

# Conantokin G-induced changes in the chemical coding of dorsal root ganglion neurons supplying the porcine urinary bladder

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## Abstract

Conantokin G (CTG), isolated from the venom of the marine cone snail *Conus geographus*, is an antagonist of *N*-methyl-d-aspartate receptors (NMDARs), the activation of which, especially those located on the central afferent terminals and dorsal horn neurons, leads to hypersensitivity and pain. Thus, CTG blocking of NMDARs, has an antinociceptive effect, particularly in the case of neurogenic pain treatment. As many urinary bladder disorders are caused by hyperactivity of sensory bladder innervation, it seems useful to estimate the influence of CTG on the plasticity of sensory neurons supplying the organ. Retrograde tracer Fast Blue (FB) was injected into the urinary bladder wall of six juvenile female pigs. Three weeks later, intramural bladder injections of CTG (120 µg per animal) were carried out in all animals. After a week, dorsal root ganglia of interest were harvested from all animals and neurochemical characterization of FB<sup>+</sup> neurons was performed using a routine double-immunofluorescence labeling technique on 10-µm-thick cryostat sections. CTG injections led to a significant decrease in the number of FB<sup>+</sup> neurons containing substance P (SP), pituitary adenylate cyclase activating polypeptide (PACAP), somatostatin (SOM), calbindin (CB) and nitric oxide synthase (NOS) when compared with healthy animals (20% vs. 45%, 13% vs. 26%, 1.3% vs. 3%, 1.2 vs. 4% and 0.9% vs. 6% respectively) and to an increase in the number of cells immunolabelled for galanin (GAL, 39% vs. 6.5%). These data demonstrated that CTG changed the chemical coding of bladder sensory neurons, thus indicating that CTG could eventually be used in the therapy of selected neurogenic bladder illnesses.

**Key words:** conantokin G, urinary bladder, sensory innervation, dorsal root ganglia neurons, immunohistochemistry, neuropeptides, pig.

## Introduction

It is well-known that one of the crucial elements of the reflex arc participating in the regulation of the urinary bladder physiological functions, is afferent nerve fibers conveying the sensory information to DRGs sensory neurons. In a recent study by Bossowska and collaborators (2009) it was demonstrated that the porcine urinary bladder receives dual afferent innervation originating from sensory neurons located in lumbar (L3-L6) and sacro-coccygeal (S3-S4 and Cq1) DRGs. It is well known that different bladder disorders in humans are associated with several distinct changes in urinary bladder functions and sensory sensation conveyed from the organ. The neuronal and mechanical events associated with bladder filling and micturition become hypersensitive and progressively painful and it has been suggested that detrusor hyperexcitability and pain sensation was mainly due to alterations in the afferent innervation of the urinary bladder. A recent study has demonstrated that a number of voltage- and ligand-gated ion channels have been implicated in pathological pain transmission (Millan 1999). Following tissue injury or inflammation, functioning of dorsal horn ligand- as well as voltage-gated ion channels is altered such that neurons exhibit persistent spontaneous firing or activation to normally non-noxious stimuli, being in part responsible for the pain and hypersensitivity. Thus, one possible method of treating pathological pain is to block cation channels at the level of the primary afferents or dorsal horn neurons.

It has been established *in vitro* that several peptide venoms isolated from *Conus* marine snails reveal a significant selectivity to particular ligand- and voltage-gated ion channels (Olivera and Teichert 2007). For example, conantokins attenuate glutamate induced neurotoxicity *in vitro* inhibiting ionotropic glutamate receptors (Olivera 1997) which are divided into three classes: NMDARs, kainate and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Dingledine et al. 1999). NMDARs are ligand-gated ion channels whose activation leads to calcium ( $\text{Ca}^{2+}$ ) influx into nerve cells and to activation processes involved in long-term potentiation (Malenka et al. 1988). NMDARs have been detected on the presynaptic terminals of afferent fibers (Liu et al. 1997) and are expressed in most dorsal root ganglia (DRG) neurons (Sato et al. 1993). For example, in the periphery NMDARs are present in afferent terminals of the urinary bladder in rats (Gonzalez-Cadavid et al. 2000). Accumulating evidence suggests that NMDARs play a pivotal role in the transmission of excitatory signals from primary sensory neurons to the brain through the spinal cord (Dickenson 1990) and

regulate synaptic plasticity by involving changes in neurotransmitter release (Bustos et al. 1992). Thus, hyperactivation of NMDARs is generally recognized as a key factor in sustaining chronic inflammatory and neuropathic pain transmission (Fisher et al. 2000).

A well-known natural antagonist of NMDARs is the best-characterized, small 17 amino acid peptide conantokin G (CTG), isolated from the fish-hunting snail *Conus geographus* (Olivera 1997). In rat hippocampal brain slices CTG can significantly and reversibly decrease the total charge transfer, peak amplitude, and frequency of NMDAR-mediated EPSCs (Barton et al. 2004). It has been shown that CTG exhibits potent anticonvulsant and antinociceptive properties (Xiao et al. 2008). In the dorsal horn, NMDARs found on primary afferent central terminals suggest that the antinociceptive effect of CTG is due to reducing neurotransmitter release (Marvizon et al. 2002) mechanism in tissue injury-associated pain and hypersensitivity (Millan 1999). The intrathecal injection of CTG also led to robust antinociceptive effects in rat models of persistent pain (Hama and Sagen 2009).

Since an increasing excitability of DRG neurons during pathological conditions of the urinary bladder leads to its hyperactivity and pain transmission, it seems to be essential to determine if CTG can trigger changes in chemical coding and plasticity of sensory DRG neurons supplying the urinary bladder in the pig, a species that can be used as a very good animal model for the human lower urinary tract. Therefore, the present study was aimed, by means of combined retrograde tracing and immunochemistry techniques, at determining the CTG-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 Ethical Committee, under deep thiopental anesthesia. All animals were pretreated with atropine (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and propionylpromazine (Stresnil, Janssen Pharmaceutica, Belgium; 0.5 mg/kg b.w., i.m.) thirty minutes before the main anesthetic, sodium thiopental (Sandoz, PL, 0.5 g per animal) was given intravenously in a slow, fractionated infusion. After a mid-line laparotomy, the urinary bladder was gently exposed and a total volume of 40  $\mu\text{l}$  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 animals were injected with CTG by aid of a cystoscope (120 µg per animal). After a week 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<sup>+</sup> neurons with clearly visible nuclei were counted in every fourth section. The number of FB<sup>+</sup> cells found in all DRGs from a 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 image Analysis software (version 3.02, Soft Imaging System, GER) and data were used to divide urinary bladder-projecting neurons into 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), calcitonin gene-related peptide-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<sub>3</sub> 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 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, however, without incubation with primary antibody.

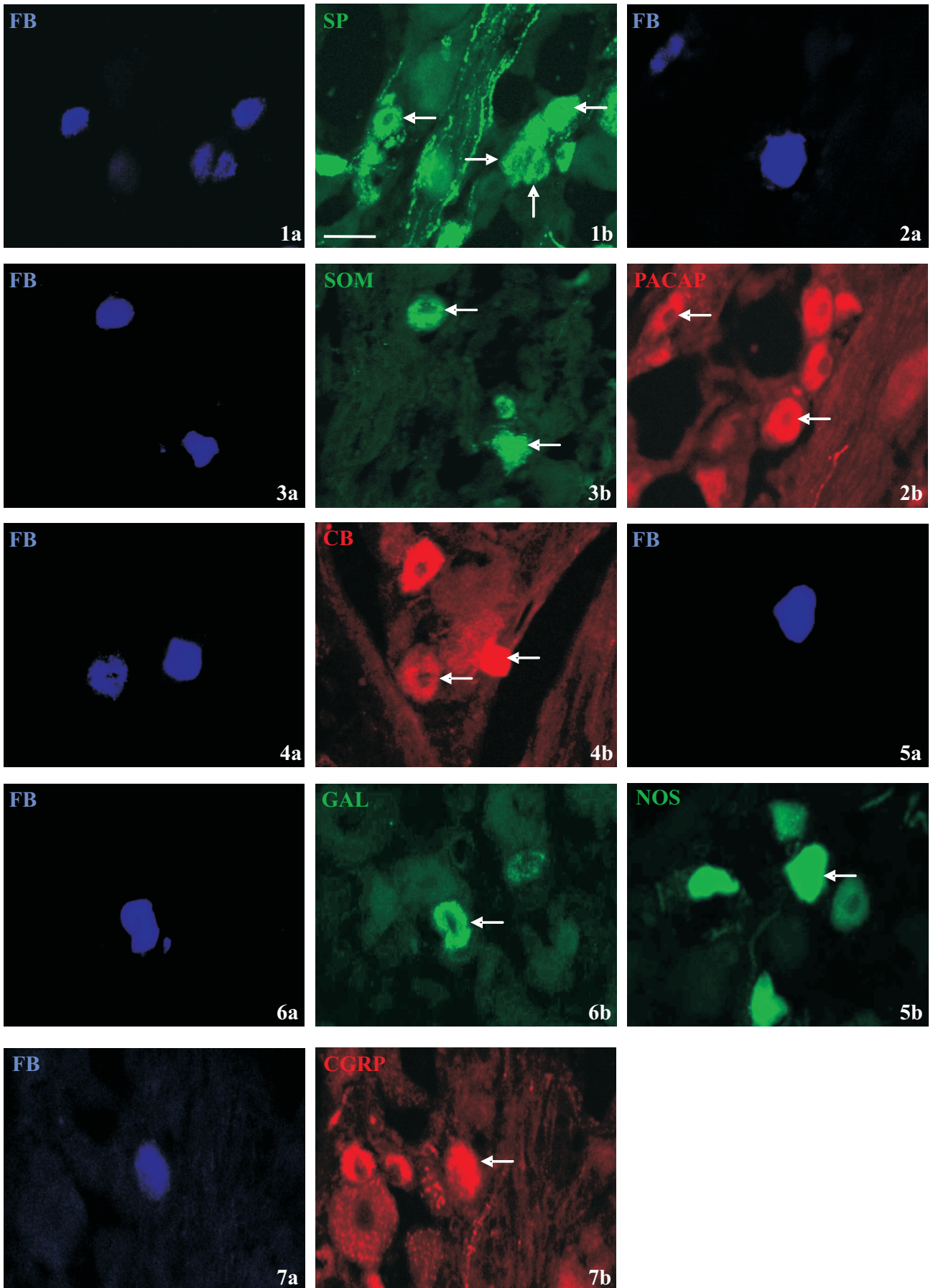
Retrograde labeled/double-immunostained perikarya were then evaluated under an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each fourth section (only neurons with a clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured 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<sup>+</sup> neurons immunolabeled for SP ( $20.3 \pm 1.2\%$  vs.  $45.2 \pm 4.4\%$  observed under physiological conditions; Bossowska et al. 2009) was found after CTG treatment, it should however be stressed that in comparison with previous data (Bossowska et al. 2009) such a decrease has been primarily observed in the sacro-coccygeal population of bladder-projecting neurons (Fig. 1b-arrows;  $17.5 \pm 1.5\%$  vs.  $46.2 \pm 6.0\%$ ) while in lumbar DRGs retrogradely labeled sensory cells responded to CTG treatment in a less pronounced manner ( $32.9 \pm 2.5\%$  vs.  $43.7 \pm 10.7\%$ ). Similarly to data obtained in the group of healthy animals (Bossowska et al. 2009), CTG-challenged SP-IR bladder sensory neurons belonged mainly to the class of small-sized perikarya (Fig. 1a;  $77.6 \pm 7.9\%$  vs.  $89.7 \pm 1.3\%$ ).

CTG bladder injections evoked a significant decrease in the number of FB-labeled PACAP-IR neurons ( $12.8 \pm 1.9\%$ ), when compared to the data 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. 2a;  $68.5 \pm 4.2\%$  vs.  $78.9 \pm 2.1\%$ ). In comparison to a previous report (Bossowska et al. 2009) CTG led to a distinct decrease only in the number of sacro-coccygeal subpopulations of PACAP-IR bladder cells (Fig. 2b-arrows;  $13.8 \pm 1.8\%$  vs.  $31.2 \pm 4.0\%$ ).

CTG treatment of the bladder led to a significant decrease in the number of FB<sup>+</sup> SOM-IR neurons ( $1.3 \pm 0.6\%$  vs.  $3.7 \pm 2.8\%$ , CTG-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. 3b-arrows); in similarity with 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 ( $2.5 \pm 0.9\%$  vs.  $6.1 \pm 4.6\%$  and  $0.4 \pm 0.9\%$  vs.  $0.3 \pm 0.3\%$ , CTG-treated vs. healthy animals, respectively). The majority of SOM-IR FB<sup>+</sup>



cells belonged to the class of small-sized perikarya ( $70.8 \pm 4.2\%$ ); however, in contrast to the animals under physiological conditions (Bossowska et al. 2009), a subpopulation of medium-sized FB<sup>+</sup> SOM-IR neurons (Fig. 3a;  $29.2 \pm 1.5\%$ ) were also found after CTG treatment.

The number of FB<sup>+</sup> neurons containing CB distinctly decreased ( $1.2 \pm 0.7\%$ ) after bladder injection of CTG, when compared to the relative frequency of such coded neurons in healthy animals ( $2.8 \pm 1.4\%$ ; Bossowska et al. 2009). In contrast to the data obtained under physiological conditions (Bossowska et al. 2009) the percentage of small-sized CB-IR neurons (Fig. 4a;  $81.5 \pm 3.1\%$ ) was higher than that of medium-sized ones ( $18.5 \pm 1.4\%$ ) and a significant increase, particularly in the number of small-sized neurons, was observed. In healthy animals the number of CB<sup>+</sup> sensory cells was very similar both in the lumbar and sacro-coccygeal subpopulation of FB-labeled neurons ( $3.1 \pm 2.5\%$  and  $3.5 \pm 1.2\%$ ; Bossowska et al. 2009) while CTG evoked a significant decrease in the number of sacro-coccygeal subpopulations of CB-IR bladder cells (Fig. 4b-arrows;  $2.1 \pm 1.4\%$ ) and the total depletion of such neurons belonging to the lumbar subpopulation of DRGs neurons.

A significant decrease in the number of nNOS-IR FB<sup>+</sup> neurons ( $0.9 \pm 0.3\%$  vs.  $5.8 \pm 2.5\%$  observed under physiological conditions; Bossowska et al. 2009) was found after CTG treatment, both in the lumbar and sacro-coccygeal subpopulation of bladder-projecting cells. In contrast to a previous report (Bossowska et al. 2009), immunoreactivity to nNOS was primarily found in small-sized retrogradely labeled DRG neurons (Fig. 5a;  $91.6 \pm 1.7\%$  vs.  $30.2 \pm 2.7\%$ ). It should be stressed that in comparison with healthy animals (Bossowska et al. 2009) CTG evoked a distinct decrease in the number of sacro-coccygeal subpopulations of nNOS-IR bladder cells (Fig. 5b-arrow;  $0.3 \pm 2.9\%$  vs.  $1.5 \pm 0.5\%$ ) and a total depletion of these cells in lumbar DRGs.

The number of GAL-containing FB<sup>+</sup> sensory neurons distinctly increased ( $38.2 \pm 0.6\%$ ) after bladder injection with CTG, when compared to the data obtained under physiological conditions ( $6.5 \pm 2.5\%$ ; Bossowska et al. 2009). The majority of GAL-IR bladder-projecting cells ( $89.3 \pm 3.6\%$ ) were small in diam-

eter; however, in contrast to the results obtained in the intact animals (Bossowska et al. 2009), a reliable population of medium-sized GAL-IR neurons was also observed after CTG treatment (Fig. 6a;  $10.7 \pm 3.6\%$ ). It has previously been found in healthy animals (Bossowska et al. 2009) that the number of GAL-IR FB<sup>+</sup> cells was distinctly higher in the lumbar ( $9.3 \pm 3.8\%$ ), than in the sacro-coccygeal subpopulation of bladder-projecting DRGs neurons ( $5.9 \pm 2.4\%$ ); a similar picture was observed after CTG administration ( $39.8 \pm 2.2\%$  vs.  $17.3 \pm 4.1\%$ , lumbar (Fig. 6b-arrow) vs. sacro-coccygeal neuronal subset, respectively). However, it should be stressed that a significant increase in the number of GAL-IR cells was observed in both these neuronal subpopulations in animals challenged by CTG injection.

There were no distinct changes in the number of CGRP-IR FB<sup>+</sup> neurons ( $32.9 \pm 8.3\%$ ) after CTG treatment, when compared to the results obtained under physiological conditions ( $36.1 \pm 4.7\%$ , Bossowska et al. 2009). In contrast to results obtained in intact animals (Bossowska et al. 2009), immunoreactivity to CGRP was overwhelmingly observed in small-sized sensory neurons (Fig. 7a;  $90.8 \pm 3.9\%$  vs.  $32.7 \pm 2.1\%$ ), while this neuropeptide was not found in large FB<sup>+</sup> cells after CTG treatment. In similarity with the healthy animal group (Bossowska et al. 2009) more FB<sup>+</sup>/CGRP<sup>+</sup> were found in lumbar ( $89.4 \pm 5.3\%$  vs.  $44.4 \pm 4.3\%$ ) than in sacro-coccygeal DRGs studied (Fig. 7b-arrow;  $35.6 \pm 5.6\%$  vs.  $23.5 \pm 5.9\%$ ) after CTG treatment.

## Discussion

In the present study CTG-induced a down-regulation in the expression of SP-, PACAP-, CB-, SOM- and nNOS-IR, and simultaneously an up-regulation of GAL immunolabelling was found in the porcine urinary bladder afferent neurons, while this neurotoxin did not evoke any distinct changes of CGRP expression in FB-labeled sensory cells. Moreover, for the first time we noted that expression of neurotransmitters in bladder afferent neurons could be down- or up-regulated differently, depending on the spinal cord segmental level at which these parental DRGs



Fig. 1. S4 DRG, ipsilateral to the site of FB injections. Four small-sized retrogradely traced bladder sensory neurons (a) containing SP-IR (b-arrows). Scale bar = 50  $\mu$ m, applies to all figures; Fig. 2. S3 DRG, contralateral to the site of FB injections. Two FB-labeled nerve cells of a small diameter (a) exhibiting PACAP-IR (b-arrows); Fig. 3. L6 DRG, contralateral to the site of FB injections. Two FB<sup>+</sup> afferent medium-sized neurons (a) immunolabeled for SOM (b-arrows); Fig. 4. Cq1 DRG, ipsilateral to the site of FB injections. Two sensory neurons of a small-diameter supplying the urinary bladder wall (a) containing CB (b-arrows); 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 L4 DRG. A medium-sized FB-positive neuron (a) immunolabeled for GAL (b-arrow); Fig. 7. Ipsilateral S3 DRG. A single, medium-sized FB-traced sensory neuron (a) containing CGRP-IR (b-arrow).

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 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 there were two distinct "sensory centers" found along the lumbo-sacro-coccygeal DRGs, it may be assumable that the functional interpretation of the alterations observed in the present study might be separately derived from the lumbar and sacro-coccygeal DRGs in which CTG-induced changes were observed.

It is well known that SP released from the bladder afferent nerves and sacral spinal cord is involved in the mechanoreceptor-mediated micturition reflex. In addition, SP observed in capsaicin-sensitive bladder afferent neurons may be involved in mediating urinary bladder hyperreflexia (Ahluwalia et al. 1994) but SP released within the bladder wall is known to trigger inflammatory responses (Chien et al. 2003). It has also been demonstrated that upon 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), it may be suggested that this neuropeptide could be involved as an excitatory neurotransmitter in several types of bladder reflexes (Mersdorf et al. 1992).

It has been shown in rats that the activation of NMDARs presenting at bladder sensory nerve terminals (Gonzalez-Cadavid et al. 2000) leads to release of SP (Ma et al. 2008). It is also known that CTG inhibits NMDARs activation (Layer et al. 2004) and exhibits potent antinociceptive properties by the reduction of neurotransmitter release from afferent nerve terminals (Marvizon et al. 2002). Thus, it may be possible that, in pigs, intravesical injections of CTG can lead to an inhibition of SP release from bladder sensory nerve terminals, causing a reduction of inflammatory responses; however, this suggestion has to be confirmed in other studies. NMDARs are also expressed on the presynaptic central terminals of afferent fibers in the dorsal horn of spinal cord and their activation directly facilitates SP release from these nerve fibers (Liu et al. 1997). In the present study, for the first time, we noted that the number of SP-IR bladder-projecting sensory neurons distinctly decreased in lumbar as well as in sacro-coccygeal DRGs after bladder injections of CTG. This may suggest that CTG can reduce the excitatory and nociceptive effect

of SP at the spinal level, not only by blockade of NMDARs activation resulting in an inhibition of SP release from primary central afferents (Marvizon et al. 2002), but also by reduction of SP expression in bladder sensory DRG neurons, which in turn, may cause an inhibition of bladder contractions and pain transmission.

Although the exact physiological function(s) of PACAP in the lower urinary tract are still unclear, Ishizuka and co-workers (1995) reported that PACAP may be involved in the facilitation of spontaneous bladder contractions in rats. It has also been shown that the expression of PACAP in DRG bladder cells increased after chronic cyclophosphamide-induced cystitis (Vizzard 2000). These results indicated that PACAP may be a principal component of bladder hyperreflexia, leading to an increase in the excitability of sensory neurons participating in the bladder reflex arc. Additionally, Oshawa et al. (2002) have postulated that PACAP released from primary afferent fibers potentiates nociceptive transmission to the dorsal horn by a direct interaction with NMDARs. Other studies have shown that upregulation of PACAP in the superficial layer of the dorsal horn increases NO formation by nNOS in neuropathic pain in mice, especially through NMDARs activation (Mabuchi et al. 2003). These results indicate that PACAP may also indirectly influence excitation and pain transmission in the dorsal horn by activation of NMDARs and increase of nNOS formation. Our observations have shown that in the pig the number of bladder sensory neurons containing PACAP distinctly decreased in sacro-coccygeal DRGs after bladder injections of CTG. Although the mechanism of CTG action on DRG neurons is not yet known, these results may suggest that this neurotoxin, down-regulating PACAP expression in bladder afferent cells, may lead to a diminishing of its release within the sacral spinal cord, which in turn, might reduce the excitatory effect of PACAP on the preganglionic neurons forming the parasympathetic nucleus and may cause an inhibition in bladder contractions and pain transmission.

After CTG treatment, a significant decrease in the number of SOM-IR bladder neurons was observed in the porcine lumbar DRGs studied. Previous studies have demonstrated that SOM is released into the spinal dorsal horn on peripheral nociceptive stimulation, and depresses the firing of dorsal horn neurons activated by noxious stimulation (Sandkuhler and Helmchen 1990). It has also been shown that SOM exerts an 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 a distinct decrease in the number of

SOM-IR neurons after challenging the bladder with CTG, remains obscure.

CB has been thought to act as a  $\text{Ca}^{2+}$  buffer, thus controlling the  $\text{Ca}^{2+}$  level within the cytoplasm of a neuronal cell. In DRG, CB has 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), which strongly suggests that CB may be involved in some aspects of pain transmission. Ng and co-authors (2004) have shown that NDMARs and CB are co-expressed in the ganglion neurons of the retina and that activation of NMDARs leads to an increase in CB expression in these neurons. In pigs the number of bladder sensory neurons containing CB distinctly decreased in lumbar as well as in sacro-coccygeal DRGs after bladder injections of CTG. This decrease of CB expression in bladder sensory neurons may be caused by CTG inhibitory action on NMDARs located on bladder afferent terminals, but this suggestion must be confirmed by other studies. These results may also suggest that a decrease in CB expression may probably lead to changes in CB-dependent  $\text{Ca}^{2+}$  homeostasis in affected neurons, which in turn, may result in a decrease in SP release rate from central afferent branches in the dorsal horn, suppressing pain transmission.

The results of our study demonstrate that bladder injections with CTG induced a significant decrease in the number of nNOS-containing, FB-labeled neurons, both in the lumbar and sacro-coccygeal subpopulation of neurons studied. Previous studies have suggested that NO plays a pivotal role in the facilitation of the micturition reflex evoked by noxious chemical irritation of the bladder and is also involved at the spinal level in the facilitation of the micturition reflex by nociceptive bladder afferents (Kakizaki and de Groat 1996). Furthermore, NO released from afferent nerve terminals of the urinary bladder (Birder et al. 2001) participates in the initiation of inflammatory responses and triggering of painful sensations (Aley et al. 1998). It is also known that increase of NO formation and its release in the superficial layers of the dorsal horn (Chen et al. 2008) depends on activation of NMDARs (Mabuchi et al. 2003). As NMDARs have been found in afferent terminals of the urinary bladder in rats (Gonzalez-Cadavid et al. 2000), one possible mechanism of antinociceptive and anti-inflammatory action of CTG may be an inhibition of NO release from bladder afferent terminals by blocking of NMDARs activation. As CTG produced a significant decrease in the number of nNOS-IR bladder sensory neurons in the pig, it may be possible that this neurotoxin also reduced the excitatory effect of NO at the spinal level, acting on the population of pregan-

glionic neurons located in the parasympathetic sacral nucleus, which in turn, may cause an inhibition of bladder contractions and pain transmission.

In the present study we noted that the number of GAL-expressing bladder-projecting sensory neurons distinctly increased in lumbar as well as in sacro-coccygeal DRGs after bladder injections of CTG. In all animal models of neuropathic pain, GAL was not only upregulated in DRG neurons, but an increase in its release has also been reported in superficial layers of the spinal dorsal horn, which in turn, leads to attenuation of pain transmission (Colvin et al. 1997). It has also been reported that GAL may counteract the action of PACAP and nNOS on bladder-projecting afferent cells (Zvarova et al. 2004) and it 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 CTG, increasing GAL expression in bladder-projecting sensory neurons, can lead to a diminishing of SP, CGRP, PACAP or nNOS release from afferent central terminals within the sacral spinal cord and to a reduction in the excitatory effect of the above-mentioned neurotransmitters on the preganglionic neurons forming the parasympathetic nucleus, which in turn, may lead to an inhibition of bladder contractions and pain transmission under pathological conditions.

Our experiments have shown that intravesical injections of CTG did not evoke any significant changes in the number of CGRP-IR bladder afferent neurons, either in the lumbar, or in sacro-coccygeal DRGs studied. The mechanism(s) of such resistance against CTG by CGRP-IR afferent neurons remains obscure.

In conclusion, the results of the present study have shown that CTG profoundly influenced the chemical coding of DRG cells supplying the porcine urinary bladder. This strongly suggests that CTG might be used in the treatment of hyperactivity of the afferent limb of reflex arcs responsible for transmission of sensory and pain information from the urinary bladder.

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