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Original article

Botulinum toxin type A-induced changes in the chemical coding of dorsal root ganglion neurons supplying the porcine urinary bladder

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Abstract

Botulinum toxin type A (BTX) is a potent neurotoxin, which in recent years has been effectively applied in experimental treatments of many neurogenic disorders of the urinary bladder. BTX is a selective, presynaptically-acting blocking agent of acetylcholine release from nerve terminals what, in turn, leads to the cessation of somatic motor and/or parasympathetic transmission. However, application of this toxin in urological practice is still in the developmental stages and the full mechanism of its action remain elusive. Thus, the present study was aimed at investigating the neurochemical characterization of dorsal root ganglion (DRG) neurons supplying the porcine urinary bladder after BTX treatment. Retrograde tracer Fast Blue (FB) was injected into the urinary bladder wall in six juvenile female pigs and three weeks later, intramural bladder injections of BTX (100 IU per animal) were carried out in all the animals. After a week, DRG from L1 to Cq1 were harvested from the pigs and neurochemical characterization of FB⁺ neurons was performed using double-labeling immunofluorescence technique on 10- μ m-thick cryostat sections. BTX injections led to a significant decrease in the number of FB⁺ neurons containing substance P (SP), calcitonin gene-related peptide (CGRP), calbindin (CB), somatostatin (SOM) and neuronal nitric oxide synthase (nNOS) when compared with that found in the healthy animals (19% vs. 45%, 18% vs. 36%, 0.6% vs. 3%, 0.4 vs. 4% and 0.1% vs. 6%, respectively) These data demonstrated that BTX changed the chemical coding of bladder sensory neurons, and therefore this drug should be taken into consideration when it planning experimental therapy of selected neurogenic bladder disorders.

Key words: botulinum toxin A, urinary bladder, sensory innervation, dorsal root ganglia neurons, immunohistochemistry, neuropeptides, pig

Introduction

Botulinum toxin type A (BTX), produced by *Clostridium botulinum*, is a potent polypeptide neurotoxin that inhibits neurotransmission through the proteolysis of proteins involved in synaptic vesicle docking and

fusion with the plasma membrane (Welch et al. 2000). Once internalized by nerve terminals, BTX cleaves specific sites of synaptosome-associated protein 25 (SNAP-25), preventing the SNARE-mediated fusion of synaptic vesicles with the neuronal membrane and thus, blocking neurotransmitter and inflammatory/

/pain mediator release (Chancellor et al. 2008). The inhibitory effect of BTX on somatic and autonomic neurotransmission is well documented. It inhibits the release of acetylcholine at the neuromuscular junction (Jankovic 2004) or acetylcholine and norepinephrine release from efferent nerve terminals in the lower urinary tract (Smith et al. 2003). This efferent effect results in suppressing muscle contractility. Apart from its therapeutic effects on muscular hypercontraction, there is a growing body of evidence that BTX may also inhibit afferent neurotransmission and has analgesic properties in animals and humans. Filippi and co-workers (1993) have revealed that local injections of BTX directly reduce afferent 1a fiber activation and thereby produce a modulatory effect on sensory feedback. BTX has been demonstrated to inhibit the release of substance P (Welch et al. 2000) and CGRP (Durham and Cady 2004), which are implicated in the genesis of pain, from rat cultured embryonic dorsal root ganglia (DRG) and trigeminal ganglion neurons, respectively. BTX may also reduce a formalin-induced release of glutamate (Cui et al. 2004), which stimulates local nociceptive neurons through activation of receptors on peripheral afferents (Coggeshall and Carlton 1998). Jabbari and collaborators (2003) reported that in humans segmental burning pain in patients suffering from spinal cord lesions was relieved by multiple subcutaneous injection of BTX.

In recent years the urological use of BTX has gained widespread popularity in the treatment of lower urinary tract symptoms (LUTS) such as frequency and urgency incontinence due to intractable neurogenic or idiopathic urinary bladder disorders. Injections of BTX have successfully been used to relieve urological conditions such as refractor detrusor hyperreflexia (Neugart et al. 2006), detrusor sphincter dyssynergia (Chen et al. 2011), overactive bladder syndrome (Kanagarajah et al. 2011) or interstitial cystitis/painful bladder syndrome (Gottsche et al. 2011). BTX appears to have an effect on both efferent and specific sensory pathways of urinary bladder innervation. It has been shown that BTX inhibits acetylcholine (ACh) and norepinephrine release from efferent nerve terminals in the lower urinary tract (Smith et al. 2003). BTX also inhibits afferent nerve-mediated bladder strip contractions (Smith et al. 2004), presumably by blocking neurotransmitter release such as SP and CGRP from peripheral afferent nerve terminals in the bladder (Chuang et al. 2004, Reitz et al. 2004), and reduces the number of sensory fibers immunoreactive to P2X3 and TRPV1 in the bladder (Apostolidis et al. 2005).

Although both direct and indirect BTX effects on the bladder sensory innervation pattern are well documented, there is, so far, no available data concerning the influence of BTX on the chemical coding of

sensory DRG neurons supplying the urinary bladder in the pig, an animal species that can be used as a very good animal model for investigations of the human lower urinary tract disorders. Therefore, the present study was aimed at determining by means of combined retrograde tracing and immunochemistry techniques, BTX-induced changes in the chemical coding of porcine urinary bladder-projecting DRG neurons.

Materials and Methods

The present study was performed on six immature Great Polish White female pigs (aged 8-12 weeks, 15-20 kg b.w.), kept under standard laboratory conditions with free access to food and water *ad libitum*. Surgical procedures were applied in agreement with the guidelines of the Local Ethics Committee under deep thiopental anesthesia. All the animals were pretreated with atropine (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and azaperone (Stresnil, Janssen Pharmaceutica, Belgium; 0.5 mg/kg b.w., i.m.) thirty minutes before the main anesthetic, sodium thiopental (Sandoz, PL) was given intravenously in a slow, fractionated infusion, not exceeding the doses of 0.5 g per animal. After a mid-line laparotomy, the urinary bladder was gently exposed and a total volume of 40 μ l of 5% aqueous solution of the fluorescent retrograde tracer FB (Dr K. Illing KG & Co GmbH, Gross Umstadt, Germany) was injected into the right side of the urinary bladder wall in multiple injections. Three weeks later all the pigs were injected with BTX by aid of a cystoscope (100 IU per animals, Botox). After a week all the animals were killed by an overdose of sodium pentobarbital and, after the cessation of breathing, perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Bilateral spinal ganglia, together with the spinal cord segments from L1 to Cq1, were collected from all the pigs, postfixed in the same fixative for 10 minutes, washed several times in 0.1 M phosphate buffer and stored in 18% buffered sucrose at 4°C until sectioning. ten- μ m-thick serial cryostat sections, prepared from all DRG studied were examined using an Olympus BX51 fluorescence microscope equipped with an appropriate filter set. Only FB⁺ neurons with clearly visible nuclei were counted in every fourth section. The number of FB⁺ cells found in all DRGs from the particular animal as well as the relative frequency of perikarya belonging to the particular neuronal classes were pooled and presented as mean \pm SEM. The diameter of perikarya studied was measured by means of an image Analysis software (version 3.02, Soft Imaging System, GER) and data were used to divide urinary bladder-projecting neurons into the three size-classes: small (average diameter up to 30

µm), medium-sized (diameter 31-50 µm) and large afferent cells (diameter > 51 µm). FB-labeled sensory neurons were processed for immunohistochemistry, applying a routine double-labeling immunofluorescence technique for biologically active substances including SP (rat monoclonal, Biogenesis, UK, 1:300), CGRP (rabbit polyclonal, Peninsula, USA; 1:9000), SOM (rat monoclonal, Biogenesis, UK; 1:60), GAL (rabbit polyclonal, Peninsula, USA; 1:1000), PACAP (rabbit polyclonal, Peninsula, USA; 1:15000), nNOS (mouse monoclonal, Sigma, USA; 1:400) and CB (rabbit polyclonal, Swant, Switzerland; 1:9000). Briefly, after immersion in a blocking and permeabilizing solution containing 1% Triton X100, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN₃ and 10% normal goat serum in 0.01M phosphate-buffered saline for 1 hour (h) at room temperature to reduce non-specific background staining, the sections were incubated overnight at room temperature with the primary antiserum in a humid chamber. Primary antisera were visualized by rat- and mouse-specific secondary antisera conjugated to FITC or rabbit-specific antibodies conjugated to biotin (all from Jackson Immunochemicals, USA). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson Immunochemicals, USA). Control slides were processed as described, however, without incubation with primary antibody. Retrograde labeled/double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filters sets, counted in each fourth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Relationships between immunohistochemical staining and FB distribution were examined directly by interchanging filters. Pictures were captured by a digital camera connected to a PC, analyzed with Analysis software (version 3.02, Soft Imaging System, GER) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results

Although a distinct decrease in the number of FB⁺ neurons immunolabeled for SP (Fig. 1b; 19.3 ± 5.1% vs. 45.2 ± 4.4%) was found after BTX treatment, it should be stressed that in comparison with previous data (Bossowska et al. 2009) such decrease was primarily observed in the lumbar subpopulation (15.8 ± 2.5% vs. 43.7 ± 10.7%) while in sacro-coccygeal DRGs retrogradely labeled sensory cells responded to BTX treatment in a less pronounced manner (21.1 ± 7.1% vs. 46.2 ± 6.0%). Similarly to data obtained in the group of healthy animals (Bossowska et al. 2009), BTX-challenged SP-IR bladder sensory neurons belonged main-

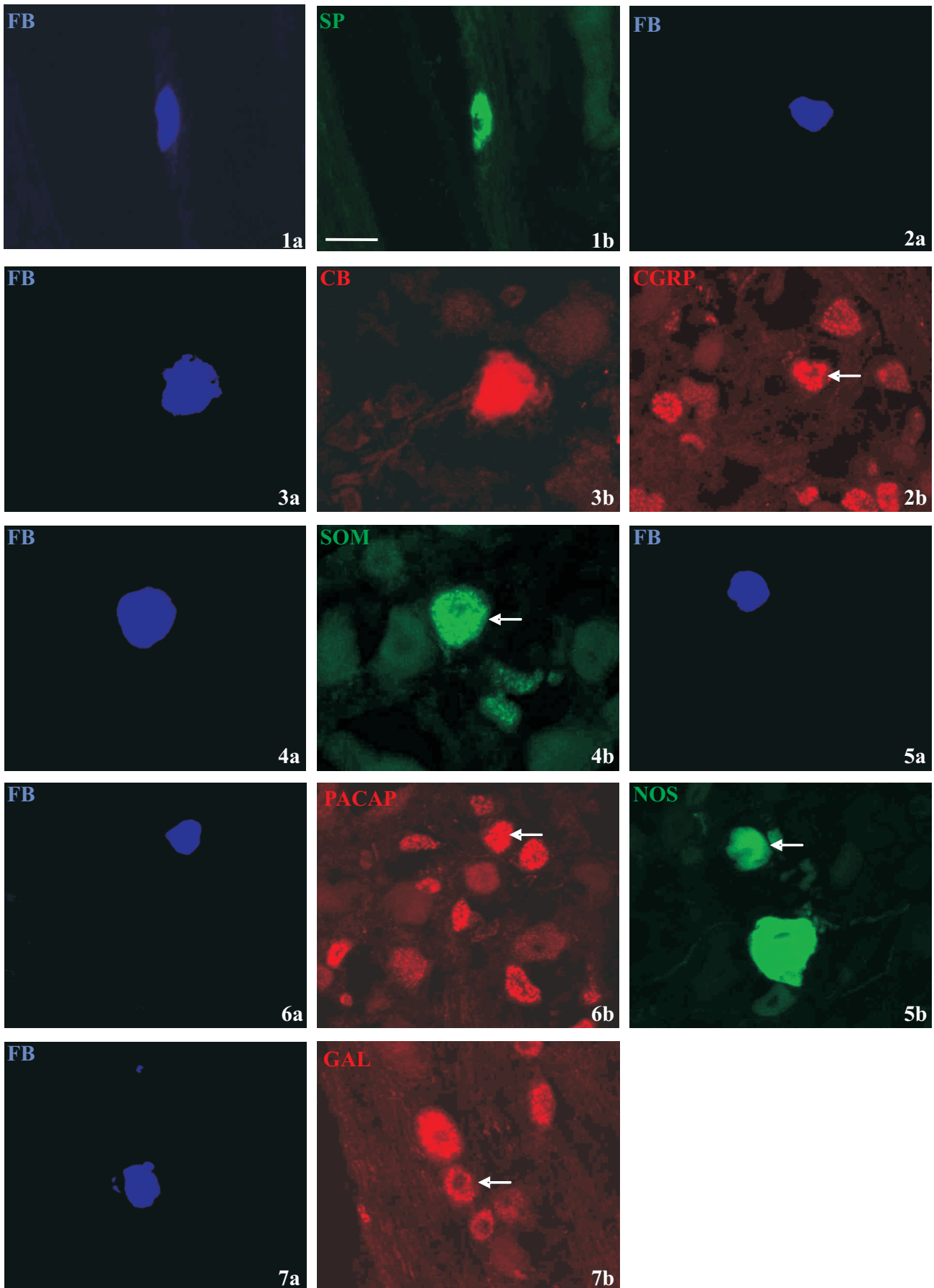
ly to the class of small-sized perikarya (Fig. 1a; 82.8 ± 2.1% vs. 89.7 ± 1.3%).

BTX injections evoked a significant decrease in the number of FB-labeled CGRP-IR neurons (17.9 ± 8.9%), when compared to data obtained under physiological conditions (36.1 ± 4.7%; Bossowska et al 2009). In contrast to the results obtained in intact animals (Bossowska et al. 2009), immunoreactivity to CGRP was overwhelmingly observed in small-sized sensory neurons (Fig. 2a; 93.9 ± 3.4% vs. 32.7 ± 2.1%), while this neuropeptide was not found in large FB⁺ cells after BTX treatment. Although in similarity to the healthy animal group (Bossowska et al. 2009) more FB⁺/CGRP⁺ neurons were found in lumbar (Fig. 2b-arrow; 38.2 ± 1.7% vs. 44.4 ± 4.3%) than in sacro-coccygeal DRGs studied (7.9 ± 5.1% vs. 23.5 ± 5.9%), after BTX treatment there was a significant decrease in the number of CGRP-IR cells in the latter population of bladder sensory neurons.

Table 1. The percentage of neurons expressing different biologically active substances among the total bladder-projecting sensory neurons in intact pigs and in the group of the animals treated with BTX.

Neurotransmitters	Intact animals	Animals after BTX treatment
SP	45.2 ± 4.4	19.3 ± 5.1
CGRP	36.1 ± 4.7	17.9 ± 8.9
CB	2.8 ± 1.4	0.6 ± 0.8
SOM	3.7 ± 2.8	0.4 ± 0.3
NOS	5.8 ± 2.5	0.1 ± 0.5
PACAP	26.1 ± 3.3	29.3 ± 0.8
GAL	6.5 ± 2.5	10.7 ± 3.4

The number of FB⁺ neurons containing CB distinctly decreased (till 0.6 ± 0.8%) after bladder injections of BTX, when compared to the relative frequency of CB-IR bladder-projecting sensory neurons in healthy animals (2.8 ± 1.4%; Bossowska et al 2009). In contrast to data obtained under physiological conditions (Bossowska et al. 2009) whole subpopulation of FB⁺/CB⁺ sensory cells were small-sized (Fig. 3a). In healthy animals the number of CB-IR sensory neurons was very similar in both the lumbar and sacro-coccygeal subpopulation of FB-labeled cells (3.1 ± 2.5% and 3.5 ± 1.2%; Bossowska et al. 2009) while BTX evoked a significant decrease in the number of sacro-coccygeal CB⁺ bladder neurons (Fig. 3b; 0.1 ± 0.1) and the total depletion of identically chemically coded cells belonging to the lumbar subpopulation of DRGs bladder-projecting cells.



BTX treatment of the bladder led to a significant decrease in the number of all the FB⁺ SOM-IR neurons (till $0.4 \pm 0.3\%$ vs. $3.7 \pm 2.8\%$, BTX-challenged vs. intact animals, respectively; Bossowska et al. 2009). However, this decline in the number was restricted exclusively to the lumbar subpopulation of bladder-projecting DRG neurons (Fig. 4b-arrow), while, similarly as in the intact animals (Bossowska et al. 2009), the lumbar subpopulation of SOM-IR bladder-projecting cells was still more numerous than that found in the sacro-coccygeal DRGs studied ($1.0 \pm 1.0\%$ vs. $0.2 \pm 0.1\%$, respectively). The majority of SOM-IR FB⁺ cells ($87.3 \pm 2.1\%$) belonged to the class of small-sized perikarya, however, in contrast to the intact animals (Bossowska et al. 2009), the subpopulation of medium-sized FB⁺ SOM-IR neurons (Fig. 4a; $12.7 \pm 1.5\%$) was also found after BTX treatment.

A significant decrease in the number of nNOS-IR FB⁺ neurons (till $0.1 \pm 0.5\%$ vs. $5.8 \pm 2.5\%$ observed under physiological conditions; Bossowska et al. 2009) was found after BTX treatment, particularly in the sacro-coccygeal subpopulation of bladder-projecting neurons (Fig. 5b-arrow). In contrast to previous report (Bossowska et al. 2009), immunoreactivity to nNOS was primarily found in small-sized retrogradely labeled DRG neurons (Fig. 5a; $88.6 \pm 1.9\%$ vs. $30.2 \pm 2.7\%$). It should be stressed that after BTX treatment nNOS-IR bladder-projecting cells were distinctly much more numerous in the lumbar ($17.9 \pm 4.6\%$) than in the sacro-coccygeal ($0.1 \pm 0.1\%$) subset of the bladder-projecting primary sensory cells.

In contrast to the decrease in the number of neurons expressing SP, CGRP, CB, SOM or nNOS immunoreactivities, BTX did not evoke any changes in the expression of PACAP and GAL in FB⁺ sensory cells (see below for details).

There were no distinct changes in the number of PACAP-IR FB⁺ neurons ($29.3 \pm 0.8\%$) after BTX treatment when compared to the results obtained under physiological conditions ($26.1 \pm 3.3\%$; Bossowska et al. 2009). Similarly to healthy animals (Bossowska et al. 2009), immunoreactivity to PACAP was found mainly in small-sized sensory cells (Fig. 6a; $82.8 \pm 2.9\%$ vs. $78.9 \pm 2.1\%$). Furthermore, no significant differences in the number of bladder sensory neurons containing PACAP were observed between lumbar

($25.9 \pm 10.7\%$) and sacro-coccygeal (Fig. 6b-arrow; $29.8 \pm 6.7\%$) DRGs studied, when compared with data obtained from animals studied under physiological conditions ($22.8 \pm 4.6\%$ and $31.2 \pm 4.0\%$; Bossowska et al. 2009).

The number of GAL-containing FB⁺ sensory neurons (Fig. 7b-arrow) was not distinctly changed ($10.7 \pm 3.4\%$) after bladder injections of BTX, when compared to the data obtained under physiological conditions ($6.5 \pm 2.5\%$; Bossowska et al. 2009). Similarly to the results obtained in the intact animals (Bossowska et al. 2009), all GAL-IR bladder-projecting sensory cells were of small diameter (Fig. 7a). It was found previously in healthy animals (Bossowska et al. 2009) that the number of GAL-IR FB⁺ cells was distinctly higher in the lumbar ($9.3 \pm 3.8\%$), than in the sacro-coccygeal ($5.9 \pm 2.4\%$) subpopulation of bladder-projecting DRG neurons; similar relationship was found after BTX administration ($19.7 \pm 4.2\%$ vs. $6.3 \pm 2.6\%$, lumbar vs. sacro-coccygeal neuronal subset, respectively).

Discussion

In the present study the BTX-induced down-regulation in the expression of SP-, CGRP-, CB-, SOM- and nNOS-IR was found in porcine urinary bladder afferent neurons, however this neurotoxin did not evoke distinct changes in GAL and PACAP expression in the bladder-projecting sensory cells. Moreover, for the first time we provided some evidence that the expression of neurotransmitters in bladder afferent neurons can be down-regulated differently, depending on the spinal cord segmental level at which the parental DRG were located. In general, it is now accepted that reflex contractions of the bladder are elicited by an activation of parasympathetic preganglionic neurons located in the sacral parasympathetic nucleus found in the sacro-coccygeal spinal cord (segments S3 to Cq1 in the pig; Bossowska et al. 2009), while an activation of sympathetic preganglionic neurons in the lumbar spinal cord (L3-L6 in the pig; Bossowska et al. 2009) has inhibitory effects on bladder smooth muscle activity (Vaughan and Satchell 1995). Therefore, as there were two distinct "sensory centers" found along the

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Fig. 1. S3 DRG, ipsilateral to the site of FB injections. A retrogradely traced small-sized bladder sensory neuron (a) containing SP immunoreactivity (b). Scale bar = 50 μm, applies to all figures; Fig. 2. L4 DRG, contralateral to the site of FB injections. A FB-labeled sensory cell of a small diameter (a) exhibiting CGRP-IR (b-arrow); Fig. 3. Ipsilateral S4 DRG. A single small-sized FB-traced sensory neuron (a) exhibiting CB-IR (b); Fig. 4. Ipsilateral L5 DRG. A medium-sized FB-positive neuron (a) expressing SOM- immunoreactivity (b-arrow); Fig. 5. S4 DRG, ipsilateral to the site of FB injections. A small-sized FB-traced sensory neuron (a) exhibiting NOS-IR (b-arrow); Fig. 6. Cq1 DRG, contralateral to the site of FB injections. A FB⁺ afferent small-sized neuron (a) immunolabeled for PACAP (b-arrow); Fig. 7. L4 DRG, ipsilateral to the site of FB injections. A small-sized sensory neuron supplying the bladder wall (a) containing GAL-immunoreactivity (b-arrow).

lumbo-sacro-coccygeal DRGs, it is assumable that the functional interpretation of the alterations observed in the present study may be separately derived from the lumbar and sacro-coccygeal DRGs in which BTX-induced changes were observed.

SP released from bladder afferent nerves is involved in the mechanoreceptor-mediated micturition reflex. In rats, systemic administration of capsaicin for depletion of SP resulted in urine retention or an increased volume/pressure threshold for micturition, implicating an excitatory role of SP in the afferent micturition pathway (Maggi 1997). It appears possible that SP, found in afferent neurons may be involved in mediating urinary bladder hyperreflexia (Callsen-Cencic and Mense 1997). Furthermore, it has been demonstrated that under noxious stimulation from the periphery of the body, SP is released from the central endings of DRG neurons (Daggan et al. 1995). Thus, as central branches of SP-IR bladder-projecting DRG neurons were shown to project to the dorsal part of sacral parasympathetic nucleus (Vizzard 2001), it may be suggested that this neuropeptide could be involved as an excitatory neurotransmitter in several types of bladder reflexes in rat (Mersdorf et al. 1992). Thus, as may be judged from the above-mentioned studies, it appears possible that also in the pig SP may be involved in the regulation of urinary bladder functions at different levels of the neuraxis. Previous studies have shown that BTX may inhibit SP release from sensory nerve endings in the urinary bladder wall (Chuang et al. 2004). It has also been reported that BTX inhibited a calcium-dependent and potassium-evoked release of SP from rat DRG neurons in embryonic primary culture system (Welch et al. 2000). In the present study, for the first time, we provided some evidence that the number of SP-IR bladder-projecting sensory neurons was distinctly decreased in lumbar as well as in sacro-coccygeal DRG after bladder injections of BTX. The mechanism inducing a decrease in SP expression after BTX treatment is unclear yet, but it is possible that a reduction in peripheral production and retrograde uptake of neurotrophic growth factor (NGF) to the DRG/spinal cord after BTX treatment could result in decreased production of neurotransmitters in DRG (Gianantoni et al. 2006). As NGF increases SP and CGRP synthesis in DRG (Averill et al. 1995) it may suggest that an inhibition in NGF production after BTX treatment can lead to the decrease in the number of SP-IR bladder-projecting sensory neurons in the pig. As the BTX injections resulted in a significant decrease in the number of SP-IR bladder sensory neurons, it may be suggested that this neurotoxin reduces the excitatory effect of SP at the spinal level, acting on the population of preganglionic neurons located in the

parasympathetic sacral nucleus, what in turn, may cause an inhibition of bladder contractions and pain transmission.

Rapp et al. (2006) reported that BTX did not alter basal CGRP release but dramatically reduced the capsaicin-evoked CGRP release from isolated bladder in rats. BTX has also been demonstrated to inhibit a release of CGRP from cultured embryonic trigeminal ganglion neurons (Durham and Cady 2004). In our study, a significant decrease in the number of FB-labeled CGRP-IR neurons, especially within the sacro-coccygeal population of these perikarya, has been observed after bladder injections with BTX. Similarly to SP this decrease in CGRP expression in bladder sensory neurons may trigger a reduction in NGF production induced by BTX treatment (Gianantoni et al. 2006). Previous studies suggested that CGRP, which *per se* has no excitatory effect on the vesico-vesical reflex pathway (Maggi et al. 1990), is able to facilitate the SP-evoked chemonociceptive reflex. CGRP acts synergistically with SP in the spinal cord (Biella et al. 1991) and such synergism may result from the CGRP-mediated inhibition of an endopeptidase that degrades SP (LeGreves et al. 1985), thus elevating the local concentration of SP at the site of release. This may suggest that CGRP is involved in the sensitization of afferent neuronal pathways in the lower urinary tract and that it plays a role in mediation of bladder overactivity. Thus, the decrease in the number of CGRP-IR bladder primary afferent neurons after BTX bladder injections, what probably leads to a diminishing of its release within the sacral spinal cord, may reduce the excitatory effect of SP on preganglionic neurons forming parasympathetic nucleus, what in turn, may lead to an inhibition of bladder contractions and pain transmission under pathological conditions.

CB has been thought to act as a Ca^{2+} buffer, thus controlling Ca^{2+} level within the cytoplasm of a neuronal cell. CB has been reported to act not only as a passive buffer but also to have an active role in neuronal activity due to its high Ca^{2+} -buffering activity (Baimbridge et al. 1992). This has been found in medium- or small-sized DRG neurons, mostly in subpopulation of muscle and visceral nociceptors (Honda 1995). Numerous CB-containing small- or medium-sized DRG neurons also contained SP (Li et al. 2005). This strongly suggests that CB is involved in some aspects of pain transmission, at least in small-sized spinal ganglion neurons. In pigs, the number of bladder sensory neurons containing CB distinctly decreased in lumbar as well as in sacro-coccygeal DRGs after bladder injection of BTX. It probably may lead to changes in CB action control on the Ca^{2+} homeostasis in affected neurons, what in turn may result in a decrease in

SP release rate from central afferent branches in the dorsal horn, suppressing pain transmission.

After BTX treatment, a significant decrease in the number of SOM-IR bladder neurons was observed in the porcine lumbar DRG studied. The previous study has shown that sciatic nerve transection causes a marked decrease of SOM (Noguchi et al. 1989) in lumbar DRGs in rats. Such changes in peptide expression following injury are thought to be initiated by a reduction in the normal retrograde transport of trophic molecules derived from the endoneurium or the target tissue (Raivich et al. 1991). It has also been shown that prolonged intrathecal treatment with glial cell line-derived neurotrophic factor (GDNF) increased the number of SOM-containing sensory neurons in DRG and the activity induced release of SOM in the dorsal horn isolated *ex-vivo* (Charbel-Issa et al. 2001). As BTX led to a reduction in NGF production in human bladders (Gianantoni et al. 2006) it is possible that this neurotoxin also inhibits GDNF production in the porcine bladder tissue, what in turn, may lead to a decrease in SOM expression in bladder-projecting sensory neurons. It has been shown that SOM is released into the spinal dorsal horn on peripheral nociceptive stimulation (Morton et al. 1989) and depresses the firing of dorsal horn neurons activated by noxious stimulation (Sandkühler and Helmchen 1990). However, the exact relevance of observed decrease in the number of SOM-IR bladder-projecting neurons after BTX treatment remains to be elucidated in detail.

Nitric oxide (NO) acts as “retrograde transmitter” in sensory pathways and plays a pivotal role in nociceptive processing in multisynaptic local circuits of the spinal cord (Meller and Gebhart 1993). It has also been suggested that NO plays a role in the facilitation of the micturition reflex evoked by noxious chemical irritation of the bladder as well as that it is involved in the facilitation of the micturition reflex by nociceptive bladder afferents at the spinal level (Kakizaki and de Groat 1996). Birder and colleagues (2001) have demonstrated that the major sites of NO release in the rat bladder are afferent nerves and that BTX treatment triggers off an increase in NO release from the bladder tissue. This may suggest that in pigs one of the possible mechanism leading to a decrease in the number of bladder-projecting sensory neurons after BTX treatment is an increasing release of NO from bladder afferent nerve terminals. As BTX produced a significant decrease in the number of NOS-IR bladder sensory neurons in the pig, it may be possible that this neurotoxin reduces the excitatory effect of NOS at the spinal level, acting on the population of preganglionic neurons located in the parasympathetic sacral nucleus, what in turn, may cause an

inhibition of bladder contractions and pain transmission.

Our study have also demonstrated that BTX was not able to evoke changes in the expression of PACAP and GAL in bladder-projecting DRG neurons. As it has been shown that PACAP (Girard et al. 2008) and GAL (Callsen-Cencic and Mense 1997) were upregulated during bladder cystitis and that these neuropeptides play very important anti-inflammatory and antinociceptive functions (Kim et al. 2000, Jimenez-Andrade et al. 2004) it may suggest that microinjections of BTX into the urinary bladder wall have not evoked any inflammatory responses.

The present study has shown that BTX profoundly influences the chemical coding of DRG cells supplying the porcine urinary bladder, causing a decrease in the number of SP-, CGRP-, CB-, SOM- and NOS-IR bladder afferent cells. It strongly suggests that BTX can be used in the treatment of hyperactivity of the afferent limb of reflex arcs responsible for the transmission of sensory and pain information from the urinary bladder and the influence of BTX on the sensory bladder innervation has to be taken into account, when this neuroactive agent is used in the experimental therapy of selected neurogenic bladder disorders.

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