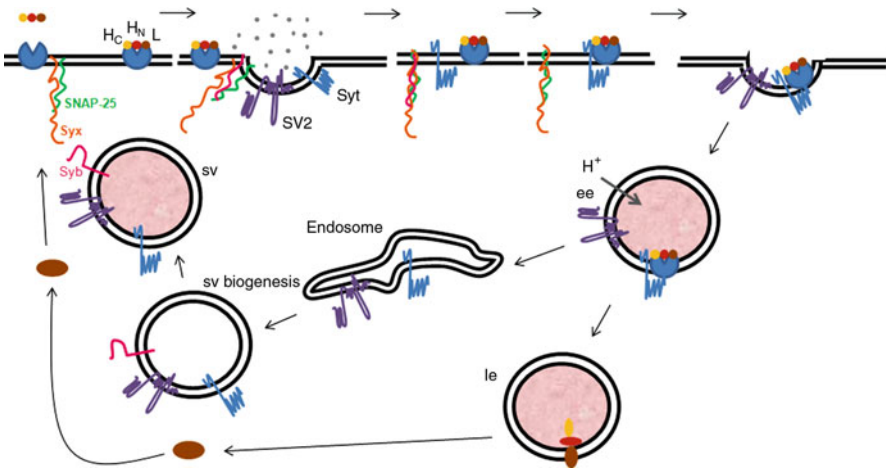

Fig. 2 Schematic model of BoNT toxin mechanism of action [adapted from Binz and Rummel (2009); with Wiley InterScience permission]

The H_{CC} fraction of BoNT/A connects to the terminal portion of the abundant low-affinity receptor NAcGalβ3-1Galβof polysialogangliosides G_{T1b}, G_{Q1b}, G_{D1a}, and G_{D1b}, maintaining the BoNT/A attached to the neuronal membrane (Rummel et al. 2004; Simpson 1984; Yowler and Schengrund 2004; Binz and Rummel 2009; Brunger and Rummel 2009). During exocytosis, the H_{CC} fraction of BoNT/A will bind to the scarce, but now available, high-affinity receptor synaptic vesicle protein 2 – SV2 (Rummel et al. 2004; Simpson 1984; Yowler and Schengrund 2004; Binz and Rummel 2009; Brunger and Rummel 2009; Dong et al. 2006; Mahrhold et al. 2006). The SV2 has three isoforms: A, B, and C, the latter having the highest affinity to the BoNT (Dong et al. 2006; Mahrhold et al. 2006). Once connected to the SV2 receptor, the toxin is internalized inside synaptic vesicles during the process of recycling (Schiavo et al. 1994).

Only the LC of the toxin is translocated from the synaptic vesicle to the neuronal cytosol, after being pulled apart from the rest of the chain following the reduction of the disulfide bridge (Galloux et al. 2008; Fischer et al. 2008). LC translocation is processed through a pore formed by H_N fragment, once this part of the BoNT/A molecule is incorporated into the synaptic vesicle membrane (Blaustein et al. 1987; Kalandakanond and Coffield 2001; Fischer et al. 2008). Although the LC dimension seems to be far superior to the pore diameter, it is believed that it passes through the pore partially unfolded (Koriazova and Montal 2003; Fischer and Montal 2007a, b; Galloux et al. 2008; Montal 2009; Cai et al. 2006). This conformation change is dependent on a low pH inside the synaptic vesicle (Koriazova and Montal 2003; Fischer and Montal 2007a, b; Galloux et al. 2008; Montal 2009; Cai et al. 2006). Before translocation, LC portion is depleted from Zn, which is regained once LC reaches the neuron cytosol (Simpson et al. 2001). Once in the cytosol, the higher pH of this cellular compartment prompts LC to regain its neutral conformation (Schiavo et al. 1994). It is known that LC can be phosphorylated by Src family of tyrosine kinases in the tyrosine-71, which will increase the thermal stability of the endopeptidase (Ferrer-Montiel et al. 1996; Ibañez et al. 2004).

2.1.1 Effect of BoNT in Neurotransmitter Release

BoNT/A prevents neurotransmitter release from intoxicated cells, much of which neurons, in a calcium-dependent manner (Schiavo et al. 1994; Sakaba et al. 2005). The basic fusion machinery that leads to synaptic vesicle and plasma membrane fusion is formed by several molecules, such as the vesicle proteins VAMP2 (synaptobrevin 2; v-SNARE; associated with synaptophysin and V-ATPase) and synaptotagmin, the soluble NSF (SNAP/NSF complex), and the membrane proteins syntaxin 1 and SNAP-25 (Schiavo et al. 1994). SNAP-25 is associated with the internal leaf of neuronal cytoplasmic membrane by palmitoylation (Schiavo et al. 1994; Prescott et al. 2009). Syntaxin and SNAP-25 form the t-SNAREs (Schiavo et al. 1994). t-SNAREs together with v-SNARE form *trans*-SNARE or *SNARE* core complex (Schiavo et al. 1994; McNew et al. 2000). In order for neurotransmission to occur, the C-terminal of syntaxin 1 binds to the C- and N-terminal of SNAP-25 and to the major part of the cytoplasmic domain of VAMP2 forming a *trans*-SNARE complex (Jahn and Scheller 2003; Jahn et al. 2003). The *trans*-SNARE complex is in association with other factors, such as α-SNAP/NSF complex (ATPase that uncouple *trans*-SNARE complex), Rab proteins, and SM protein (such as Munc18 or Munc 13, which organizes spatial and temporal *trans*-SNARE complex) and complexin (Brunger et al. 2008; Vassilieva and Nusrat 2008; Wickner and Schekman 2008). The free energy released during the formation of this complex (35 k_BT, ≈20 kcal/mol) together with calcium-induced synaptotagmin activation can allow the fusion of the two membranes (Chen et al. 1999; Südhof and Rothman 2009). In fact, it is necessary 50–100 k_BT for the fusion to occur, which is obtained with the formation of three or more *trans*-SNARE complexes (Südhof and Rothman 2009).

In spite of SNARE complex cleavage by BoNT/A, synaptic vesicles are still able to bind the cytoplasmic membrane (Humeau et al. 2000; Muller et al. 1987; Marsal et al. 1989). However, this binding might occur far from voltage-sensitive calcium channels (Ibañez et al. 2004; Atlas et al. 2001). This will prevent the calcium-dependent fusion between synaptic vesicles and the cytoplasmatic membrane (Ibañez et al. 2004; Wonnacots et al. 1978; López-Alonso et al. 1995). This observation is confirmed in experiments in which an increase in calcium influx allows this ion to be available to the fusion process even in vesicles primed away from the places of calcium entry (Ibañez et al. 2004; de Haro et al. 2003).

This same mechanism might hold true for afferent neurons. It was recently showed that in rat trigeminal ganglia, CGRP is coexpressed with SNAP-25, syntaxin 1, VAMP, and synaptotagmin, VAMP being extremely important for CGRP release (McVary et al. 1998). Vanilloid-induced or bradykinin-induced CGRP release was only partially abolished by BoNT/A (McVary et al. 1998). This ineffectiveness of BoNT/A could be explained by the massive calcium entry to the cell caused by capsaicin stimulation (McVary et al. 1998).

A less well discussed mechanism of action of BoNT/A deals with its effect on the energetic machinery of the cells. Neuronal ATP and creatine phosphate levels decrease in cholinergic neurons after BoNT/A administration (Dunant et al. 1987,

1988, 1990). In consequence, one might expect that the nerve terminal affected by the toxin will be impaired.

2.2 *BoNT/A and the Urinary Bladder*

BoNT/A injections in the bladder wall may induce detrusor paralysis, provided that appropriate doses of neurotoxin are administered. In humans, detrusor paralysis and urinary retention were observed in patients with NDO and IDO (Apostolidis et al. 2005b, 2006b; da Silva and Cruz 2009). In animal experiments, normal animals will also display detrusor paralysis, provided that adequate doses of BoNT/A are injected in the bladder.

BoNT/A was first thought to induce bladder paralysis by preventing acetylcholine release in the neuronal–muscular junction (Edmunds and Long 1923; Dickson and Shevky 1923; Ambache 1949; Burgen et al. 1949). In the skeletal muscle, BoNT/A causes muscular paralysis that recovers within 2–4 months time after BoNT/A injection. During this period, a decrease of extrajunctional acetylcholinesterase can be detected and axons develop lateral sprouts that eventually disappear once synaptic transmission recovers (Thesleff et al. 1990; de Paiva et al. 1999; Meunier et al. 2002). Curiously, neuronal sprouting during detrusor paralysis was never documented in the urinary bladder (Haferkampa et al. 2004). In addition, no changes could be detected in the detrusor smooth muscle cells or nerve fibers coursing the urinary bladder (Haferkampa et al. 2004). On the other end, fragments of cleaved SNAP-25 seem to last much longer in the human bladder than in striated muscle (Schulte-Baukloh et al. 2007) and the average duration of BoNT/A in the bladder, around 9 months, largely exceeds that of skeletal muscle. Altogether, these facts indicate that additional mechanisms of action are probably operative in the bladder.

Among possible mechanisms, interruption of sensory input from the bladder, has received great attention. About 50% of the human bladder afferents express SV2 and SNAP-25, indicating their sensitivity to the neurotoxin (Coelho 2009). BoNT/A decreases the release of both substance P and CGRP from bladder afferent peripheral terminals in the bladder wall (Apostolidis et al. 2006b; Welch et al. 2000b; Chuang et al. 2004; Rapp et al. 2006; Lucioni et al. 2008), which is expected to limit neurogenic inflammation in the bladder. In addition, BoNT/A also inhibits the spinal cord release of glutamate, substance P, and CGRP by sensory nerves (Meng et al. 1997; Purkiss et al. 2000; Duggan et al. 2002; Aoki 2005), decreasing the excitation of second-order spinal cord sensory neurons.

BoNT/A was shown to prevent TRPV1 trafficking to the membrane during bladder inflammation (Morenilla-Palao et al. 2004). This process is expected to not only affect the noxious transmission in the bladder, but also prevent the activation of micturition reflex by bladder filling. In accordance with these experimental findings, TRPV1 and P2X3 immunoreactivity in the urinary bladder of patients with detrusor overactivity was shown to decrease after BoNT/A administration (Apostolidis et al. 2005b).

Another mechanism by which BoNT/A might interfere with the sensory arm of micturion reflex deals with the decrease of ATP release after BoNT/A injection in the bladder wall in animal models of spinal cord injury (Khera et al. 2004; Smith et al. 2008). ATP activates P2X3 receptors in the suburothelium, which was shown to be essential for bladder contractions (Cockayne et al. 2000). The decrease of ATP release was initially suggested to be dependent on a direct effect of BoNT/A on urothelial cells (Khera et al. 2004; Smith et al. 2008). However, rodents and human urothelial cells do not express SV2 or SNAP-25 (Coelho 2009). The decrease of ATP release should therefore be interpreted as either a direct effect of BoNT/A on ATP producing machinery (Dunant et al. 1987, 1988, 1990), a possible effect on other urothelial proteins involved in ATP release, such as connexins, nucleoside transporters, or the ABC cassette system (Grossman et al. 1994; Wang et al. 2005), or an indirect consequence of the intravesical pressure decrease following detrusor paralysis (Vlaskovska et al. 2001).

A marked decrease in urine concentration of nerve growth factor (NGF) was observed in NDO and IDO patients after BoNT/A injections (Giannantoni et al. 2006a; Liu et al. 2009). The mechanism of NGF release impaired by BoNT/A is unknown at this moment. Although an effect on urothelial cells could be suggested on the basis of NGF released from those cells (Birder et al. 2007), a recent observation from the same group seems to exclude such hypothesis. In fact, in contrast with urine levels (Kim et al. 2005; Yokoyama et al. 2008; Kuo et al. 2009), NGF concentration in mucosal samples of patients with detrusor overactivity is not superior to those of normal subjects (Birder et al. 2007). Whatever the mechanisms of NGF decrease in urine, the lack of this neurotrophin is expected to reduce excitability of bladder sensory fibers (Dmitrieva and McMahon 1996). Quite recently a decrease of brain-derived nerve factor concentration was also found after BoNT/A administration (Pinto et al. 2010).

The impairment of bladder nociceptive input following BoNT/A application in the bladder (Smith et al. 2004) opened the opportunity of BoNT/A injection in patients with bladder pain syndrome/interstitial cystitis (BPS/IC) (Giannantoni et al. 2006a; Liu and Kuo 2007a). Due to the concentration of bladder nociceptive fibers in the trigonal region, BoNT/A injections restricted to the trigone were recently evaluated in BPS/IC (Pinto et al. 2010). Pain alleviation proved to be highly effective in spite of the limited area of injection (Pinto et al. 2010). In addition, the risk of micturition dysfunction was minimized when compared with whole bladder injection (Pinto et al. 2010).

2.3 BoNT/A and the Prostate

The effect of BoNT/A was recently investigated in the prostate. Injections in the rat, dog, and human prostate were shown to decrease prostate volume (Chuang et al. 2005; Silva et al. 2008b, c). In a study carried out in rats, the prostate volume

reduction was correlated with an activation of apoptotic mechanisms (Silva et al. 2008b). Interestingly, prostate apoptosis was shown to be dependent on sympathetic nerve impairment and consequent decrease of the adrenergic stimulation of gland (Silva et al. 2008b). In addition to prostate volume changes, BoNT/A was shown to induce a dose-dependent decrease on the contractile function of the dog prostate (Schaible et al. 2005), further reinforcing the importance of sympathetic nerve signaling.

These findings suggest for intraprostatic BoNT/A, a totally different mechanism than proposed for intradetrusor BoNT/A (see Sect. 2.2), where parasympathetic impairment seems to play the key role. However, sympathetic fibers are the most numerous nerves in the prostate, whereas parasympathetic nerves predominate in the bladder (McVary et al. 1998; Schaible et al. 2005). Indirectly the differences between the bladder and the prostate indicate that BoNT/A effect in a particular organ depends on the impairment of the most important/numerous nerve fibers coursing through it.

2.4 Clinical Implications in the Usage of BoNT/A

The use of BoNT/A has become the treatment of excellence for patients with NDO (Cruz and Silva 2006), since it is simple and reproducible. It consists of BoNT/A injection (200–300 U of Botox® or 500–1,000 of Dysport®) distributed by 30 detrusor sites of injections under cystoscopic control. The treatment resulted in a reduction of incontinence episodes in neurogenic detrusor overactive patients (Reitz et al. 2004; Kalsi et al. 2007; Schurch et al. 2005; Ehren et al. 2007), with a mean duration between 6 and 9 months. More complete studies comparing BoNT/A with a placebo will prompt the definitive approval of this drug for NDO treatment.

Several studies, although also limited in size, have demonstrated clinical and urodynamics improvement in patients with refractory IDO after BoNT/A administration (Schmid et al. 2006; Sahai et al. 2007; Schulte-Baukloh et al. 2005; Brubaker et al. 2008). Furthermore, patients with benign prostate hyperplasia (Maria et al. 2003; Kuo 2005b; Chuang et al. 2005; Silva et al. 2008c; Doggweiler et al. 1998) and patients with BPS/IC (Giannantoni et al. 2006b, 2008; Pinto et al. 2009) presented an improvement in their symptoms after treatment with BoNT/A.

3 Future Directions

The key role of motor and sensory nerve fibers of somatic and autonomic origin for the LUT function makes the nervous system a foreseeable target for the control of micturition dysfunction. TRPV1 receptors are the first example of these targets that

are expected to be more and more relevant as new antagonist will come into therapeutic use. BoNT/A represents a nonspecific, albeit extremely potent, neurotoxin. However, the capacity to impair LUT innervation for long periods of time makes this toxin extremely attractive for the treatment of LUT dysfunction refractive to standard treatment. Future directions in BoNT/A will inevitably pass through the definition of doses and choice of injection places according to pathologies to treat, taking into consideration the different distribution of somatic, autonomic, and sensory fibers throughout the LUT.

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