行政院國家科學委員會專題研究計畫　成果報告

睡眠剝奪與呼吸中止對交感與副交感神經節相關神經化學物質表現之影響

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中 文 摘 要

睡眠障礙為一顯著之壓力來源，常伴隨交感神經系統活性上升並增加罹患心血管疾病之機率。先前研究顯示，一氧化氮 (nitric oxide, NO) 於調控交感神經系統活性功能中扮演相當重要的角色；緣此，本實驗目的即在偵測嚴重睡眠剝奪後，負責心血管訊息初級傳遞之結狀神經節 (nodose ganglion, NG) 內，一氧化氮合成酶 (nitric oxide synthase, NOS) 之活性表現情形。睡眠剝奪之實驗模式採用水上轉盤法 (disc on water, DOW method)。成年大鼠置於水上轉盤並接受五日睡眠剝奪後，將結狀神經節取下並進行一氧化氮合成酶免疫組織化學反應 (NOS immunohistochemistry)。實驗結果顯示，在正常大鼠結狀神經節內，約有 43% 之神經元具一氧化氮合成酶免疫組織化學反應正向標誌。電腦影像分析結果亦發現，具一氧化氮合成酶正向標誌神經元之染色相對視密度值 (relative optical density, ROD) 為 143 ± 3%。然而，經睡眠剝奪處理後，結狀神經節內具一氧化氮合成酶正向標誌之神經元數目從 43% 劇降至 20%，其染色相對視密度值亦從 143 ± 3% 驟降至 109 ± 2%。一氧化氮為重要之交感神經活性抑制物質，本研究證明睡眠剝奪將導致一氧化氮合成酶活性顯著降低，此一變化將嚴重干擾一氧化氮之生合成過程，並引發交感神經系統過度興奮，繼而可能致使心血管疾病發生之機率大增。

ABSTRACT

Sleep disorder is a prevalent stress associated with increased sympathetic activity and potentiates the occurrence of cardiovascular disease. Since nitric oxide (NO) may play an important role in the regulation of sympathetic activity, the present study is aimed to determine the NO synthase (NOS) expression in the nodose ganglion (NG), which carrying the primary cardiovascular afferents to the lower brainstem, after total sleep deprivation (TSD). The TSD was performed by the disc on water (DOW) method. Adult rats subjected to five days of TSD were processed for quantitative nicotinamine adenine dinucleotide phosphate-diaphorase (NADPH-d, a co-factor of NOS) histochemistry and neuronal NOS immunohistochemistry. The present results indicated that in the untreated and control rats, about 43% of the nodose neurons were positively stained for NADPH-d/NOS reactivity. Quantitative image analysis revealed that the staining intensity [relative optical density (ROD)] of NADPH-d/NOS positive neurons were 143 ± 3%. However, following TSD, both the percentage and ROD of NADPH-d/NOS reactive neurons were drastically decreased from nearly 43% to 20% as well as from 143 ± 3% to 109 ± 2%, respectively. Considering that NO could serve as an important messenger in the sympatho-inhibition response, a decrease of NOS expression in the NG following TSD would impair the NO production, which might serve as a potential mechanism responsible for the development of neuropathogenesis in TSD relevant cardiovascular disturbances.

INTRODUCTION

A healthy amount of sleep is paramount to leading a vigorous and productive lifestyle [1,2]. In subjects suffering from sleep deprivation there is a desynchronization of the autonomic nervous system, which unavoidably leads to cardiovascular dysfunction [3-6]. Previous studies have demonstrated that information concerning the haemodynamic fluctuations of the cardiovascular system is transmitted by pseudounipolar neurons of the nodose ganglion (NG) [7-10]. The vagal afferents derived from NG are crucial in relaying diverse information such as alterations in blood pressure, changes in blood oxygenation, and mechanical stimulation of the thoracic and abdominal organs to the nucleus tractus solitarius (NTS) for reflex maintenances of autonomic function [7-10]. During the past few decades, numerous neurotransmitters and/or neuromodulators were identified in the NG (for review, see [11]). However, among the numerous neurochemicals involved in the transmission of cardiovascular inputs, nitric oxide (NO) has attracted special interest because of its divergent biochemical roles in the regulation of sympathetic activity [12-16].

NO is a free radical gas synthesized from L-arginine by nitric oxide synthase (NOS) [17]. Three isoforms of NOS have been characterized in the mammalian tissue. Type I is found in neurons (neuronal NOS, nNOS), Type II is best characterized in macrophages, and type III is found in endothelial cells [18]. Neuronal and endothelial NOS are constitutively expressed and are dependent on Ca^{2+}/calmodulin for NO production, whereas type II NOS is Ca^{2+} independent and is expressed after immunological stimulation [17-19]. Numerous studies have shown that NO could mediate a multitude of physiological and pathophysiological activities ranging from synaptic plasticity to neurodegeneration (for review, see [20,21]). Pharmacological evidence also suggested that NO could serve as a retrograde messenger in the vagal afferents and participate in the regulation of cardiovascular function [22-25]. Ruggiero *et al.* had indicated that NO released from vagal afferent terminals of NG neurons may play an important role at the level of NTS or medullary reticular formation in the central

regulation of sympathetic outflow and arterial blood pressure [26]. Reports by Tseng *et al.* and Vitagliano *et al.* further demonstrated that microinjection of NO-donor into the brainstem can increase the neuronal activity of NTS neurons and depress the sympathetic tone [12,27]. Similar finding was also observed in pathological condition in which a reduced activity of neuronal NOS was detected in the brainstem of pre-hypertensive stage of spontaneous hypertensive rats [28]. Based on these findings, it is reasonable to suggest that NO, either endogenous generated as released from NG neurons or produced by extra-application, could mediate a tonic inhibition of sympathetic activity [15]. However, although keeping the proper levels of NO in the cardiovascular inputs may participate in the homeostatic regulation of autonomic function [26,29], it is still unclear whether the NOS expression in the NG would actually alter following total sleep deprivation (TSD). As an attempt to answer this question and provide the morphological manifestation that impaired NO production generated by NG may be one of the factors leading to TSD relevant clinical dysfunction, this study is aimed to determine the neuronal NOS expression in the NG of total sleep deprived rats by the use of nicotinamine adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry and NOS immunohistochemistry. Moreover, in order to assess the staining intensity objectively, all histochemical reactive sections were further processed for computerized quantitative image analysis.

MATERIALS AND METHODS

Treatments of experimental animals

Adult male Wistar rats (n = 21, weighing 200 ~ 250 g) obtained from the Laboratory Animal Center of the National Taiwan University were used in this study. The experimental animals were divided equally into three groups. Rats in the first group were subjected to TSD for five days (TSD group), while those in the second group were housed in the TSD apparatus but were permitted to sleep (control for sleep deprivation, TSC group). For animals in the third group, they were kept in the plastic cage placed aside from the TSD apparatus and

served as normal untreated controls (Untreated group). TSD was performed by the disc-on-water (DOW) method modified after that of Rechtschaffen [30]. Briefly, the apparatus was composed of two rectangular clear plastic chambers (60 × 20 × 60 cm in each) placing side by side. A single plastic disc (40 cm in diameter) serving as the rat-carrying platform was built in the lower quarter of the two chambers. Under the disc and extending to the walls of the two chambers was filled with water to a depth of 5 cm. The disc was controlled by a motor set to rotate the disc for 8 s at a moderate speed of 3.5 rev/min and then stop for 15 s. Before experiment begins, animals in the first two groups were resided in the TSD apparatus for at least 7 days of adaptation. During this period, the chambers were installed of solid mat instead of water. Sleep deprivation depends on the rat's aversion to water, since rats rarely entered the water spontaneously. As sleep deprivation begins, rats in the TSD group placing on the disc had to keep awake and walk against the direction of disc rotation to avoid being forced into the water. For TSC group, rats were received the same physical activity as that of the TSD group except that they were allowed to sleep from 06:00 ~ 18:00 wherein no disc movement was initiated. All experimental animals were exposed to an automatically regulated light:dark cycle of 12:12 at a constant temperature of 25℃ ± 1℃. Food and water were made available through grids placed on top of the chambers. In the care and handling of all experimental animals, the Guide for the Care and Use of Laboratory Animals (1985) as stated in the United States NIH guidelines were followed. All the experiments with sleep deprivation were also approved by the Laboratory Animal Center Authorities of the Chung Shan Medical University.

Perfusion and tissue preparation

After five days of TSD, all rats were deeply anesthetized with 7 % chloral hydrate (0.4 ml/kg, i.p.) and perfused transcardially with 0.9 % saline followed by 300 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Both sides of the NG were then removed and post-fixed in the same fixative for 2 h, followed by overnight immersion in 30

% sucrose buffer at 4℃ for cryoprotection. Serial 20 μm thick sections of the NG were cut longitudinally with a cryostat on the following day and were alternatively placed into three wells of a cell culture plate. Sections collected in the first well were processed for NADPH-d histochemistry, and those in the second well were processed for neuronal NOS immunohistochemistry. For sections collected in the third well, the neuronal NOS immunofluorescence along with NADPH-d histochemistry was carried out.

NADPH-d histochemistry

Tissue samples processed for NADPH-d histochemistry were first rinsed in 0.1 M PB and then treated in NADPH-d reaction medium containing 0.1 mg/ml nitroblue tetrazolium, 1 mg/ml β-NADPH and 0.3 % Triton X-100 (all from Sigma, St Louis, MO, USA) in 0.1 M PB (pH 7.4) for 1 h at 37℃. After incubation, the sections were washed several times in 0.1 M PB to terminate the reaction. For quantitative neuronal counting, half of the reacted sections were further counterstained with neutral red before being rapidly dehydrated through a series of graded alcohol, cleared with xylene, and coverslipped with Permount.

Neuronal NOS immunohistochemistry

For neuronal NOS immunohistochemistry, tissue sections collected in the second well were first placed in 0.01M phosphate buffer saline (PBS), pH 7.4, containing 10% methanol and 3% hydrogen peroxide for 1 h to reduce the endogenous peroxidase activity. Following this, sections were incubated in the blocking medium containing 0.1% Triton X-100, 3% normal goat serum and 2% bovine serum albumin (all from Sigma, St. Louis, MO, USA) for 1 h to block nonspecific binding. After several washes in PBS, the sections were incubated in the rabbit polyclonal anti-nNOS (Chemicon AB1552, Temecula, CA, USA) antiserum at the dilution of 1:500 with the blocking medium for 48 h at 4℃. After the incubation in primary antibody, sections were further incubated with a biotinylated secondary antibody (1:200) (Vector Labs, Burlingame, CA, USA) at room temperature for 2 h. The reaction product was revealed by the standard avidin-biotin complex (ABC) procedure (Vector Labs, Burlingame,

CA, USA) with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) as a substrate of peroxidase.

Colocalization of neuronal NOS immunofluorescence and NADPH-d histochemistry

For neuronal NOS immunofluorescence, tissue sections were first placed in 0.01M phosphate-buffered saline (PBS) containing 0.1 % Triton X-100, 3 % normal goat serum, and 2 % bovine serum albumin (all from Sigma, St Louis, MO, USA) for 1 h to block non-specific binding. After rinses in PBS, the sections were incubated in rabbit polyclonal anti-nNOS (Chemicon AB1552, Temecula, CA, USA) at the dilution of 1:500 for 48 h at 4℃. Subsequent detection of the reaction was carried out by using the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antiserum at the dilution of 1:80 for 2 h at room temperature. After several rinses, the sections were mounted on gelatinized slides and coverslipped with buffered glycerin in order to retard fading. FITC-labeled (nNOS-immunoreactive) neurons were examined with a fluorescence microscope (Zeiss) equipped with an appropriate filter (450 ~ 490 nm for excitation). After photomicrography, the sections were carefully floated off the slides and processed for NADPH-d histochemistry following the methods described above. Incubation without primary or secondary antibodies was used as negative controls (data not shown).

Quantitative image analysis

The general approach for all quantitative image analysis was similar to our previous study [31]. Cell counting was carried out in sections processed for NADPH-d histochemistry and counterstained with neutral red (first well). A total of 10-15 sections per animal were used. Only cell profiles with a visible nucleus on the focal plane were included in the count. The total number of neurons of NG was the sum of both the NADPH-d positive [NADPH-d (+)] and negative cells. The percentage value of NADPH-d (+) neurons was then obtained using the total number of neurons in the same section as a reference of 100%. The neuronal NOS staining intensity was assessed in sections processed for NADPH-d histochemistry as

neuronal NADPH-d is a reliable marker for neuronal NOS [32]. On the other hand, the sections carried out for NADPH-d histochemistry usually yield better results in neuronal staining which in turn could facilitate the computer-assisted quantitative evaluation. The staining intensity was then quantified with a computer based image analysis system (MGDS) and Image-Pro Plus software. A digital camera mounted on the ZEISS microscope imaged sections at 50× magnification in bright field and displayed them on a high-resolution monitor. All images were captured on the same day in order to maintain uniform settings adjusted at the beginning of capturing. The optical density (OD) of the NADPH-d (+) neurons was measured at the rostro-caudal extent of NG. All densitometric readings taken from each section were then combined and averaged to obtain the total OD (TOD) of each section. The background staining (BOD) of each section was measured by averaging ten random rectangles (area of rectangle = 150 μm^2) of the neuropil in the NG. The relative OD (ROD) for each section was then expressed as the percentage of TOD/BOD, so that ROD of 200% means that the value of TOD is two-folded than that of BOD. All parameters were carefully controlled following the recommended procedures for gray level adjustment, histogram stretch and minimal OD [33]. Comparison for the values obtained from untreated, TSC, and TSD rats were subjected to one-way ANOVA analysis. The effect of each challenge compared with untreated group was further analyzed using the Bonferroni *post hoc* test. The statistical difference was considered significant if $P<0.05$.

RESULTS

1. Cell counts

Quantitative evaluation of the total neuronal numbers had revealed that about 6193 ± 210 neurons were present in the NG of untreated rats, and nearly 6166 ± 287 as well as 6187 ± 294 neurons were examined in this ganglion in both TSC and TSD rats, respectively (Table 1). No obvious neuronal loss was observed in the NG after 5 days of TSD. Subsequent statistical analysis also demonstrated that no significant difference of the neuronal populations among

these experimental groups was found.

2. Neuronal NADPH-d/NOS expression in the NG of untreated and TSC rats

In the untreated rats, numerous NADPH-d/NOS (+) neurons were detected in both sides of the NG (Figs. 1A; 2A). The NADPH-d/NOS (+) neurons were of various sizes and exhibited various staining intensities (Figs. 1A,C; 2A). No clear relationship was observed between the neuronal size and staining intensity (Figs. 1A; 2A). All NADPH-d/NOS (+) neurons were homogeneously distributed throughout the NG without preferential concentration (Figs. 1A; 2A). In addition, the NADPH-d histochemical reaction product was deposited mainly in the neuronal perikarya and processes but not in the cell nuclei (Fig. 1C). There was no detectable difference in the staining pattern of NADPH-d/NOS (+) neurons between untreated and TSC NGs with regard to both frequency and staining intensity (Figs. 3; 4). Quantitative analysis revealed that about 43% and 41% of the nodose neurons was positive stained for NADPH-d/NOS reactivity in the untreated and TSC group, respectively (Fig. 3). Densitometric measurement displayed that the ROD of the NADPH-d (+) nodose neurons were 143 ± 3% in the untreated group and 140 ± 4% in the TSC group (Fig. 4).

3. Neuronal NADPH-d/NOS expression in the NG of TSD rats

However, in rats subjected to five days of TSD, both the percentage and staining intensity of NADPH-d/NOS reactivity was drastically reduced in both sides of the NG (Figs. 1B; 2B). The reduction of NADPH-d/NOS staining was randomly distributed throughout the NG without preference for any specific neuronal size or intra-ganglionic regions (Figs 1B,D; 2B). Quantitative evaluation revealed that the percentage of NADPH-d/NOS (+) neurons in the NG was significantly decreased from 43% to nearly 20% after TSD treatment (Fig. 3). Densitometric analysis also shown that the ROD of the NADPH-d (+) neurons was markedly declined from 143 ± 3% to 109 ± 2% following TSD (Fig. 4). The colocalization study further demonstrated that despite the experimental groups, nearly all NADPH-d (+) neurons were double labeled with NOS immunofluorescence (Fig. 2C,D).

DISCUSSION

The present study is the first report employing quantitative methods to provide the morphological evidence that TSD would significantly reduce the neuronal NADPH-d/NOS expression in the NG. The attenuated effect of TSD on NADPH-d/NOS expression was evident in both labeling frequency and staining intensity (Figs. 1A,B; 2A,B). Since the adequate NOS reactivity is thought to be essential for normal amounts of NO production [17], the reduction of NOS expression in the NG induced by TSD may suggest a decrease of NOS production, and subsequently interfere with the normal neurotransmission activity. It is well known that the pseudounipolar neurons of the NG would transmit numerous visceral and haemodynamic information from specialized sensory endings of the vagus nerve to the NTS [7-10]. The information transmitted by the vagal afferents of NG were then integrated in the NTS and further processed to relevant autonomic centers such as parvocellular, parabrachial, caudal and rostral ventrolateral reticular areas within the brainstem [34,35]. Previous studies have indicated that the terminal processes of the NG would generate NO as a transmembrane neuronal messenger in the transmission of vagal inputs [23,24,26]. Recent studies have further demonstrated that manipulating NO levels would significantly alter the neural activity of NTS, which can subsequently lead to concurrent changes of cardiovascular responses [12,22,25,27,29,36-41]. It has been reported that local application of L-arginine, a NOS substrate, can enhance the firing properties of NTS neurons [22,27,36,37]. The work of Wu et al. [38] also demonstrated that by the use of real time electrochemical analysis, increasing extracellular levels of NO would excite the NTS neurons and tonically inhibit the blood pressure and sympathetic outflow. In contrast, NO blockade with NOS inhibitor L-NAME or NOS gene knockout has been shown to attenuate the extrinsic excitability of NTS neurons and contribute to the elicitation of hypertension and tachycardia [37-40]. Based on these findings, it is suggested that endogenous NO may act as a powerful excitatory neuromodulator in the vagal afferents [41]. Through both pre- and post-synaptic modulation,

NO could enhance the discharge rate of NTS, which could further provide depressive effects on pre-sympathetic vasomotor nuclei [41-43]. With regard to this viewpoint, inadequate NO production in the NG would cause deficient excitation of the NTS neurons and result to over-activation of the sympathetic tone. On the other hand, in addition to regulate NTS excitability via neuronal transmission, biochemical study has demonstrated that NO can act as an autocrine regulator in baroreceptor neurons within the NG [44]. It has been reported that administration of NO scavenger to the NG would noticeably activate the baroreceptor neuron activity [44]. Recent neuroanatomical studies have further provided evidence to support that prolonged baroreceptor activation would produce sustained hypertension [45,46]. Our present results are thus parallel to these biochemical findings in which by the use of quantitative histochemical and immunohistochemical methods, we have provided the first morphological evidence to show that the neuronal NADPH-d/NOS expression was markedly reduced in the NG following TSD (Figs. 1A,B; 2A,B). In light of the critical importance of appropriate NO generation in maintenance of NTS activation and baroreceptor regulation [41,43,44], lacking or diminishing of NOS expression in the vagal afferent neurons following TSD may cause either improper depression of NTS or abnormal activation of baroreceptor activity. Through the consequent connection of the pre-sympathetic autonomic centers, the enzymatic reduction was suggested to play an essential role in the formation of TSD relevant cardiovascular dysfunction.

However, although TSD is a well established and widely adopted model in clinical research, it should be taken into consideration that whether any results obtained from this regime is directly specific for or attributed from TSD. Since our TSD paradigm is based on the DOW method, the accompanying effects of psychological, physiological or metabolic changes associated with the increased level of physical activity should not be overlooked. From this point of view, we have designed one group that served as the internal control for total sleep deprivation (TSC) to evaluate the possible effects of physical activity on the

enzymatic reactivity. The present results showed that there was no significant difference of NADPH-d/NOS expression between the TSC and untreated groups in both terms of labeling frequency and staining intensity (Figs. 3; 4). Given that the TSC rats were housed in the same experimental apparatus and receiving equal exercise as that of TSD ones, the similar pattern of NADPH-d/NOS expression observed in the TSC and untreated group may indicate that the possible influence of physical or metabolic debilitation produced by the current experimental procedure may be insignificant to affect the enzymatic expression. Based on this viewpoint, the marked decrease of NADPH-d/NOS expression in the NG following TSD may directly result from lack of sleep seeing that the solely difference between TSD and TSC rats is that owning a daily opportunity to sleep in the later group.

On the other hand, it is noteworthy that the neuronal numbers in the NG was not altered significantly following TSD (Table 1). This result means that the decreased labeling of NADPH-d/NOS reaction in the NG of TSD rats was a consequence of physiological changes in enzyme levels and was not due to neuronal dying. The reduced expression of NADPH-d/NOS (+) nodose neurons may directly result from either substantial decrease of NOS synthesizing or extensive acceleration of NOS exhaustion. Although we did not know which event is really occurred in the current paradigm, facilitated release of monoamines and their subsequent breakdown during sleep deprivation has already been verified [47]. However, it is not clear whether NOS expression is a reversible change after recovery from TSD. Since the cardiovascular disturbance is a prominent dysfunction following TSD [3,4], it would thus be interesting to explore whether the NOS expression will still be descended in the prolonged post-TSD periods.

In summary, the present study provides the first morphological evidence to show that the neuronal NADPH-d/NOS expression would drastically down-regulate in the NG following severe TSD. Although the detailed mechanisms regarding the formation of TSD relevant cardiovascular diseases are not fully understood, the current findings have provided important

insights concerning the significance of NOS deficiency within the primary visceral afferent neurons in the development of cardiovascular dysfunction.

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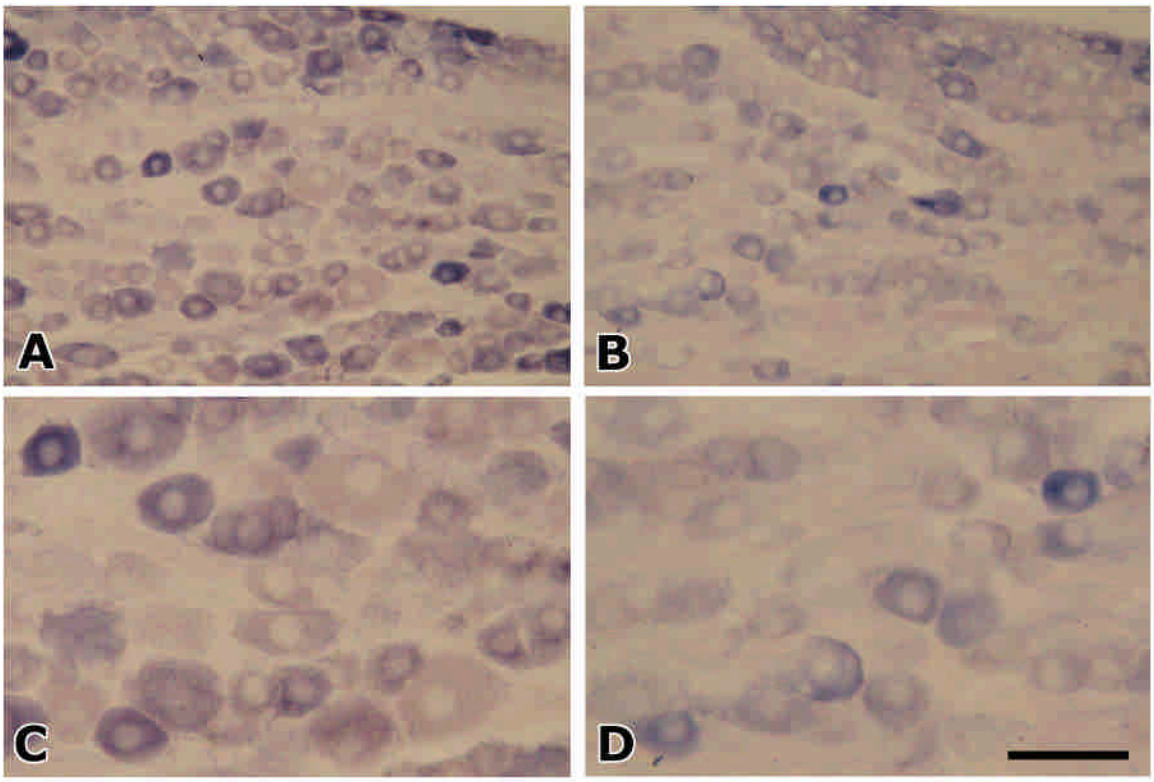

Fig. 1. Light photomicrographs showing NADPH-d histochemistry in the nodose ganglion (NG) of untreated (A, C) and total sleep deprived (TSD) rats (B, D). Note that numerous NADPH-d (+) neurons with various staining intensities were scattered throughout the NG of untreated rats (A). However, following TSD (B), the NADPH-d reactivity was drastically reduced in the NG in terms of labeling frequency and staining intensity. C: Higher magnification showed that the NADPH-d reaction product was mainly deposited in the cytoplasm of NG neurons. D: Following TSD, the reduction of NADPH-d reactivity was easily identified by the diminished labeling of the histochemical reaction. Scale bar = 100 μm in A, B, and 50 μm in C, D.

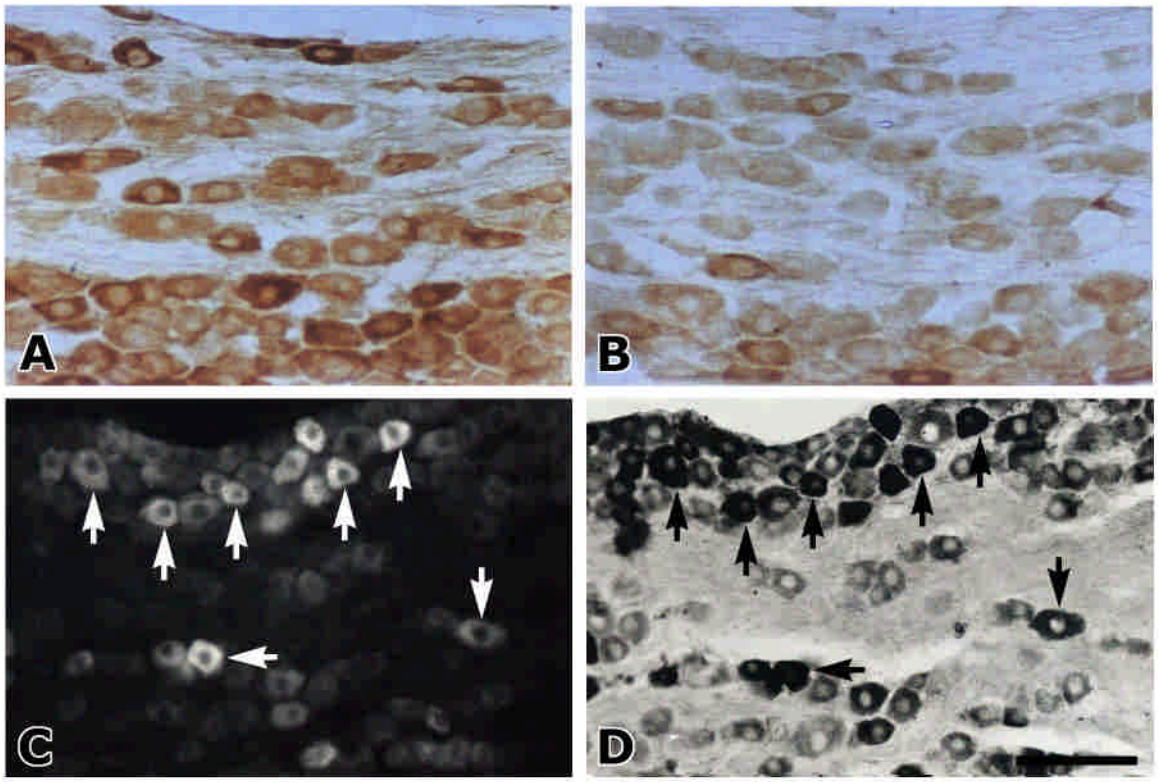

Fig. 2. Light (A,B,D) and fluorescence (C) photomicrographs showing neuronal NOS immunohistochemistry (A,B), neuronal NOS immunofluorescence (C) and NADPH-d histochemistry (D) in the nodose ganglion (NG) of untreated (A,C,D) and total sleep-deprived (TSD) rats (B). Note that numerous NOS (+) neurons were scattered throughout the NG in the untreated rats (A). However, following TSD (B), the NOS reactivity was drastically reduced in the NG with regard to both labeling frequency and staining intensity. It is worthy to note that a majority of NOS immunofluorescence neurons were co-localized with NADPH-d reactive cells (arrows in C,D). Scale bar = 75 μm in A, B, and 100 μm in C, D.

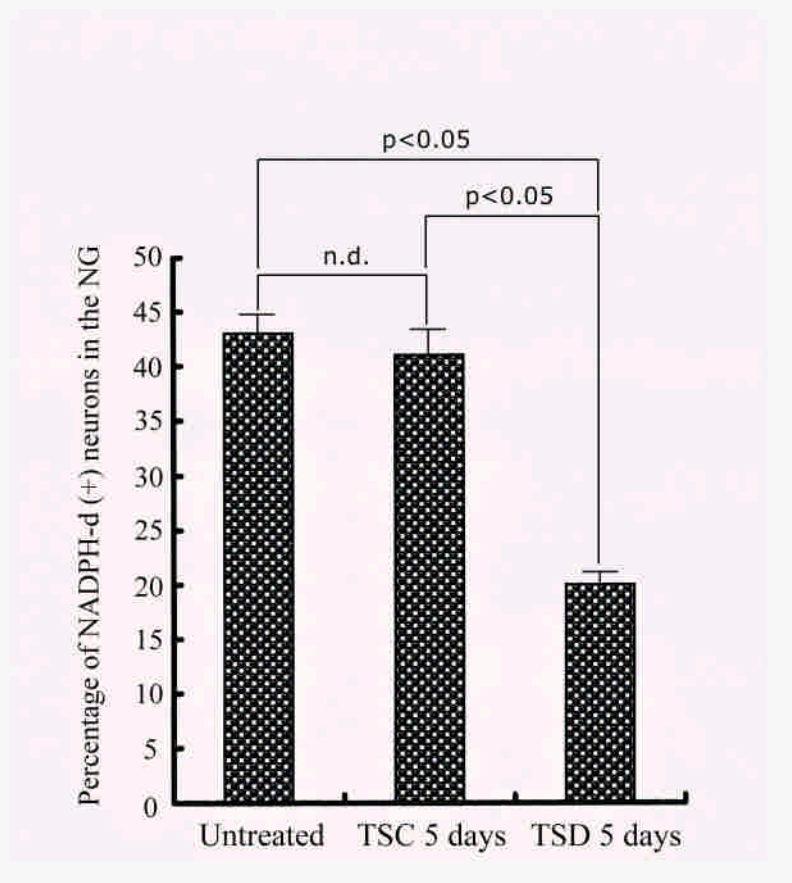

Fig. 3. Histograms showing the percentage of NADPH-d-positive [NADPH-d (+)] neurons in the nodose ganglion (NG) of untreated, control for total sleep deprived (TSC) and total sleep-deprived (TSD) rats. Note that about 43% and 41% of the nodose neurons were positively stained for NADPH-d histochemistry in the untreated and TSC groups, respectively. Also note that no significant difference (n.d.) was observed between the untreated and TSC groups. However, following 5 days of TSD, the percentage of NADPH-d (+) neurons were markedly decreased to nearly 20% as compared with that of untreated value.

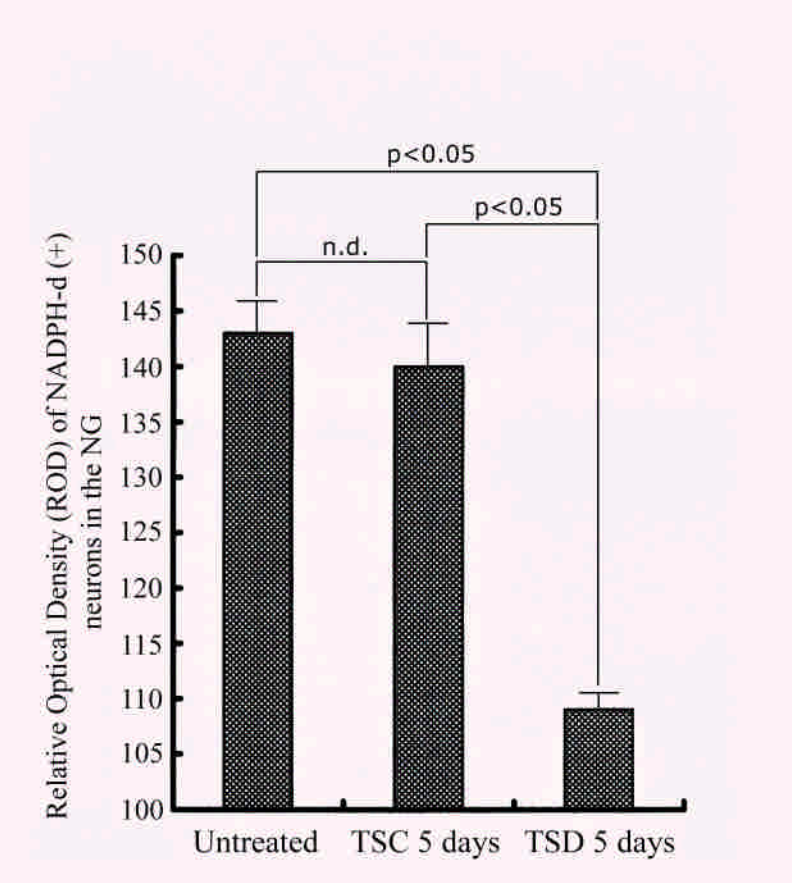

Fig. 4. Histograms showing the relative optical density (ROD) of NADPH-d-positive [NADPH-d (+)] neurons in the nodose ganglion (NG) of untreated, control for total sleep deprived (TSC) and total sleep-deprived (TSD) rats. Note that the ROD of NADPH-d (+) neurons in the NG was no significant difference between the untreated and TSC groups. Also note that following 5 days of TSD, the staining intensity of NADPH-d (+) neurons were markedly decreased.

Table 1 Total neuronal numbers in the NG of untreated, TSC and TSD rats (n = 7 in each group)[1]

Untreated rats	TSC (5 days) rats	TSD (5 days) rats
6193 ± 210	6166 ± 287	6187 ± 294

[1] Numerical data are expressed as means ± S.E.M.. NG, nodose ganglia; TSC, control for total sleep deprivation; TSD, total sleep deprivation.