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Original article

Tetrodotoxin induced changes in the chemical coding of dorsal root ganglion neurons supplying the porcine urinary bladder

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Abstract

Tetrodotoxin (TTX) mode of action is based on a blocking of fast sodium channels in nerve cell membrane what, in turn, abolishes the propagation of the action potential along the nerve fibers. TTX is currently used in experimental therapies focused on neoplastic or neurogenic pain, however, as for now there is no data concerning the influence of TTX on dorsal root ganglion (DRG) sensory neurons function. Thus, the present study was aimed at characterization of neurochemical coding of porcine sensory bladder-projecting cells after bladder instillation with TTX. Retrograde tracer Fast Blue (FB) was injected into the urinary bladder wall of six juvenile female pigs and three weeks later bladder instillation with TTX (12 μg per animal) was carried out in all animals. A week later, DRGs of interest were harvested from all animals and the neurochemical characterization of FB^+ neurons was performed using routine double-immunofluorescence labeling technique on 10-μm-thick cryostat sections. In TTX-treated animals the number of FB^+ cells containing galanin (GAL), nitric oxide synthase (NOS), somatostatin (SOM) and calbindin (CB) was 2.5%, 2%, 0.25% and 0.2%, respectively and that of pituitary adenylate cyclase-activating polypeptide (PACAP)-immunoreactive (IR) cells was 43%. These data when compared with previous reports, demonstrated that TTX profoundly changed the chemical coding of porcine bladder-projecting sensory neurons thus implicating that it may be used in case of hypoactivity of afferent part of reflex arc responsible for transmission of sensory information from the urinary bladder.

Key words: tetrodotoxin, urinary bladder, sensory innervation, dorsal root ganglia neurons, immunohistochemistry, neuropeptides, pig

Introduction

Tetrodotoxin (TTX) is a potent neurotoxin isolated for the first time in 1950 from toxic pufferfish by Yokoo (Yokoo 1950). This biological active substance inhibits nerve and muscle conduction by selective blockade of sodium (Na^+) channels (Narahashi 2001), resulting in respiratory paralysis that causes death. TTX blocks the action potentials in nerves by binding to the voltage-gated fast Na^+ channels in nerve cell

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membranes (Hwang and Noguchi 2007). The binding site of this toxin is located at the pore opening of the volt age-gated Na^+ channel. The use of this toxin as a biochemical probe has elucidated two distinct types of voltage-gated Na^+ channels present in humans: a tetrodotoxin-sensitive (TTX-s) and a tetrodotoxin-resistant (TTX-r) voltage-gated Na^+ channel. Tetrodotoxin binds to TTX-s Na^+ channels with a binding affinity of 5-15 nanomolar, while the TTX-r Na^+ channels bind TTX with low micromolar affinity. Nerve cells containing TTX-r Na^+ channels are located primarily in cardiac tissue, while nerve cells containing TTX-s Na^+ channels dominate in the rest of the body (Hwang and Noguchi 2007). At present blocking of fast Na^+ channels has a potential medical use in treating of some cardiac arrhythmias and TTX has also proved useful in the treatment of neurogenic pain accompanying such diverse problems as terminal cancer (Hagen et al. 2008) or heroin withdrawal (Stimmel 2002).

It is well known that afferent innervation, originating from dorsal root ganglion (DRG) sensory neurons, participates in the regulation of the urinary bladder physiological functions. In a recent study by Bossowska and collaborators (2009) it has been shown that the porcine urinary bladder receives dual afferent innervation originating from sensory neurons located in lumbar (L3-L6) and sacro-coccygeal DRGs (S3-S4 and Cq1), with the majority of bladder-projecting afferent cells located in S3 and S4 DRGs (85%). Furthermore, it has also been documented that the bladder-projecting sensory neurons expressed a broad array of transmitter substances, including substance P (SP), calcitonin gene-related peptide (CGRP), SOM, GAL, PACAP, NOS and CB (Bossowska et al. 2009).

In 1999 Yoshimura and de Groat showed that the majority of bladder-projecting afferent neurons exhibited TTX-r Na^+ channels and action potentials, and that they are capsaicin-sensitive (Yoshimura and de Groat 1999). There are at least two TTX-r Na^+ channels in adult DRG neurons and they are preferentially found on small nociceptive neurons playing a central role in neurotransmission of nociceptive signaling in DRG sensory cells (Hille 2001).

Thus, as there is still no available data concerning the influence of TTX on the chemical phenotype of bladder-projecting sensory neurons, we decided to investigate how this neurotoxin may change the chemical coding of DRG cells, what in turn may reflect changes in their function. Therefore, the present study was aimed, by means of combined retrograde tracing and immunochemistry techniques, at determining the TTX-induced changes in the chemical coding of porcine urinary bladder-projecting DRG neurons.

Materials and Methods

Present study was performed on six immature Great White Polish female pigs (aged 8-12 weeks, 15-20 kg b.w.), kept under standard laboratory conditions with a free access to food and water. Surgical procedures were applied in agreement with the guidelines of the Local Ethical Committee under deep thiopental anesthesia. All 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 dose of 0.5 g per animal. After a mid-line laparotomy the urinary bladder was gently exposed and a total volume of 40 µl 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 urinary bladder wall in multiple injections. Three weeks later a bladder instillation with TTX (12 µg per animal) was carried out in all animals. Seven days later all 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 of interest, together with the spinal cord, were collected from all animals, 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. 10-µm-thick serial cryostat sections, prepared from all DRGs 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 particular animal as well as the relative frequency of perikarya belonging to the particular neuronal classes were pooled and presented as mean ± SEM. The diameter of perikarya studied was measured by means of an image analysis software (Analysis 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% NaN_3 and 10% normal goat serum in 0.01M phosphate-buffered saline for 1 hour at room temperature to reduce non-specific background staining, sections were incubated overnight at room temperature with particular 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 above, however, without incubation with primary antibody. FB-positive and simultaneously immunolabeled 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 the 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

As shown in detail in our previous study (Bossowska et al. 2009), the porcine bladder-projecting DRG neurons expressed, under physiological conditions, a broad spectrum of neurotransmitters or their synthesizing enzymes, including SP, CGRP, PACAP, GAL, nNOS, SOM and CB. Analysis of the relative frequency of particular differently-coded neuronal subpopulations, performed on consecutive sections, showed that SP-, CGRP-, PACAP-, GAL-, nNOS-, SOM- or CB-containing cells constituted on average 45%, 36%, 26%, 6.5%, 6%, 4% and 3% of all retrogradelly traced DRG neurons, respectively. However, approximately 36% of all bladder-projecting sensory cells were immunonegative to all substances studied.

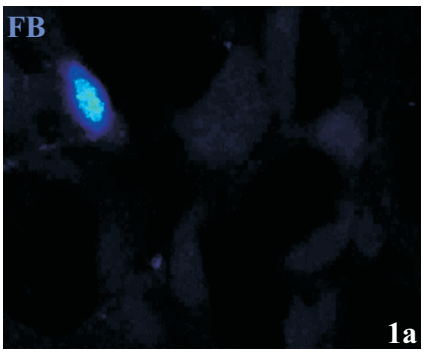
There were no distinct changes in the number of SP- or CGRP-IR FB^+ sensory neurons (43.8 ± 6.8% and 30.8 ± 1.2%) after TTX treatment, when compared to the results obtained under physiological conditions (45.2 ± 4.4% and 36.1 ± 4.7%; Bossowska et al. 2009). Similarly to the healthy animals (Bossowska et al. 2009) immunoreactivity to SP (Fig. 1b-arrow) was found mainly in small-sized FB^+ sensory cells (Fig. 1a; 84.9 ± 4.5% vs. 89.7 ± 1.3%). Furthermore, no significant differences in the number of bladder afferent neurons containing SP were observed between lumbar (41.7 ± 3.7% vs. 45.9 ± 4.6%) and sacro-coccygeal (44.8 ± 5.0% vs. 44.2 ± 2.9%) DRGs studied, when compared to the data obtained from animals under physiological conditions (Bossowska et al. 2009). In contrast to results observed in control animals (Bossowska et al. 2009), after TTX treatment immunoreactivity to CGRP was significantly more often observed in the cytoplasm of small FB^+ sensory neurons (87.8 ± 5.6% vs. 32.7 ± 2.1%), than in medium-sized ones (Fig. 2a; 12.2 ± 1.7% vs. 68.3 ± 1.9%), while none of large neurons contained this neuropeptide. Similarly to animals under physiological conditions (Bossowska et al. 2009) more $FB^+/CGRP^+$ sensory cells were found in the lumbar (Fig. 2b-arrow; 65.0 ± 15.0% vs. 44.4 ± 4.3%) than in the sacro-coccygeal DRGs studied (30.8 ± 1.2% vs. 23.5 ± 5.9).

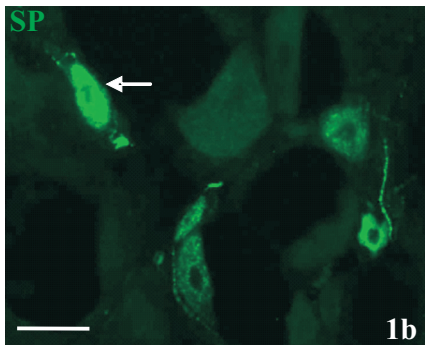
Table 1. The percentage contents of particular neurotransmitters in the bladder sensory neurons in intact animals and in the group of the animals treated with TTX.

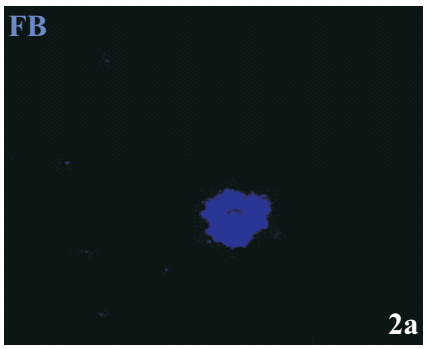
Neurotransmitters	Intact animals	Animals after TTX treatment
SP	45.2 ± 4.4	43.8 ± 6.8
CGRP	36.1 ± 4.7	30.8 ± 1.2
PACAP	26.1 ± 3.3	43.3 ± 0.2
GAL	6.5 ± 2.5	2.5 ± 3.3
NOS	5.8 ± 2.5	2.1 ± 1.6
SOM	3.7 ± 2.8	0.25 ± 0.2
CB	2.8 ± 1.4	0.2 ± 0.2

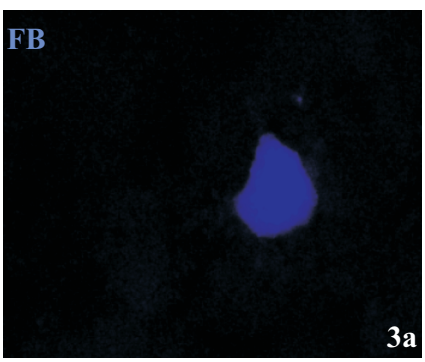
Although a distinct increase in the number of FB^+ afferent neurons immunolabeled for PACAP (43.3 ± 0.2% vs. 26.1 ± 3.3%, TTX-challenged vs. intact animals, respectively; Bossowska et al. 2009) was found after TTX treatment, it should however be stressed that in comparison with previous data (Bossowska et al. 2009) such increase was primarily observed in the lumbar subpopulation (38.5 ± 11.5% vs. 22.8 ± 4.6%) while in the sacro-coccygeal DRGs retrogradely labeled sensory cells responded to TTX treatment in a less pronounced manner (Fig. 3a,b – arrow; 37.3 ± 0.2% vs. 31.2 ± 4.0%). Similarly to the data obtained in the group of healthy animals (Bossowska et al. 2009), TTX-challenged PACAP-IR bladder sensory neurons belonged mainly to the class of small-sized perikarya (82.1 ± 7.7% vs. 78.9 ± 2.1%).

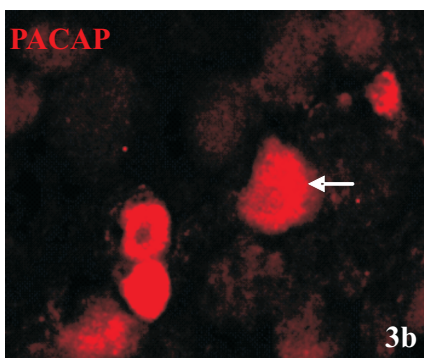
In contrast to the increase in the number of PACAP-IR bladder-projecting neurons, TTX treat-

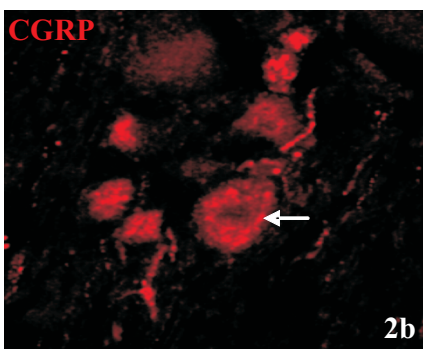

FB
1a

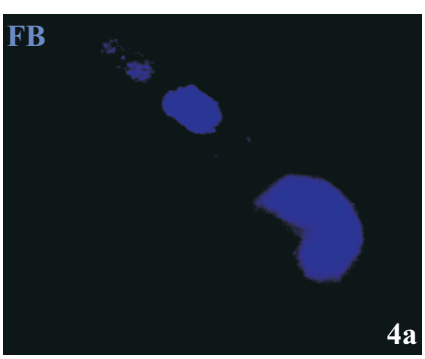

SP
1b

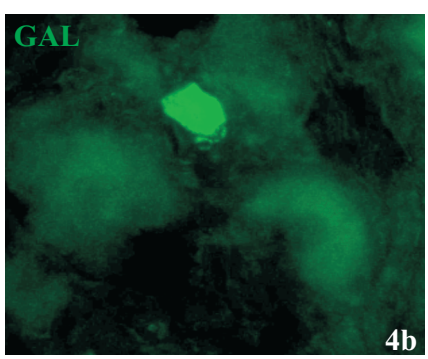

FB
2a

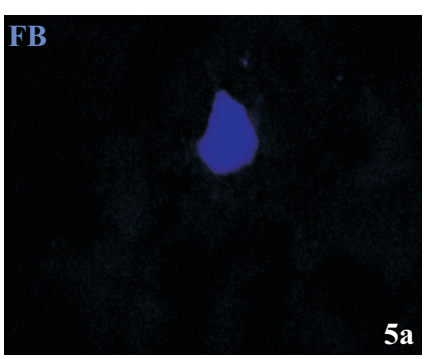

FB
3a


PACAP
3b

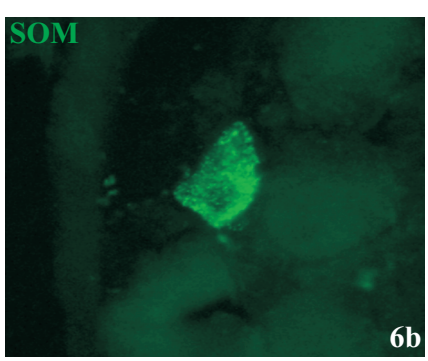

CGRP
2b

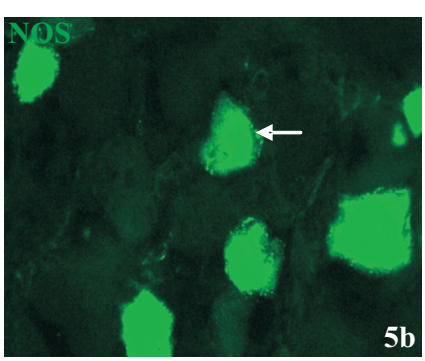

FB
4a

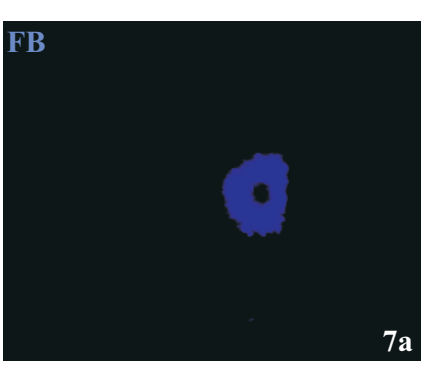

GAL
4b

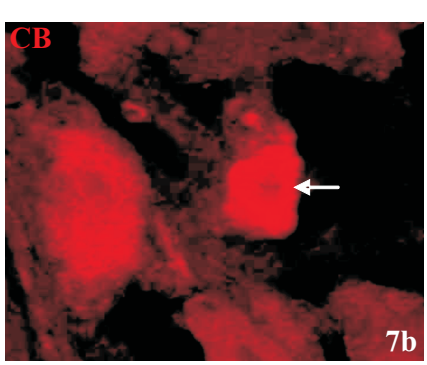

FB
5a


FB
6a


SOM
6b


NOS
5b


FB
7a


CB
7b

ment led to a significant decrease in the number of FB$^+$ sensory cells containing GAL, nNOS, SOM and CB (see below for details).

The number of GAL-containing FB$^+$ sensory neurons distinctly decreased (2.5 ± 3.3%) after bladder instillation with TTX, when compared to the data obtained under physiological conditions (6.5 ± 2.5%; Bossowska et al 2009). The majority of GAL-IR bladder-projecting cells were small in diameter (Fig. 4a; 76.4 ± 4.3%) however, in contrast to the results obtained in the previous study (Bossowska et al. 2009), a small population of medium-sized GAL-IR neurons (23.6 ± 4.3%) was observed after TTX treatment. It has previously been found in healthy animals (Bossowska et al. 2009) that the number of GAL-IR FB$^+$ cells was distinctly higher in the lumbar (9.3 ± 3.8%), than in the sacro-coccygeal subpopulation of bladder-projecting DRG neurons (5.9 ± 2.4%). A similar picture was observed after TTX administration where the values were 3.5 ± 1.2% for lumbar (Fig. 4b) and 1.8 ± 2.1% for sacro-coccygeal neuronal subset. However, it should be stressed that a significant decrease in the number of FB$^+$ GAL-IR cells was observed in both those neuronal subpopulations in animals challenged by TTX instillation.

A significant decrease in the relative number of nNOS-IR FB$^+$ neurons (till 2.1 ± 1.6% vs. 5.8 ± 2.5% observed under physiological conditions; Bossowska et al. 2009) was found after TTX 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 the small-sized retrogradelly labeled DRG neurons (Fig. 5a; 63.7 ± 9.7% vs. 30.2 ± 2.7%). It should be stressed that similarly to healthy animals (Bossowska et al. 2009), after TTX treatment, nNOS-IR bladder-projecting cells were distinctly much more numerous in the lumbar (9.0 ± 1.7% vs. 8.6 ± 2.8%) than in the sacro-coccygeal (0.5 ± 1.5% vs. 1.5 ± 0.5%) subset of the bladder-projecting primary sensory cells.

TTX treatment of the bladder has led to a significant decrease in the number of FB$^+$ SOM-IR DRG neurons (till 0.25 ± 0.2% vs. 3.7 ± 2.8%, TTX-challenged vs. intact animals, respectively; Bossowska et al. 2009). Although, this decline in the number was restricted exclusively to the lumbar subpopulation of bladder-projecting DRG cells. Similarly to healthy animals (Bossowska et al. 2009), the lumbar subpopulation of SOM-IR bladder-projecting cells (Fig. 6b) was still more numerous than that found in the sacro-coccygeal DRGs studied (2.5 ± 4.5% vs. 6.1 ± 4.6% and 0.25 ± 0.9% vs. 0.3 ± 0.3%, respectively). Furthermore, all SOM-IR FB$^+$ cells observed after TTX instillation were small in size (Fig. 6a).
The number of FB$^+$ afferent neurons containing CB distinctly decreased (till 0.2 ± 0.2%) after bladder instillation with TTX, when compared to the relative frequency of such neurons in healthy animals (2.8 ± 1.4%; Bossowska et al 2009). In contrast to the data obtained under physiological conditions (Bossowska et al. 2009) the percentage content of CB-IR bladder sensory neurons in medium- and small-sized was very similar (46.5 ± 3.3% and 43.8 ± 3.4%) in TTX-challenged animals but a significant increase, particularly in the number of small-sized FB$^+$ sensory cells (Fig. 7a), was observed. In healthy animals the number of CB-IR sensory neurons was very similar both in the lumbar and sacro-coccygeal subpopulation of FB-labeled cells (3.1 ± 2.5% and 3.5 ± 1.2%; Bossowska et al. 2009) while TTX evoked a significant decrease in the number of sacro-coccygeal CB$^+$ bladder neurons (Fig. 7b; 0.8 ± 1.6) and the total depletion of the CB-positive cells belonging to the lumbar subpopulation of DRGs bladder-projecting cells.

Discussion

In the present study the TTX-induced up-regulation in the expression of PACAP and down-regulation of nNOS-, SOM-, GAL- and CB expression in the bladder afferent neurons has been shown, however, this neurotoxin did not evoke any distinct changes, either in SP, or in CGRP expression in the bladder-projecting sensory cells. Moreover, we provided, for the first time, evidences that the expression of these substances was dissimilarly up- or down-regulated in bladder afferent neurons located in the lumbar and sacro-coccygeal DRGs. It is now widely accepted that reflex contractions of the bladder are elicited by an activation of parasympathetic preganglionic

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Fig. 1. S3 DRG, ipsilateral to the site of FB injections. A small-sized retrogradely traced bladder sensory neuron (**a**) containing SP immunoreactivity (**b-arrow**). Scale bar = 50 µm, apples to all figures; Fig. 2. L4 DRG, contralateral to the site of FB injections. FB-labeled nerve cell of a medium diameter (**a**) exhibiting CGRP-IR (**b-arrow**); Fig. 3. S3 DRG, contralateral to the site of FB injections. A FB$^+$ afferent medium-sized neuron (**a**) immunolabeled for PACAP (**b- arrow**); Fig. 4. L6 DRG, ipsilateral to the site of FB injections. Two sensory neurons supplying the urinary bladder wall (**a**), one of which contained GAL-immunoreactivity (**b**); Fig. 5. S4 DRG, ipsilateral to the site of FB injections. A single, small-sized FB-traced sensory neuron (**a**) exhibiting NOS-IR (**b-arrow**); Fig. 6. Ipsilateral L5 DRG. A small FB-positive neuron (**a**) expressed SOM- immunoreactivity (**b**); Fig. 7. Ipsilateral S3 DRG. A single, small-sized FB-traced sensory neuron (**a**) exhibiting CB-IR (**b-arrow**).

neurons located in the sacral parasympathetic nucleus at the sacro-coccygeal spinal cord (segments S3 to Cq1 in the pig; see Bossowska et al. 2009), while an activation of sympathetic preganglionic neurons in the lumbar spinal cord (L3-L6 in the pig; see Bossowska et al. 2009) has inhibitory effects on bladder smooth muscle activity (Vaughan and Satchell 1995). Therefore, as we have demonstrated the existence of two distinct "sensory centers" formed by the lumbar and sacro-coccygeal DRGs (Bossowska et al. 2009), it is assumable that the functional and clinical interpretations of alterations observed in the present study should be separately formulated for the lumbar and sacro-coccygeal DRGs in which TTX-induced changes were observed.

Our experiments have shown that the intravesical administration of TTX did not evoke any significant changes in the number of SP- and CGRP-IR bladder afferent neurons, either in the lumbar, or in sacro-coccygeal DRGs studied.

SP released from the bladder afferent nerves and sacral spinal cord is involved in the mechanoreceptor-mediated micturition reflex. In rats, systemic administration of capsaicin for depletion of SP resulted in the urine retention or an increased volume/pressure threshold for micturition, implicating an excitatory role of SP in the afferent micturition pathway (Maggi 1997). SP present in capsaicin-sensitive bladder afferent neurons may be involved in mediating urinary bladder hyperreflexia (Ahluwalia et al. 1994) but the release of SP within the bladder wall is known to trigger inflammatory responses, including plasma extravasation, vasodilatation and immune cell activation (Chien et al. 2003). Furthermore, it has also been demonstrated that upon the noxious stimulation in the periphery, 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 the sacral parasympathetic nucleus (Vizzard 2001) and intrathecally applied SP facilitated normal micturition, 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). On the other hand, SP released for the central endings of afferent neurons located in thoraco-lumbar DRGs has also been shown to facilitate the impulse activity of corresponding sympathetic preganglionic neurons of the intermediolateral nucleus (Cammack and Logan 1996), which are the source of the sympathetic (inhibitory) innervation of the bladder. 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. However, the physiological relevance and the exact mechanism(s) and place(s) of action(s) of this neuropeptide in the domestic pig remains to be addressed in detail.

CGRP has been shown to act synergistically with SP within the spinal cord (Biella 1991), what results from the CGRP-mediated inhibition of a SP-degrading endopeptidase (Le Greves et al. 1985) elevating the local concentration of SP at the site of release. These data may indicate that CGRP, which by itself has no excitatory effect on the vesico-vesical reflex pathway (Maggi 1990), could be involved into facilitation of SP-evoked chemonociceptive reflexes.

As it has been shown in the present study, TTX instillation was not able to change the number of SP- and/or CGRP-expressing bladder afferent neurons, what most probably reflected the fact that in healthy animals the vast majority of bladder-projecting SP- and CGRP-IR DRG neurons belonged to the subset of TTX-r Na^+ channels-bearing cells (Yoshimura and de Groat 1997), thus the studied agent was not able to influence their physiology. However, it should be stressed that a distinctly different situation could probably be observed in DRG from animals suffering from various bladder disorders associated with inflammation or spinal cord injury, as in such cases the majority of bladder afferent cells exhibited TTX-s Na^+ channels (Yoshimura and de Groat 1997) what may allow TTX to profoundly change their (patho)physiological properties; however, this hypothesis must be verified in detail.

Although the exact physiological role of PACAP in the lower urinary tract is still unclear, the study of Ishizuka and co-workers (1995) suggests that PACAP is involved in the facilitation of spontaneous bladder contractions in control rats by an excitatory action on the spinal micturition pathways. It has also been shown that the expression of PACAP in bladder afferent cells in rats increased during chronic cystitis induced by cyclophosphamide treatment (Vizzard 2000) or after spinal cord injury (Zvarova et al. 2005). These results indicate that PACAP may represent a principal component of bladder hyperreflexia by increasing excitability of sensory neurons in the bladder reflex arc. Additional roles for PACAP in micturition reflex pathways in these cases include modulation of nociceptive transmission through interaction with NMDA receptors (Oshawa et al. 2002) or modulation of inflammatory responses (Kim et al. 2000) by down-regulation of proinflammatory cytokine(s) synthesis and/or release. In the present study we have found that TTX has led to a significant increase in the number of PACAP-IR bladder sensory neurons in pigs both in the lumbar and in the sacro-coccygeal DRGs. Although the exact mechanism of TTX-evoked increase of the number of PACAP-IR

bladder-projecting neurons is still unclear, these results may suggest that this toxin can be used in experimental therapies of afferents hypoactivity within the reflex arcs. However, this suggestion needs to be verified in detail.

Results of the present study clearly demonstrated that TTX, instilled into the porcine urinary bladder, was able to evoke a dramatic decrease in the number of GAL-, nNOS-, SOM- and CB-IR sensory neurons supplying the organ. It should, however, be stressed that changes in the relative frequencies of particular subpopulations of afore mentioned neurons varied depending on the spinal cord level at which their parental DRGs were located. Thus, an equally strong decrease in the number of GAL- or CB-IR bladder-projecting neurons was observed both in the lumbar and sacro-coccygeal DRGs studied, while TTX-induced diminishing in the number of nNOS- and SOM-expressing perikarya was more pronounced in the sacro-coccygeal and lumbar DRGs, respectively.

It has previously been shown that GAL, released in the superficial dorsal horn from central processes of DRG neurons, activated inhibitory GAL1 receptors expressed by putatively glutamate-containing excitatory dorsal horn neurons, attenuating pain transmission (Liu and Hokfelt 2002). Furthermore, a reduction in galaninergic transmission within dorsal horn appears to increase pain (Liu and Hökfelt 2000), underlining an inhibitory role of high amount of endogenous GAL under pathological conditions, like in nerve injury. On the other hand, GAL has been proposed not only to counteract the action of PACAP and nitric oxide (NO) on bladder-projecting afferent cells (Zvarova et al. 2004) but it also inhibits presynaptically the release of SP and CGRP from capsaicin-sensitive primary afferents (Callsen-Cencic and Mense 1997). Thus, as may be judged from the above-mentioned data, GAL may have a potent modulatory function within the bladder-controlling neural circuits which participates in the urinary bladder facilitation and pain transmission. Finally, the results of our study may suggest that TTX, reducing the GAL expression in the bladder-projecting sensory neurons, can lead to the increase of the urinary bladder activity.

It has previously been shown that NO may act as a "retrograde transmitter" in the sensory pathways and may play a crucial role in nociceptive processing in the spinal cord (Meller and Gebhart 1993). It has also been suggested to facilitate the micturition evoked by chemical irritation of the bladder (Kakizaki and de Groat 1996). Furthermore, NO released by afferent nerves and epithelial cells of the urinary bladder (Birder et al. 2001) participates in the initiation of inflammatory responses and triggering of painful sensations (Aley et al. 1998). Renganathan and co-workers (2000) have provided evidences that NO is able to suppress fast and slow Na^+ currents in injured neurons, suggesting that NO may be an autocrine regulator of Na^+ currents in C-type DRG neurons, leading, by suppression of both fast and slow Na^+ currents, to DRG hypoexcitability (Renganathan et al. 2000).

Previous studies have demonstrated that SOM is released into the spinal dorsal horn on peripheral nociceptive stimulation (Morton et al. 1988) and depresses the firing of dorsal horn neurons activated by noxious stimulation (Sandkühler and Helmchen 1990). It has also been shown that SOM exerts a systemic antinociceptive effect (Helyes et. al. 2000) and inhibitory action on acute neurogenic and non-neurogenic inflammatory reactions (Than et al. 2000). However, the role of SOM in the maintaining of bladder functions, as well as the physiological relevance of distinct decrease in the number of SOM-IR neurons after challenging the bladder with TTX, remains obscure yet.

CB has been thought to act as a Ca^{2+} store, thus controlling Ca^{2+} level within the cytoplasm of a neuronal cell. CB has been reported to act not only as a passive store but also to have an active role in neuronal activity due to its high Ca^{2+}-binding activity (Baimbridge et al. 1992). In DRG, CB has predominantly been found in large-sized neurons whose peripheral terminals innervate muscle spindles (Philippe and Droz 1989). It has also been found in a subpopulation of medium- or small-sized muscle and visceral nociceptors (Honda 1995). Numerous CB-containing small- or medium-sized DRG neurons also contained SP (Li et al. 2005), what strongly suggests, that CB may be involved in some aspects of pain transmission, at least in small spinal ganglion neurons.

Results of the present study have clearly shown that TTX is able to profoundly change the chemical coding of DRG cells supplying porcine urinary bladder, leading to a drastic decrease in the number of neurons containing GAL, SOM, NOS and CB and a simultaneous increase in the number of PACAP-IR cells. However, TTX was not able to influence SP and CGRP expression rate in the bladder-projecting sensory cells. Further studies are necessary to elucidate in detail the mode of action and physiological/clinical relevance of this neurotoxin in the bladder-supplying DRG neurons.

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