SAGE-Hindawi Access to Research
International Journal of Hypertension
Volume 2011, Article ID 216464, 9 pages
doi:10.4061/2011/216464

Research Article

Modulation of Tyrosine Hydroxylase, Neuropeptide Y, Glutamate, and Substance P in Ganglia and Brain Areas Involved in Cardiovascular Control after Chronic Exposure to Nicotine

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Received 11 February 2011; Revised 3 June 2011; Accepted 14 June 2011

Academic Editor: Thomas Unger

Considering that nicotine instantly interacts with central and peripheral nervous systems promoting cardiovascular effects after tobacco smoking, we evaluated the modulation of glutamate, tyrosine hydroxylase (TH), neuropeptide Y (NPY), and substance P (SP) in nodose/petrosal and superior cervical ganglia, as well as TH and NPY in nucleus tractus solitarii (NTS) and hypothalamic paraventricular nucleus (PVN) of normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) after 8 weeks of nicotine exposure. Immunohistochemical and *in situ* hybridization data demonstrated increased expression of TH in brain and ganglia related to blood pressure control, preferentially in SHR, after nicotine exposure. The alkaloid also increased NPY immunoreactivity in ganglia, NTS, and PVN of SHR, in spite of decreasing its receptor (NPY1R) binding in NTS of both strains. Nicotine increased SP and glutamate in ganglia. In summary, nicotine positively modulated the studied variables in ganglia while its central effects were mainly constrained to SHR.

1. Introduction

Cardiovascular effects of tobacco smoking are primarily attributed to the presence of nicotine in cigarettes. This alkaloid may promote decrease in baroreflex sensitivity, increase in heart rate and blood pressure, atherosclerosis, coronary heart disease, and myocardial infarction [1].

Nicotine potentiates sympathetic nervous system leading to increase in plasma and brain catecholamine levels [2, 3]. In addition, we have previously demonstrated the acceleration of onset and the exacerbation of hypertension in genetic hypertension predisposed rats after nicotine exposure [4].

The activation of sympathetic neurotransmission by nicotine may be based on its direct effect on the central nervous system, on sympathetic ganglia to increase the efferent nerve activity, and/or on peripheral sympathetic nerve endings and adrenal medulla stimulating catecholamine release [5]. Nevertheless, the activated sympathetic system might promote the reflex parasympathetic response composing an elaborated physiological effect after nicotine administration.

The alkaloid instantly interacts with the central nervous system binding to nicotinic acetylcholine receptors in the hypothalamus, hippocampus, midbrain, and medulla oblongata [6, 7] modulating norepinephrine, dopamine, vasopressin, glutamate, neuropeptide Y (NPY), and other neurotransmitter systems [8].

Nicotine acts also on chemoreceptors afferents [9], enteric nervous system [10], and visceral sensory afferents (for a review about nicotinic mechanisms in the autonomic control of organ systems refer to De Biasi [11]).

Peripheral areas involved in cardiovascular control are also under the influence of nicotine, such as the nodose/petrosal ganglia [12]. It is well documented the presence

of several neurotransmitters such as NPY, substance P (SP), catecholamines, and glutamate in nodose and petrosal ganglia, which are related to baroreflex [13, 14]. Furthermore, baroreflex afferents to nucleus tractus solitarii (NTS) contain glutamate as the main neurotransmitter involved in cardiovascular control, which may be regulated by several other neurotransmitters such as catecholamines, NPY. and SP.

Superior cervical ganglion is part of the sympathetic chain located next to carotid bifurcation that participate in generating the vasomotor tonus of head and neck [15]. The study of superior cervical ganglion is useful to evaluate the function of sympathetic autonomic nervous system. It is well established that the presence of the enzyme tyrosine hydroxylase (TH) in this ganglion [16], which participates in catecholamines synthesis, as well as NPY and SP immunoreactive profiles and nicotinic acetylcholine receptors [17] that may influence superior cervical ganglion activity [18].

In view of this, the aim of the present study is to evaluate the modulation of glutamate, TH, NPY, and SP in nodose/petrosal and superior cervical ganglia and as well as TH and NPY in nucleus tractus solitarii and hypothalamic paraventricular nucleus of normotensive and spontaneously hypertensive rats after prolonged nicotine exposure delivered through subcutaneously implanted pellets.

2. Material and Methods

All the procedures and protocols were performed in accordance with institutional and international guidelines for animal care and use [19].

2.1. Animals and Nicotine Exposure. Male normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR), 1 month old, from the Institute of Biosciences, University of São Paulo (São Paulo, Brazil), had either nicotine or placebo pellets (Innovative Research of America, Ohio) implanted subcutaneously during 8 weeks. Initially, 1-month-old rats had 10 mg/21 days pellets implanted in the lateral side of their neck, which were replaced for pellets of 100 mg/60 days until the end of treatment. Pellets released nicotine continuously at the average rate of 8 $mg \cdot kg^{-1} \cdot day^{-1}$ along treatment [20]. It was previously demonstrated that exposure to this concentration of nicotine accelerated and intensified the hypertension in SHR although the alkaloid promoted no cardiovascular effects in WKY rats [4].

Six rats of each strain and treatment were submitted either to transcardiac perfusion for immunohistochemistry, or decapitation for binding analysis and *in situ* hybridization. Brain, nodose/petrosal and cervical superior ganglia were excised to the analysis of glutamate, tyrosine hydroxylase, neuropeptide Y, and substance P.

2.2. Immunohistochemistry. Rats were deeply anaesthetized and perfused with 50 mL of saline (0.9%) followed by 300 mL of a fixative solution consisted of 4% paraformaldehyde (w/v) and 0.2% picric acid (v/v) in 0.1 mol/L PBS, pH 6.9, at 4°C.

Brain sections (14 μm) were obtained in a cryostat (Leica, CM 3050, Germany) at rostrocaudal levels from −13.68 to −14.08 mm for the analysis of the NTS and at −1.80 to −2.12 mm to access the PVN according to the atlas of Paxinos and Watson [21]. Nodose/petrosal and superior cervical ganglia were sectioned longitudinally.

Immunoreactivity was detected by the avidin-biotin peroxidase technique. Sections were incubated for 24 hours at 4°C with rabbit polyclonal antisera against either glutamate (1 : 2000, Sigma), TH (1 : 400, Eugene Tech), NPY (1 : 2000, Sigma), or SP (1 : 1000, Peninsula) followed by incubation with a biotinylated antirabbit immunoglobulin (1 : 230, Vector, USA) for 90 min. Sections were next incubated with an avidin-biotin peroxidase complex (1 : 120, Vectastain, Vector, USA) for 45 min. Immunoreactivity was visualized using 3-3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen and H_2O_2 (0.05%) after 6 minutes of reaction.

Immunoreactivities were analyzed by semiquantitative microdensitometry, expressed as spMGV (specific mean gray value), using a KS 400 image analyzer (Zeiss, Germany) linked to a CCD 72 camera (Dage; MTI, Michigan City, Ind., USA) mounted on a Zeiss microscope (40x objective), as previously described by Zoli and coworkers [22]. The value for each animal was obtained by taking the mean of two adjacent sections of each analyzed region.

2.3. In Situ Hybridization. Brain sections were obtained as specified for immunohistochemistry and thaw-mounted onto poly-L-lysine coated slides that were postfixed with cold 4% parafolmaldehyde in phosphate buffered saline (PBS) solution, dehydrated in ethanol and processed for hybridization procedures.

Probes complementary to nucleotides 1351–1398 of TH mRNA were employed for the detection of this mRNA [23] and NPY was labeled with a probe corresponding to nucleotides 3146–3194 of rat NPY mRNA [24].

Probes were synthesized by Life Technologies, labeled with (35S)dATP using terminal deoxynucleotidyl transferase (Roche, Germany) at the 3′-end, and purified according to the previously described protocol [25]. Sections were incubated overnight at 42°C in a humidified chamber with the labeled probe (500000 dpm/50 μL/section) in a hybridization buffer [25]. Slides containing the sections were extensively rinsed in saline-sodium-citrate (SSC), dehydrated in ethanol and exposed to a radioactivity sensitive film (Kodak Biomax MR, Kodak, USA) for four weeks. For control experiments, radioactive probes were mixed with an excess (100x) of nonradioactive probe.

Films were analyzed using a computer-assisted image analyzer and software developed by Imaging Research (Brock University, Canada).

2.4. Receptor Binding Analysis. NPY1R were evaluated in the NTS and PVN through binding assay performed in the same group of rat brains used for in situ hybridization.

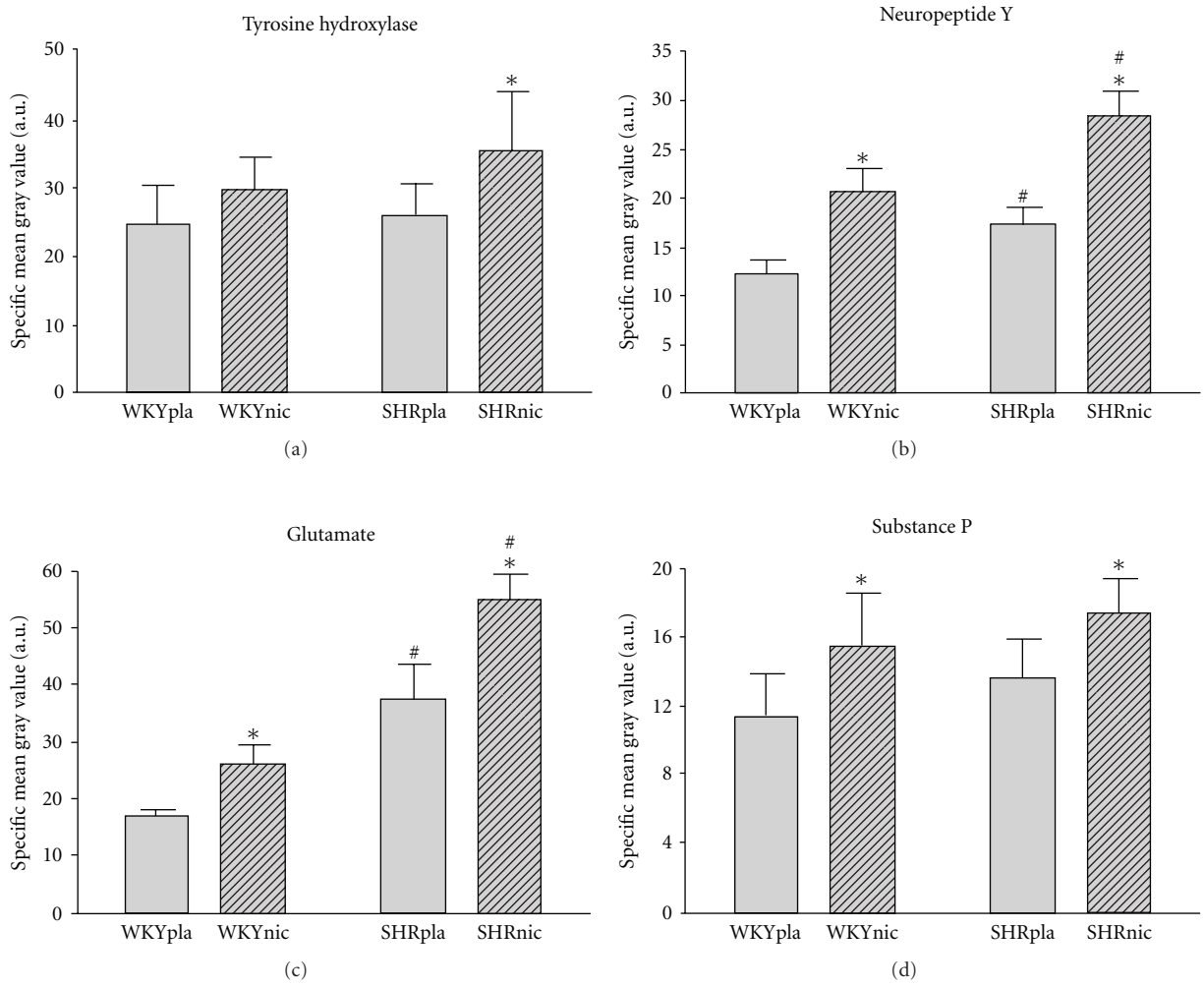

Figure 1: Immunoreactivity of tyrosine hydroxylase, neuropeptide Y, glutamate, and substance P in nodose/petrosal ganglia of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats exposed either to nicotine or placebo delivered through subcutaneously implanted pellets during 8 weeks. Values are presented as mean ± SD, $^{*}P < 0.05$ as compared to the same strain placebo treated rat; $^{\#}P < 0.05$ as compared to WKY on the same experimental condition, according to 2-way ANOVA followed by Bonferroni posttest, $n = 6$.

NPY receptors were labeled with 0.25 nM of (125I)PYY (4000 Ci/mmol, Amersham Biosciences) in a buffer containing HEPES (20 mM), NaCl (137 mM), KCl (5.4 mM), KH_2PO_4 (0.44 mM), $CaCl_2$ (1.26 mM), $MgSO_4$ (0.81 mM), bacitracin (0.1%), and bovine serum albumin (BSA, 0.3%). Ligand concentration, which is near to K_D of NPY receptors, was chosen based upon our previous results [26]. After incubating for 60 minutes, sections were rinsed with trizma 50 mM (pH 7.4) followed by distilled water, and exposed to Biomax MR film (Kodak) during 3 days.

Binding to NPY1R was calculated by displacing the radioactive ligand with 10 mM of NPY1R specific agonist (leu31-Pro34) NPY (Peninsula).

2.5. Statistics. Results were evaluated by 2-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc., version 3.00, CA). A P value < 0.05 was considered to indicate statistically significant differences. Values are shown as mean ± standard deviation (SD).

3. Results

3.1. Petrosal/Nodose Ganglia. Nicotine increased TH immunoreactivity by 40% only in SHR petrosal/nodose ganglia as compared to placebo-treated SHR (Figure 1). However, NPY, glutamate, and SP were increased in these ganglia of both strains after chronic nicotine exposure, as compared to placebo-treated rats (Figure 1). Moreover, SHR presented increased levels of NPY and glutamate as compared to WKY rats in absence of nicotine (Figure 1).

The results revealed an association between nicotine exposition and the genetic background to increase TH immunoreactivity, suggesting the interaction among gene, environment, and physiology. The interaction of these parameters was already previously demonstrated by our group when the alkaloid exacerbated the hypertension in SHR while no alteration in blood pressure was observed in the WKY [4]. In contrast, NPY and glutamate immunoreactivities depend only on the nicotine or strain, and SP immunoreactivity

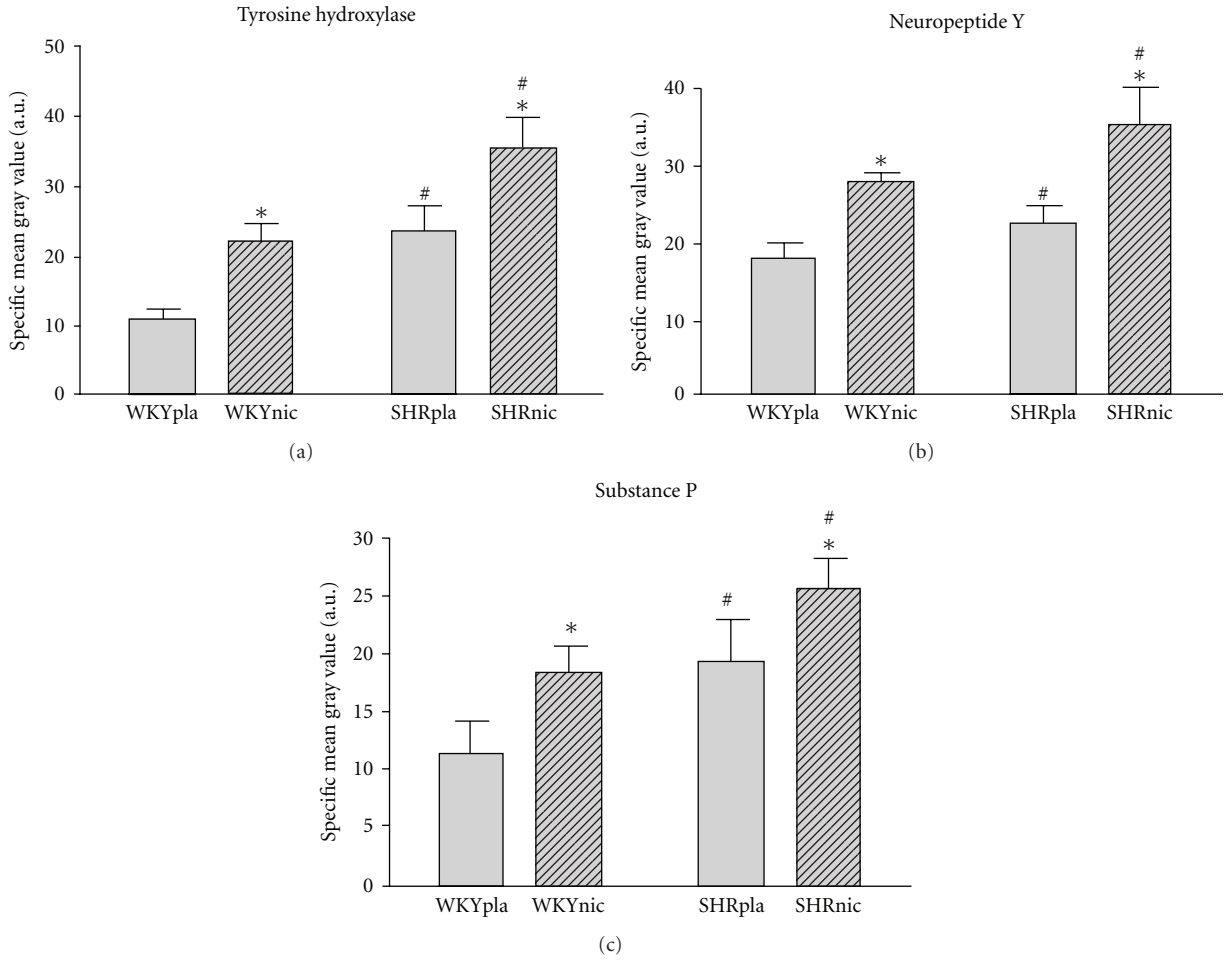

Figure 2: Immunoreactivity of tyrosine hydroxylase, neuropeptide Y, and substance P in superior cervical ganglion (SCG) of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats exposed either to nicotine or placebo delivered through subcutaneously implanted pellets during 8 weeks. Values are presented as mean ± SD, $^{*}P < 0.05$ as compared to the same strain placebo treated rat; $^{\#}P < 0.05$ as compared to WKY on the same experimental condition, according to 2-way ANOVA followed by Bonferroni posttest, $n = 6$.

is influenced by nicotine, without the participation of the genetic predisposition to hypertension.

3.2. Superior Cervical Ganglion. TH, NPY, and SP immunoreactivities were increased in SCG of SHR as compared to WKY rats (Figure 2). Prolonged nicotine exposure augmented the expression of these neurotransmitters/modulators in both strains. Alteration in the expression of these immunoreactivities were dependent on the interaction between rat strain and nicotine treatment, since the SHR presented elevated levels of these substances and nicotine exacerbated them in SCG as can be observed in Figure 2.

3.3. TH Levels in NTS and PVN. TH immunoreactivity and mRNA expression in the NTS did not differ between WKY and SHR rats without nicotine exposure. However, the PVN of SHR presents 3.5x the amount of TH mRNA of WKY rats (Figure 3), without any equivalent increase in its immunoreactivity at basal levels. Nicotine increased TH immunoreactivity and mRNA in the NTS of SHR (Figures 3(a) and 3(b)). The alkaloid also increased TH immunoreactivity in the PVN of both strains although TH mRNA in this nucleus was not altered by nicotine exposure (Figures 3(c) and 3(d)).

TH immunoreactivity and mRNA expression in the NTS are influenced by the interaction between nicotine exposure and rat strain, since only SHR responded to nicotine exposure. This might be of relevance considering the elevation of blood pressure exclusively in this strain. In the PVN, TH mRNA is influenced by strain while the treatment with nicotine is able to increase TH immunoreactivity, without interference of rat strain.

3.4. NPY in NTS and PVN. SHR presented decreased levels of NPY immunoreactivity in the NTS whereas NPY1R binding in this area is increased when compared to WKY (Figures 4(a) and 4(b)). In the presence of nicotine, NPY immunoreactivity increased in the NTS of SHR only while NPY1R binding decreased 50% in this area of both strains (Figures 4(a) and 4(b)). NPY immunohistochemistry increased in the PVN after chronic nicotine exposure only in the hypertensive

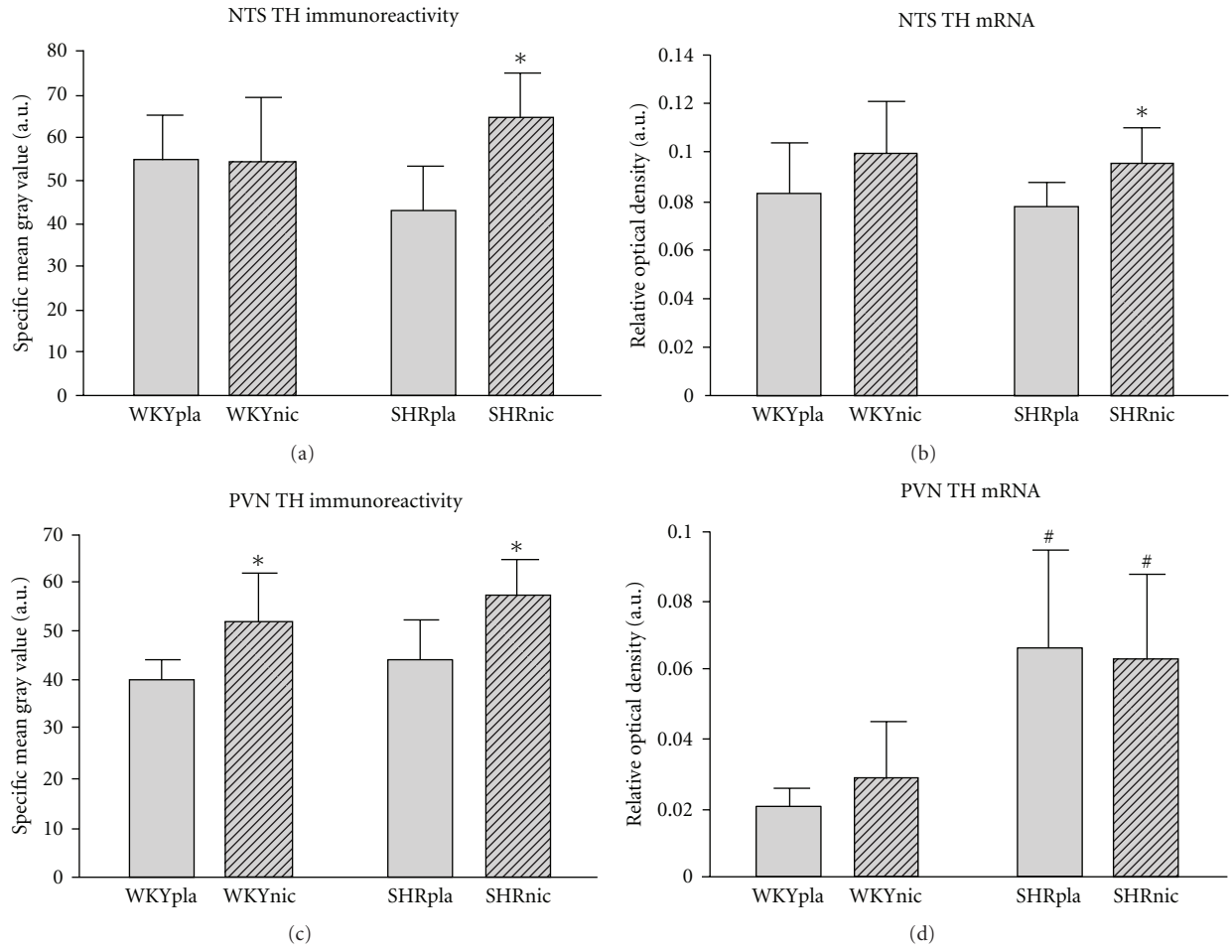

Figure 3: Immunoreactivity and mRNA expression of tyrosine hydroxylase in the nucleus tractus solitarii (NTS) and paraventricular hypothalamic nucleus (PVN) of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats exposed either to nicotine or placebo delivered through subcutaneously implanted pellets during 8 weeks. Values are presented as mean ± SD, $^{*}P < 0.05$ as compared to the same strain placebo-treated rat; $^{\#}P < 0.05$ as compared to WKY on the same experimental condition, according to 2-way ANOVA followed by Bonferroni posttest, $n = 6$.

Table 1: Relative optical density (ROD, arbitrary units) of neuropeptide Y mRNA radiolabeling in the nucleus tractus solitarii (NTS) of spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rats after prolonged exposure to nicotine or placebo delivered through subcutaneously implanted pellets.

WKY	*Placebo*	0.1053 ± 0.0295
	Nicotine	0.1035 ± 0.0211
SHR	*Placebo*	0.1174 ± 0.0218
	Nicotine	0.1130 ± 0.0249

Values are presented as mean ± SD following 2-way-ANOVA ($n = 6$).

strain (Figures 4(c) and 4(d)). NPY1R binding is found 100% increased in placebo SHR as compared to control WKY (Figure 4), nicotine seems not to modify binding parameters in the PVN.

The interaction between rat strain and nicotine exposure influenced NPY immunoreactivity in NTS and PVN. This immunoreactivity is increased by nicotine only when the genetic background of SHR is present. NPY1R binding in NTS is affected by rat strain and treatment with nicotine, separately, since the amount of NPY1R binding is increased in SHR and nicotine decreased it in the same magnitude in both strains. NPY1R binding in the PVN is modulated only by the rat strain. Nicotine had no effect over this parameter. NPY mRNA expression was not altered in SHR as compared to WKY rats in either nuclei analyzed in the presence or absence of nicotine (Table 1).

4. Discussion

In the present study we demonstrated that prolonged nicotine administration through subcutaneously implanted pellets increased TH immunoreactivity in brain and ganglia related to blood pressure control, preferentially in SHR. This is in agreement with our previous study that showed the increased responsiveness of SHR to nicotine when compared to WKY [4]. The alkaloid also increased NPY

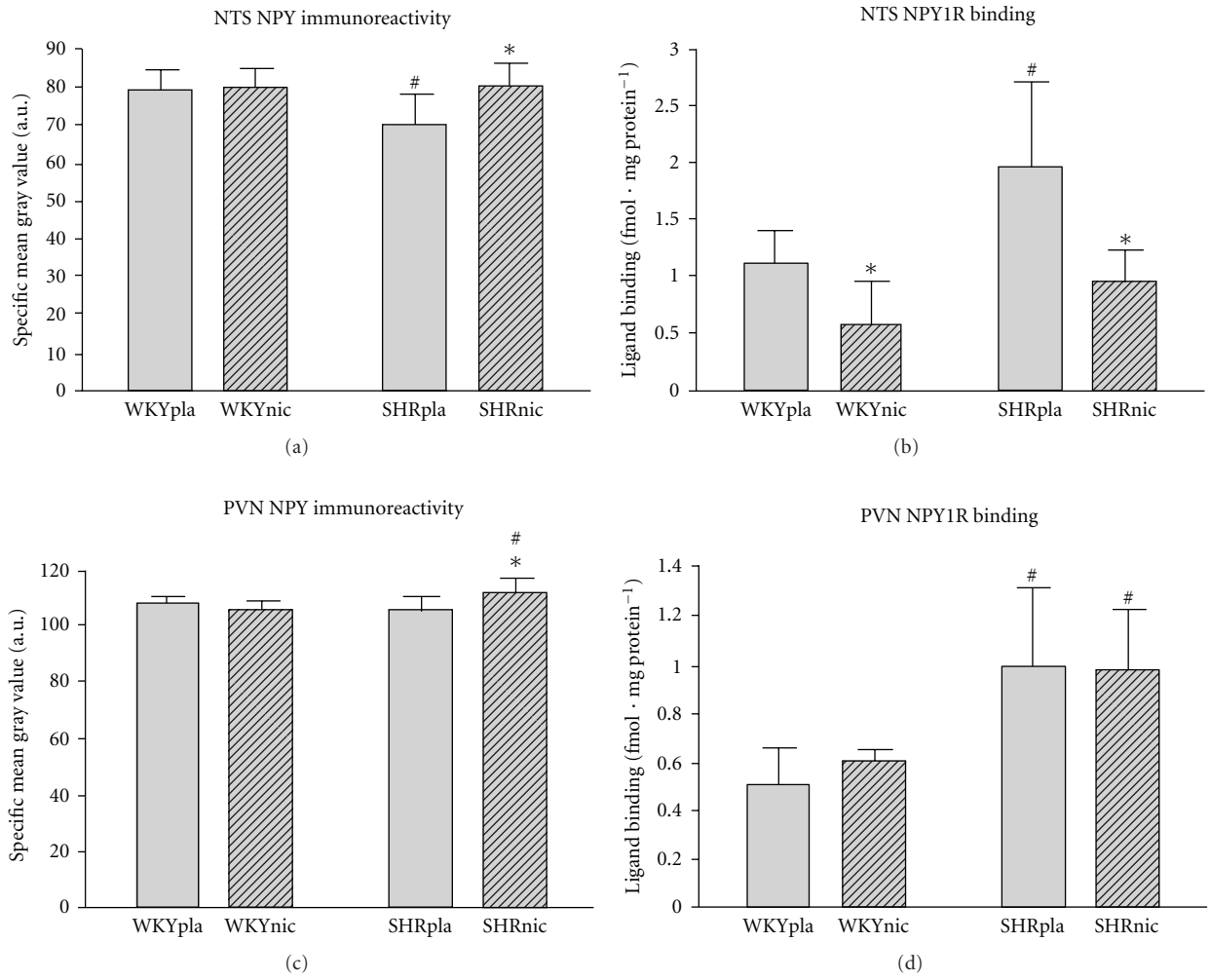

Figure 4: Neuropeptide Y immunoreactivity and NPY1R binding in the nucleus tractus solitarii (NTS) and paraventricular hypothalamic nucleus (PVN) of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats exposed either to nicotine or placebo delivered through subcutaneously implanted pellets during 8 weeks. Values are presented as mean ± SD, $^{*}P < 0.05$ as compared to the same strain placebo-treated rat; $^{\#}P < 0.05$ as compared to WKY on the same experimental condition, according to 2-way ANOVA followed by Bonferroni posttest, $n = 6$.

immunoreactivity in ganglia, NTS, and PVN of SHR, in spite of decreasing NPY1R binding in NTS of both strains. SP and glutamate were increased in ganglia after nicotine prolonged exposure.

The effects of nicotine encountered at both central and peripheral levels might be due to its action directly upon the brain as well as on the sympathetic autonomic nervous system and on smooth muscle of arterioles. It is important to point out that a recent revision written by Zanutto and collaborators [27] emphasized the long-term blood pressure regulation also by the NTS in addition to the renal mechanisms, which validate our study using prolonged nicotine administration to analyze areas of central cardiovascular control.

Glutamate immunoreactivity was evaluated only in nodose/petrosal ganglia in the present study since the analysis of this neurotransmitter in brain cardiovascular areas was conducted previously by our group [4] and the presence of glutamate as neurotransmitter in the SCG was not described yet, although the expression of glutamate receptors is found in SCG cells [28].

We have demonstrated an increase in glutamate immunoreactivity in nodose/petrosal ganglia of placebo SHR as well as after nicotine exposure in both rat strains as compared to WKY control rats. The increased glutamate in the ganglia might be released in the NTS by baroreceptor afferents to reduce blood pressure [29] since glutamate is a hypotensive agent in this nucleus. Several studies have described the sensitivity of neurons from petrosal and nodose ganglia to nicotine [30–33] and it seems that a minor part of barosensory neurons are responsive to nicotine [34]. In view of this, the response observed in SHR may be due to the raise in blood pressure, since the effects of nicotine related to glutamate immunoreactivity over the SHR were more pronounced. Moreover, nicotine may act directly upon these ganglia cells to determine integrated system responses.

Because the specificity of the ganglia neurons was not investigated, the effects of these immunoreactivities may be also related to chemoreception [35], pain [36], interaction with glial cells [37], and other physiological processes [38, 39].

Nicotine also acts upon TH and NPY immunoreactivities in nodose/petrosal ganglia of SHR compared to WKY rat ganglia which may be of relevance to the neural mechanisms of blood pressure regulation. Since these molecules are involved in the hypotensive response within the NTS it could also be that the increase in TH and NPY immunoreactivities are secondary in response to the raise in blood pressure induced by nicotine. The possible effects over neurotransmission seem to be more pronounced in SHR that are more responsive to the effects of nicotine exposure [4].

On the other hand, increased TH and NPY immunoreactivities in sympathetic ganglia such as the superior cervical ganglion may be indicative of increased activation of the sympathetic nervous system, which ends up with the release of norepinephrine into blood contributing to the development of hypertension [40, 41].

The presence of substance P immunoreactivity in nodose/petrosal ganglia is related to sensory afferents from ventricular myocardium [42], chemoreception [43, 44] and it is released as a cotransmitter by baroreceptor afferents into the NTS where it may promote an increase in blood pressure [45]. The present findings demonstrated an increase in substance P expression in nodose/petrosal ganglia of both strains after nicotine exposure which indicates a direct action of nicotine in these ganglia. As previously demonstrated [4], nicotine did not increase blood pressure in WKY rats, revealing the participation of substance P in complex regulatory mechanisms other than only blood pressure control.

At the SCG level, basal SP immunoreactivity level was increased in SHR compared to WKY rats such as previously demonstrated by Gurusinghe and Bell [46]. Nicotine treatment induced an additional increase in SP immunoreactivity in the SHR, as observed with the other transmitters, suggesting again a relation to the high blood pressure level of this strain.

Matta and colleagues [47] demonstrated the ability of peripherally administered nicotine to stimulate NPY and TH expression in the NTS and PVN associated with the release of adrenocorticotropic hormone. The present study contributes with the understanding of cardiovascular control associated with nicotine abuse, since the results demonstrated the increased responsiveness of hypertension genetic-susceptible rats to nicotine considering NPY and TH, although the cause and consequence remains to be further investigated.

Within the central nervous system, the results also suggest an association between the direct effects of nicotine and a reflex response due to the increased blood pressure of the SHR strain, an increased TH immunoreactivity only in the NTS of SHR rats was observed. The analysis of tyrosine hydroxylase possibly indicates the tentative of homeostasis maintenance in response to blood pressure elevation, as previously demonstrated by our group in a model of experimental hypertension [48]. In contrast, we demonstrated that NPY1R binding is decreased after nicotine exposure which may lead to increase in blood pressure as described by Narvaez and colleagues [49].

TH mRNA and NPY1R binding are about 100% increased in the PVN of SHR regardless of nicotine exposure. This is in agreement with previous data that demonstrated the increased sensitivity of SHR to stress related to augmented NPY expression in brain [50]. Moreover, Yu and Sharp [51] recently demonstrated the association between noradrenergic regulation of the PVN neurons and the sensitization to stress induced by nicotine administration, which is in line with the present results considering the SHR strain.

In conclusion, findings of the present study suggest that nicotine is able to modulate neurotransmitter systems especially in peripheral ganglia, while its central effects are preferentially encountered in the SHR, which may be linked to the elevation of blood pressure encountered in this strain. Whether these effects are direct or in response to alteration in blood pressure remains to be determined.

Acknowledgments

This paper was supported by research grants from Fundacao de Amparo a Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico.

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