

# Alterations in porcine intrahepatic sympathetic nerves after bisphenol A administration

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## Abstract

**Introduction.** Bisphenol A (BPA) is used in the chemical industry for manufacturing plastics which are used as food packaging. Data indicate that BPA is released from such products and is widely present in the environment and the human body. So far, the EFSA and the US FDA have determined “safe” BPA oral exposure levels, and a large amount of data indicates that BPA is harmful even at low-doses. Our previously performed analyses concerning BPA exposure demonstrated the impact of this substance on parasympathetic and peptidergic nerve fibers present within the liver. Therefore, this study concerns BPA exposure and sympathetic intrahepatic innervation in reference to several neuropeptides which modulate neuronal responses: cocaine and amphetamine regulated transcript (CART), galanin (GAL), calcitonin gene-regulated peptide (CGRP) and substance P (SP). **Materials and methods.** Fifteen young swine at 8 weeks of age were used as experimental models of the juvenile human liver. The pigs were divided into 3 groups and received capsules orally with bisphenol at a dose of 0.05 mg/kg b.w./day; a dose of 0.5 mg/kg b.w./day and placebo capsules as a control. After 28 days of oral BPA intake, the animals were euthanized, perfused with 4% paraformaldehyde (PFA), and livers were collected and fixed in PFA. The cryostat sections were subjected to a routine double-labeling immunofluorescence technique. The primary antibodies were directed against dopamine beta hydroxylase (D $\beta$ H), which is a marker for sympathetic nerves, and one of the investigated neuropeptides: CART, GAL, CGRP and SP, which co-localized the investigated nerves. Immunoreactive nerves were counted in the liver and the percentage presence of each neuronal combination in particular samples of each experimental group were determined and analyzed statistically. **Results.** The BPA oral intake at low and ten times higher dosage caused an increase of the number of sympathetic nerve fibers within the porcine liver by 48.6% and 63.7%, respectively. Moreover, BPA exposure caused an increased presence of sympathetic nerve fibers in these two experimental groups, which were co-localized with CART and GAL up to 65.9%/173.2% and 147.4%/126.3%, respectively. At the lower BPA doses of 50  $\mu$ g/kg b.w./day, the percentages of SP+/D $\beta$ H+ and CGRP+/D $\beta$ H+ nerve fibers were similar to the control.

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However at a ten times higher dose, BPA caused an increased number of SP+/ D $\beta$ H+ and CGRP+/ D $\beta$ H+ nerve fibers in the liver, up to 46.4% and 73.5% respectively.

**Conclusions.** BPA caused an increase in the number of sympathetic nerve fibers as well as sympathetic nerve fibers which co-localized with neuropeptides in the porcine liver. The increase in CART and GAL were exceptionally high even at low BPA doses. BPA food contamination may dysregulate liver sympathetic innervation, and thereby may change the oxygenated blood supply, alter metabolism and disrupt the activity of hepatic parenchymal cells. (*Folia Histochemica et Cytobiologica* 2018, Vol. 56, No. 2, 113–121)

**Key words:** BPA; intrahepatic innervation; sympathetic nervous system; D $\beta$ H; CART; GAL; CGRP; SP

## Introduction

Bisphenol A (BPA) is an organic chemical compound with applications in the chemical industry for manufacturing many types of plastic which are mostly used as food packaging: household food containers, plastic water bottles and the lining of aluminum beverage cans [1]. A large amount of data indicates that BPA is released from plastic and polymer products because it is widely found in the environment: air, soil and water. Moreover BPA is also present in food, especially in cans, which most likely causes food exposure to BPA or its metabolites. This exposure has been reported in human serum, milk, urine and feces [2, 3].

BPA is considered to be an endocrine disrupting compound (EDC) because at the cellular level it acts as an agonist of  $\alpha$  and  $\beta$  subtype estrogen receptors. It is significantly (1000–2000-fold) weaker than 17 $\beta$  estradiol, but acts similarly to estrogens causing a migration of the cytoplasmic receptor domain and activation of the estrogen nuclear receptor [4]. Plastic or polymer products with a BPA component were originally deemed to be safe, since they exhibit low concentrations of BPA. The European Food Safety Authority as well as the US Food and Drug Administration have determined BPA safe oral exposure levels to be below 4  $\mu$ g/kg body weight/day and 5000  $\mu$ g/kg body weight/day, respectively [5, 6]. At the same time, the safety of the exposure level of this EDC has been extensively discussed and much data has shown that BPA is harmful even at low-doses [7, 8]. Experimental studies performed so far concerning BPA and its effect on the nervous system have mostly demonstrated its impact as disrupted sexual differentiation and memory impairments, as well as changes in behavior such as hyperactivity and aggressiveness [9]. Moreover, a large amount of data has shown that BPA may not only change physiological processes in adults, but also could be destructive and cause alterations in nervous system development with accompanying morphological changes. *In vitro*, BPA significantly impairs the proliferation of neural progenitor cells and at high-doses it is even cytotoxic to them [10].

The *in vivo* experiments have demonstrated that BPA disrupts enteric innervation within particular parts of the porcine small intestine [11, 12].

Our previously performed analyses demonstrated the influence of BPA exposure on intrahepatic innervation components. Significant changes and increases in the number of parasympathetic and peptidergic nerve fibers present within the liver have been correlated with high BPA dosage levels [13, 14]. To complete the series of experimental studies concerning BPA and intrahepatic innervation, we decided to assess the potential changes of the sympathetic part of the autonomic nervous system with reference to several neuropeptides which modulate the neuronal response: cocaine- and amphetamine-regulated transcript (CART), galanin (GAL), calcitonin gene-regulated peptide (CGRP) and substance P (SP).

The assumption was made that experimental oral BPA exposure is similar to food BPA intake and could simulate BPA contamination in children and adolescents. The study describes the changes of intrahepatic innervation, because the small intestine digests food material and absorbs molecules which are transported directly to the liver through the hepatic portal vein.

As in previous analyses, we used young swine at the age of 8 weeks as a potential model of the juvenile human liver. The anatomical and physiological similarity between swine and human make the porcine liver one of the most useful experimental models for toxicological and pathophysiological studies [15, 16].

Using an innervated hepatic tissue to test ingested BPA exposure is reasonable since the liver is the first major organ exposed to dietary toxins. By testing alterations in the expression of nerve fibers in the liver, some suggestions concerning the impact of the above-mentioned alterations on liver function may be proposed. In this study we used an immunofluorescence technique to demonstrate the presence of dopamine beta hydroxylase (D $\beta$ H), a marker specific for sympathetic nerve fibers, and four neuropeptides (CART, GAL, CGRP and SP) which can co-localize with D $\beta$ H in the liver.

**Table 1.** Primary and secondary antibodies used in this study

PRIMARY ANTIBODIES				
Antisera	Code	Host species	Dilution	Supplier
D $\beta$ H	MAB 308	mouse	1:1000	Chemicon International Inc, UK; <a href="http://www.chemicon.com">www.chemicon.com</a>
CART	H-003-61	rabbit	1:22000	Phoenix Europe <a href="http://www.phoenixpeptide.com">www.phoenixpeptide.com</a>
GAL	RIN7153	rabbit	1:10000	Peninsula Labs, US; see Bachem AG; <a href="http://www.bachem.com">www.bachem.com</a>
CGRP	11189	rabbit	1:10000	MP Biomedicals; <a href="http://www.mpbio.com">www.mpbio.com</a>
SP	8450-0505	rabbit	1:10000	Biogenesis Inc, <a href="http://www.biogenesis.co.uk">www.biogenesis.co.uk</a>
SECONDARY ANTIBODIES				
			Dilution	Supplier
Donkey anti-mouse IgG (H+L) conjugated with FITC			1:800	715-095-151; Jackson IR Lab, US; <a href="http://ww.jacksonimmuno.com">ww.jacksonimmuno.com</a>
Biotinylated goat anti-rabbit immunoglobulins			1:1000	E0432, DAKO Corporation, US, <a href="http://www.dakousa.com">www.dakousa.com</a>
Biotin conjugated F(ab)' fragment of affinity Purified anti-rabbit IgG (H+L)			1:1000	711-1622, BioTrend, Germany; <a href="http://www.biotrend.com">www.biotrend.com</a>
CY3- conjugated Streptavidin			1:9000	016-160-084, Jackson IR Lab, US; <a href="http://www.jacksonimmuno.com">www.jacksonimmuno.com</a>

Abbreviations: CART — cocaine- and amphetamine-regulated transcript; CGRP — calcitonin gene- regulated peptide; D $\beta$ H — dopamine beta-hydroxylase; FITC — fluorescein isothiocyanate; GAL — galanin; SP — substance P. CY3- conjugated Streptavidin was used for detecting biotinylated antibodies (Cy3 excitation at 550 nm induces maximal emission at 570 nm).

## Materials and methods

**Animals and design of experiment.** The study was performed on 15 Large White Polish juvenile female pigs at the initial age of 8 weeks and about 18–20 kg body weight (b.w.). Pigs were kept under typical laboratory conditions adapted for this animal species at the Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn (Poland). The experiment was performed according to the instructions of the Local Ethical Committee for Experiments on Animals in Olsztyn, decision number (28/2013).

After a 3-day adaptive period, the pigs were randomly divided into three experimental groups and received bisphenol A (BPA) (cat. no. 239658, SigmaAldrich, St. Louis, MO, USA) or placebo in gelatin capsules for 28 days of feeding.

- 1) Control group — placebo (empty gelatin capsules);
- 2) Experimental group I — capsules with BPA at a dose of 0.05 mg/kg b.w./day;
- 3) Experimental group II — capsules with BPA at a dose 10 times higher — 0.5 mg/kg b.w./day.

All animals were weighed in order to determine their body weight and calculate the proper dosage of BPA every four days before the morning feeding.

**Tissue sampling.** After 28 days of oral BPA intake, the animals were premedicated with azaperone (Stressnil, Janssen Pharmaceutica, Bruxelles, Belgium; 2.0 mg/kg body weight, *i.m.*). After about 30 min. the animals were euthanized using an overdose of sodium thiopental intravenously

(50 mg/kg b.w.; Sandoz, Kundl-Rakúsko, Austria) and perfused transcardially with 4% buffered paraformaldehyde (PFA). Tissues were collected from all animals. Sections of liver tissue were fixed in the same PFA solution, rinsed in phosphate buffered saline (PBS, pH 7.4) for three days and kept in 18% sucrose at 4 °C. After two weeks, the tissue samples were frozen at –23°C and cut into 10  $\mu$ m-thick sections using a microtome (Microm, HM 525, Walldorf, Germany).

### Immunofluorescent labeling and histomorphometry.

The sections were subjected to a routine double-labeling immunofluorescence technique according to the method described previously by Wojtkiewicz *et al.* [16]. In short, overnight incubation was performed at 37°C with a mixture of two primary antibodies directed towards the dopamine beta hydroxylase (D $\beta$ H) marker for sympathetic nerves and one of the studied neuropeptides: CART, GAL, CGRP or SP. The specification of primary and secondary antibodies which were used in the present study is presented in Table 1. Afterwards, the samples were incubated for 1 h (37°C) with species-specific antisera conjugated to FITC or biotin, which was visualized by streptavidin-CY3.

During the present investigation, the standard controls for the specificity of “primary” antibodies were performed. These included pre-absorption of the particular antisera with appropriate antigens, as well as “omission” and “replacement” tests that completely eliminated immunofluorescent signals.

Initially, the determination of the average number of D $\beta$ H-positive nerve fibers per microscopic field of view

(0.1 mm<sup>2</sup>) was made. Nerve fibers were counted in five sections per animal (in five view fields per section). Subsequently, the number of D $\beta$ H-LI nerve fibers which were immunoreactive with the other studied substances was counted. 300 D $\beta$ H immunoreactive nerve fibers in particular sections from each animal were counted for the presence of co-localized neuropeptides, and these numbers were considered to represent 100% for each combination. This was performed in order to determine what percentages of D $\beta$ H-positive nerve fibers were immunoreactive with CART, GAL, CGRP or SP.

The measurements of the number of immunoreactive nerve fibers were performed by two investigators. Double-labeled nerve fibers were visualized under an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with epi-fluorescence and appropriate filter sets. To prevent double counting of the same nerve fibers, the evaluated sections were located at least 100  $\mu$ m apart.

**Statistical analysis.** The obtained results were pooled and presented as mean  $\pm$  SEM. Statistical analysis was performed using Student's t test (Graphpad Prism v. 6.0; GraphPad Software Inc., San Diego, CA, USA), and the differences were considered statistically significant at  $p \leq 0.05$ .

## Results

### *BPA caused an increase of sympathetic nerve density within the liver*

In comparison to the control group, there was a significant increase in the number of observed sympathetic nerves (D $\beta$ H+), analyzed in the viewfield area, for the group of animals which were fed a BPA oral exposure at 0.05 mg/kg b.w./day, and the group which was fed 0.5 mg/kg b.w./day; 48.6% and 63.7% respectively (Table 2, Fig. 1).

***BPA caused an increased presence of sympathetic nerves co-localized with the analyzed neuropeptides*** CART/D $\beta$ H immunoreactive nerve fibers, at an initial BPA oral exposure of 0.05 mg/kg b.w./day, were 65.9% more abundant than in the control, and statistically significant (Table 2, Fig. 1). At an exposure ten times higher, the CART/D $\beta$ H immunoreactive nerve presence was 173.2% higher, in comparison to the control.

Furthermore at the low and high BPA dosage, the percentage of GAL/D $\beta$ H immunoreactive nerve fibers was higher (147.4% and 126.3%, respectively) than in the control group (Table 2, Fig. 2).

At a BPA oral intake of 0.05 mg/kg b.w./day, the percentages of SP+/D $\beta$ H+ and CGRP+/D $\beta$ H+ nerve fibers were similar to the control. However at a ten times higher exposure level, the expression of SP+/D $\beta$ H+ was 46.4% and CGRP+/D $\beta$ H+ was 73.5% higher than in the control group (Table 2, Fig. 3, 4, statistically significant,  $p \leq 0.05$ ).

The results reported above are presented in Table 2 and representative images of the immunocytochemical co-localization of D $\beta$ H with the chosen neuronal markers are shown in Figures 1–4.

## Discussion

Anatomical studies have described that porcine liver in the portal area and in the parenchyma is innervated by abundant nerve fibers of the autonomic nervous system [18]. In the portal triad, the sympathetic nerves innervate the interlobular branches of the hepatic artery, portal vein and the bile duct. In the hepatic parenchyma, the sympathetic nerve fibers run from the portal area, and their terminal is in close vicinity to hepatocytes as well as other cells located in this area [18]. Comparison among higher mammalian species such as pig, dog, monkey and human demonstrate a high anatomic resemblance in the hepatic innervation between them [18, 19].

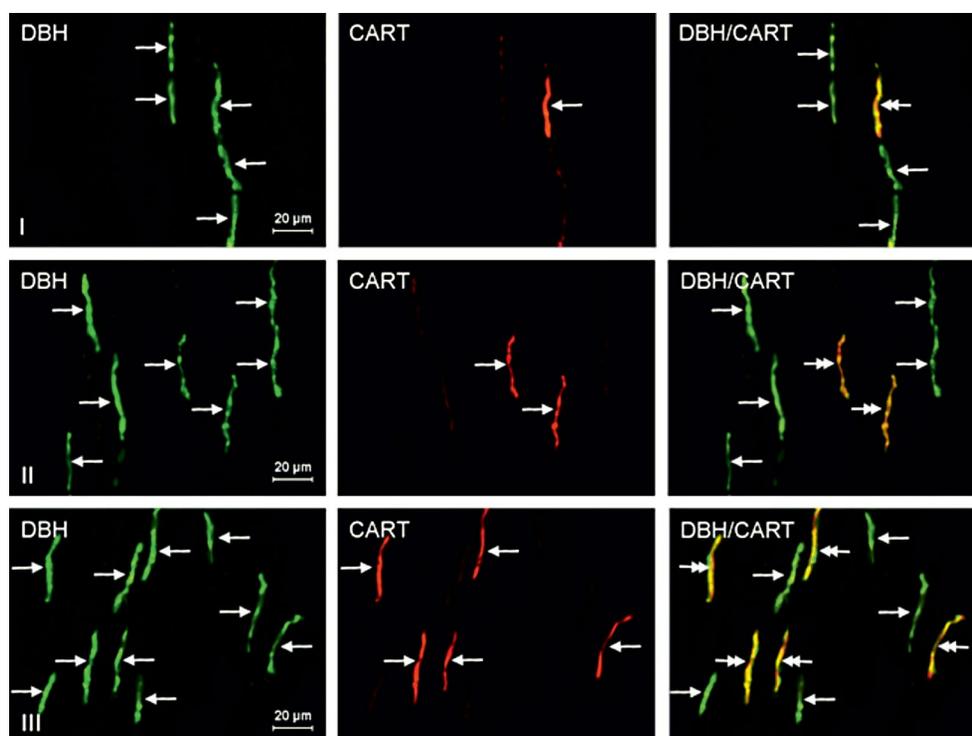
The results of this study show an increase in the presence of D $\beta$ H immunoreactive nerve fibers and this is comparable to our previous analyses which have also demonstrated an overexpression of parasympathetic and peptidnergic nerves fibers within porcine liver after BPA oral intake [13, 14]. Such results suggest that BPA food contamination may disrupt proper innervation and balance between main components of the autonomic nervous system within liver. It could be assumed that sympathetic nerve fiber overexpression may cause improper blood flow within the portal area due to potential constriction of the interlobular artery. Similarly, within hepatic parenchyma such an increase of innervation may impair blood supply within the sinusoidal area and also could have an impact on hepatocyte metabolism; the activity of Kupffer cells and hepatic stellate cells [20]. Sympathetic regulation within the parenchyma of hepatic lobules is not only involved with the regulation of liver physiology, but it could also be related with several pathophysiological processes such as liver repair and regeneration [21].

Our results also show an increase in D $\beta$ H nerves which were co-localized with SP and CGRP neuropeptides in response to BPA exposure in a dose related manner. It is known that SP- and CGRP-immunoreactive (IR) nerve fibers have an afferent course to the spinal sensory ganglia and have sensory function in the alimentary tract and its glands [22, 23]. Comparative anatomical studies concerning SP nerve fibers have been conducted in many species including human but not in porcine. These studies have demonstrated numerous SP immunoreactive nerve fibers, which are present around the interlobular veins and arteries, with a lack of such innervation around the interlobular

**Table 2.** Neurochemical characteristics and changes in neuropeptide expression in porcine intrahepatic sympathetic nerves under physiological conditions and after bisphenol A (BPA) administration. Nerve fibers were counted in five sections per animal (in five view fields per section). \*These results are statistically significant at  $p \leq 0.05$

Neurochemical characteristic of fibers	Experimental group		
	Control	Low-dose BPA	High-dose BPA
D $\beta$ H <sup>+</sup>	21.2 $\pm$ 2.08	31.5 $\pm$ 1.85*	34.7 $\pm$ 1.61*
D $\beta$ H <sup>+</sup> /CART <sup>+</sup>	8.2 $\pm$ 1.65	13.6 $\pm$ 1.57*	22.4 $\pm$ 3.26*
D $\beta$ H <sup>+</sup> /GAL <sup>+</sup>	3.8 $\pm$ 0.96	9.4 $\pm$ 2.01*	8.6 $\pm$ 1.57*
D $\beta$ H <sup>+</sup> /CGRP <sup>+</sup>	9.8 $\pm$ 1.59	10.6 $\pm$ 1.77	17.0 $\pm$ 2.81*
D $\beta$ H <sup>+</sup> /SP <sup>+</sup>	5.6 $\pm$ 1.63	6.2 $\pm$ 1.88	8.2 $\pm$ 1.98*

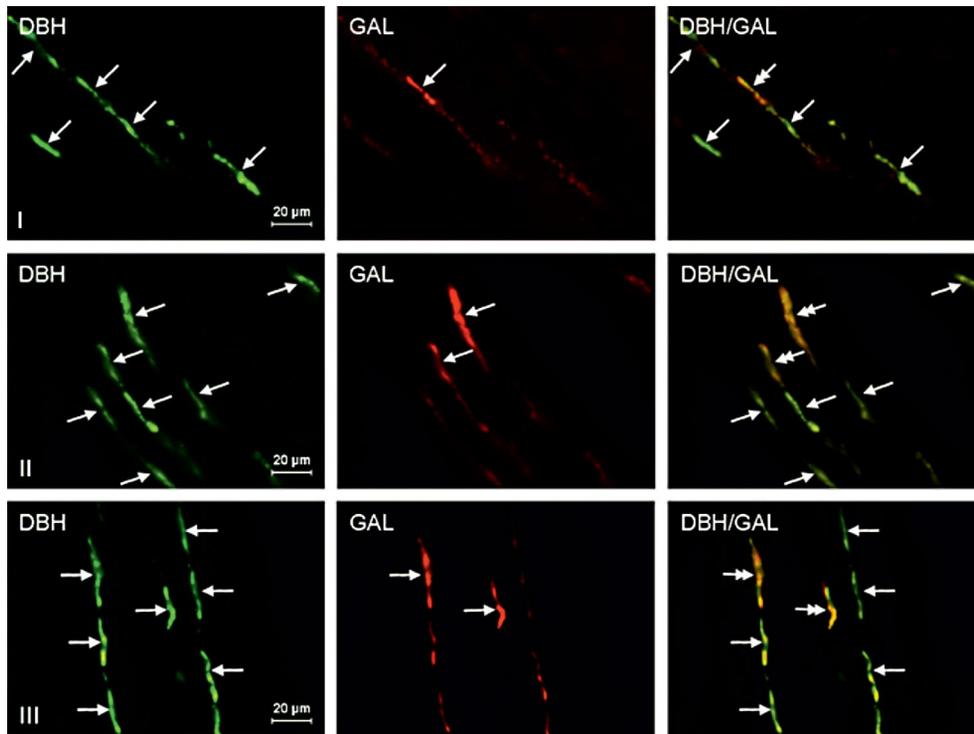
Abbreviations: BPA — bisphenol A; C — control animals; E1 — low-dose BPA group, 0.05 mg/kg b.w./day for 28 days); E2 — high dose BPA group (0.5 mg/kg b.w./ day for 28 days)



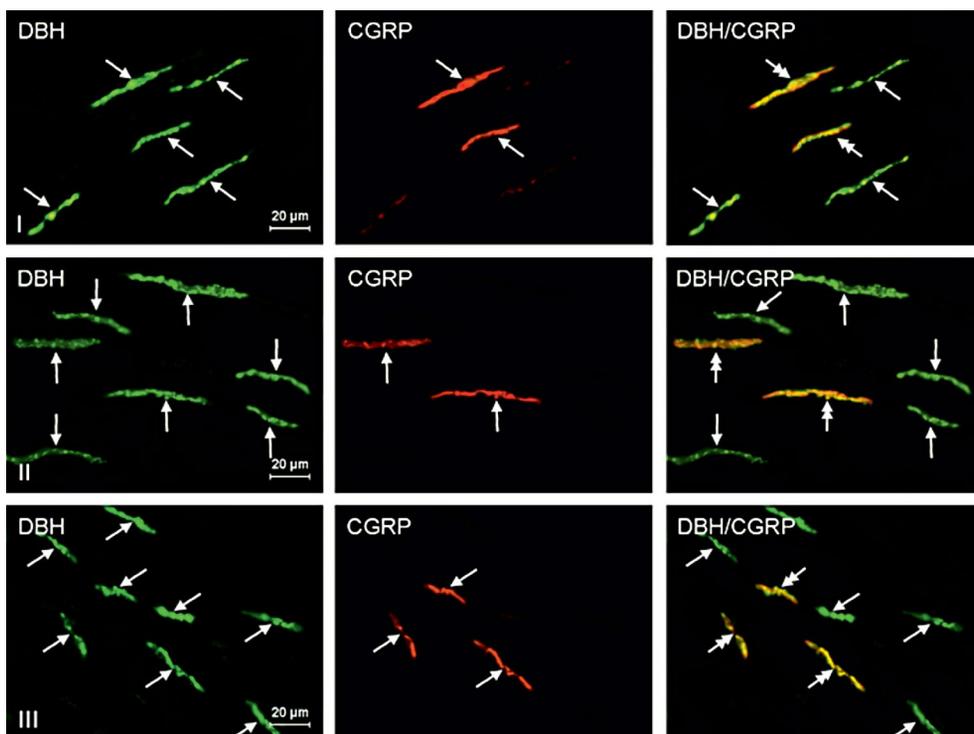
**Figure 1.** Representative images of immunofluorescent detection of D $\beta$ H-, and CART-immunoreactive nerve fibers in the livers of control pigs (I) or pigs exposed to low (II) or high doses (III) of bisphenol A, as described in Methods. D $\beta$ H- or CART-immunoreactive (IR) nerve fibers are indicated with arrows, D $\beta$ H- and CART-IR nerve fibers are indicated with double-headed arrows. Scale bars in all figures: 20  $\mu$ m.

bile duct. Furthermore, SP-IR nerve fibers are also observed in the hepatic parenchyma. So far, these have been classified as peptidergic nerve fibers [19]. Our previous studies, as well as the study presented above, have shown that SP neuropeptide within the porcine liver is colocalized with parasympathetic and sympathetic nerve fibers as well as nerve fibers alone [13, 14]. Other data have demonstrated that SP-IR nerves fibers could cause vasodilation and modulate microcirculation in hepatic sinusoids [20].

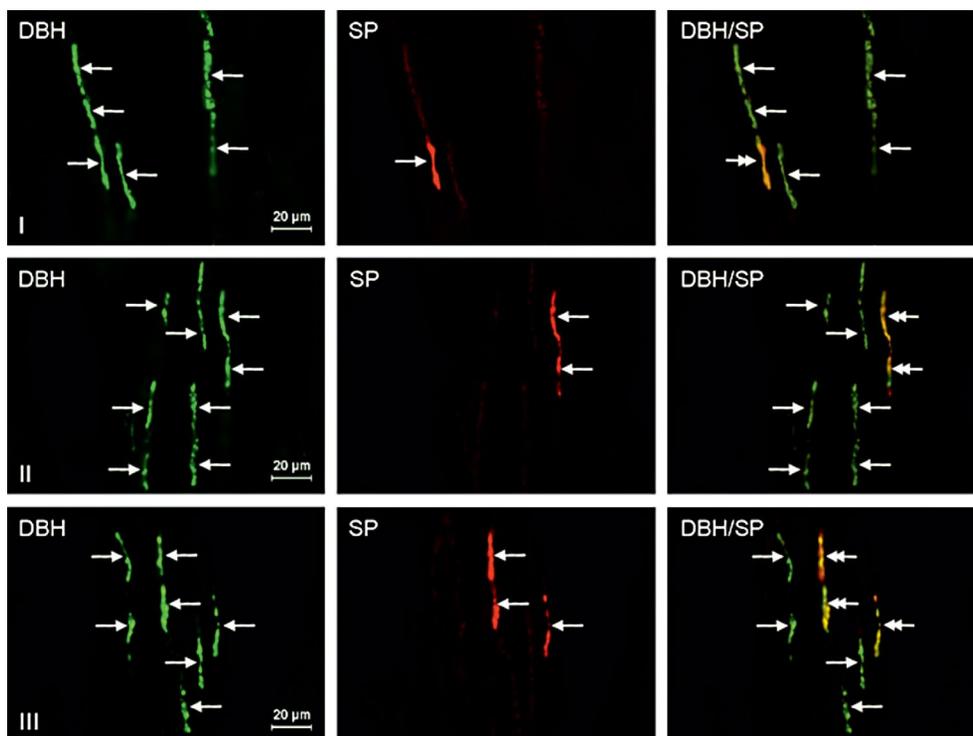
The CGRP-IR nerve fibers innervate the interlobular artery and bile duct; however, they are not present in the intralobular region of animal and human liver [19]. Other data concerned with skin innervation and internal microcirculation demonstrate that SP and CGRP are simultaneously released from sensory fibers and could cause short- and long-term vasodilation and plasma extravasation [24]. On the basis of the results of our study, it might be suspected that dose related BPA exposure changes the normal pattern of hepatic



**Figure 2.** Representative images of immunofluorescent detection of D $\beta$ H- and GAL-immunoreactive nerve fibers in the livers of control pigs (I) or pigs exposed to low (II) or high doses (III) of bisphenol A, as described in Methods. D $\beta$ H- or GAL-immunoreactive (IR) nerve fibers are indicated with arrows, D $\beta$ H- and GAL-IR nerve fibers are indicated with double-headed arrows. Scale bars in all figures: 20  $\mu$ m.



**Figure 3.** Representative images of immunofluorescent detection of D $\beta$ H- and CGRP-immunoreactive nerve fibers in the livers of control pigs (I) or pigs exposed to low (II) or high doses (III) of bisphenol A, as described in Methods. D $\beta$ H- or CGRP-immunoreactive (IR) nerve fibers are indicated with arrows, D $\beta$ H- and CGRP-IR nerve fibers are indicated with double-headed arrows. Scale bars in all figures: 20  $\mu$ m.



**Figure 4.** Representative images of immunofluorescent detection of D $\beta$ H- and SP-immunoreactive nerve fibers in the livers of control pigs (I) or pigs exposed to low (II) or high doses (III) of bisphenol A, as described in Methods. D $\beta$ H- or SP-immunoreactive (IR) nerve fibers are indicated with arrows, D $\beta$ H- and SP-IR nerve fibers are indicated with double-headed arrows. Scale bars in all figures: 20  $\mu$ m.

innervation, which could disturb blood and bile flow in the portal area and microcirculation within hepatic sinusoids.

So far, BPA is considered to be an endocrine disrupting compound which reacts with nuclear estrogen receptor [4]. Moreover, it has also been described that BPA could react with membrane epithelial growth factor receptor (EGFR) and via ERK1/2 and AKT signaling pathways. This could activate cellular growth and cell proliferation; especially in cancer cells [2]. Nevertheless, there is a lack of experimental data which could directly demonstrate how BPA could impair the components of the peripheral nervous system. There is much data concerned with brain physiology and the impact of BPA exposure which causes disruptive patterns of behavior [9].

Another result of our study clearly demonstrates the presence of sympathetic nerves which are co-localized with galanin in porcine liver. The distribution of such nerves in this organ has not been clearly evidenced, so far. Our results show an increase in the density of immunoreactive D $\beta$ H<sup>+</sup>/GAL<sup>+</sup> nerve fibers, and this alteration was visible even at low BPA doses. Data from the literature indicate that current *in-vitro* studies are mostly concerned with different hormone

dependent cells but not with hepatocytic cell cultures. These experiments demonstrate the impact of BPA on estrogen receptor up-regulation which causes cell activation and cell proliferation even at very low BPA concentrations [8]. Other data show galanin co-localization in sympathetic nerve fibers, but this was performed in dog liver. Most likely, galanin in these nerves acts as a neuromodulator and has a potential role in hepatic glucose release [25, 26].

The results of our study also demonstrate a significant increase in the presence of sympathetic nerve fibers which were co-localized with CART within the liver. Although CART is widely present within the enteric innervation of the alimentary tract, the exact function of this neuropeptide still seems to be unclear. It is possible that CART within the enteric neurons fulfils a neuroprotective role [27]. However, the widespread presence of CART in intestinal innervation more likely suggests that CART could be a modulator of motility and secretion within the alimentary tract [28, 29]. It should be emphasized that CART co-expression within sympathetic nerve fibers in mammalian liver has been demonstrated for the first time. Furthermore, a distinct increase of CART immunoreactive nerve fibers caused by BPA

exposure even at low doses is worth underlining. It is known that CART is not only a neurotransmitter in the central nervous or autonomic nervous system, but CART most likely plays a role as a hormone, because this presence has been documented in the endocrine cells of the adrenal medulla and the C-cells of the thyroid gland [30].

In summary, it should be highlighted that each neuropeptide that was studied demonstrated increased levels of expression within the sympathetic nerves of hepatic tissue. The increase of the density of CART- and GAL-IR sympathetic nerve fibers was high even at low BPA doses.  $D\beta H$  expression was also increased, which indicates an over-activation of the sympathetic nervous system. Compiling the results of our studies with previously performed analyses concerning BPA exposure and the parasympathetic and peptidergic parts of liver innervation [13, 14]; we come to the conclusion that BPA causes a general dysregulation of nerve fibers. This evidence indicates that BPA exposure even at recommended safe levels is still far too high. Furthermore, it is hoped that this study may be an early step in the determination of linking BPA exposure to pathological disease states.

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