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# The underestimated role of a somatosensory neural network on thyroid gland morphology: an experimental subarachnoid hemorrhage model study

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## Abstract

**Objectives:** Innervation of the thyroid gland has been attributed to the autonomic nervous system. Although peripheral sympathetic and parasympathetic innervations of the thyroid gland are well known, little is known about the somatosensory innervation of the thyroid gland. In this study, alterations on the somatosensory neural network of the thyroid gland following an experimental subarachnoid hemorrhage were investigated in rabbits.

**Methods:** Experiments were conducted on 23 rabbits under no medical intervention. Five rabbits were used as control group. Five rabbits were used as the sham group and serum physiologic (SF) was injected into their cisterna magna. The remaining 13 animals were used as the subarachnoid hemorrhage (SAH) group; their own blood (1 ml) was re-injected into the cisterna magna. Thyroid hormone levels of animals were measured at the end of one month. Then, histological sections of the middle parts of the thyroid glands were stained with haematoxylin-eosin (H&E) for investigation of SAH-related damage. The total follicle volume (TFV) per cubic millimeter of the thyroid gland was estimated by stereological methods. Comparison of degenerated neuronal density (DND) in the C4 dorsal root ganglia (DRG) was examined bilaterally using H&E and TUNEL stainings.

**Results:** Following SAH, neuronal degeneration in the cervical DRG caused somatic innervation deficiency, follicular atrophy and thyroid hormone depletion in the thyroid gland. T3 and T4 hormone levels of the SAH group (T3: 61±8 µg/dl; T4: 1.01±0.12 µg/dl) were significantly ($p<0.005$) lower than those of control (T3; 103±6 µg/dl, T4: 1.37±0.36 µg/dl) and sham (T3; 94±10 µg/dl; T4: 1.24±0.87 µg/dl) groups. In control groups, mean TFV was 41% / $mm^3$ and DND of C4 DRG was 6±2 / $mm^3$. These values were significantly lower than those in sham (TFV: 35%/$mm^3$ and DND: 22±7/$mm^3$) and experimental SAH (TFV: 23%/$mm^3$ and DND: 253±49/$mm^3$) groups ($p<0.0005$ and $p<0.0001$, respectively).

**Conclusion:** Thyroid follicle growth and its secretory activity are under the control of a quite complex, multi-originated, yet incompletely understood innervation pattern. We propose the presence of an underestimated role of a somatosensory neural network - an interganglionary link between the superior cervical, thyroid, laryngeal, nodose, trigeminal and dorsal root ganglia - on thyroid gland morphology.

**Keywords:** cisterna magna; dorsal root ganglion; rabbit; subarachnoid hemorrhage; thyroid gland

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## Introduction

The bulk of the parenchymal tissue of the thyroid gland is composed of the follicular cells, producing the thyroid hormones, generally under the control of the suprachiasmatic nucleus.[1] A small portion of the parenchyma is parafollicular cells which have their origins in the ultimobranchial bodies derived from epithelial clusters of the

fourth pharyngeal pouches invaded by the neural crest cells. The distribution and origin of the nerve fibers innervating the thyroid gland of rats arise from the cell bodies in the thyroid, laryngeal, superior cervical, jugular-nodose, trigeminal and dorsal root ganglia (DRGs) of C2-C5 spinal nerves.[2] Removal of the superior cervical ganglia network causes several neuroendocrine dysfunctions.[3,4] Aging process reduces thyroid hormone levels secondary to sympathetic hypofunction.[5] The thyroid gland and blood vessels have dual innervation arising from the sympathetic and parasympathetic fibers.[6] They are located around blood vessels, under the fibrous capsule and in the close vicinity to the secretory vesicles.[7] Follicular cells are not as enriched with sympathetic nervous system fibers as with cholinergic fibers.[8] There may be an undescribed interganglionary link among superior cervical, thyroid, laryngeal, nodose, dorsal root and trigeminal ganglia. Parasympathetic innervation of the thyroid gland is managed by the inferior laryngeal branches of the vagal nerve. Stimulation of the parasympathetic nerves increases the blood flow of the thyroid gland by dilating thyroidal vessels. Parasympathetic vasodilation has supplementary role in both regulation of the secretion of thyroid hormone and alteration of the thyroidal blood flow.[9,10] Extirpation of the nodose ganglion results in decrease of parasympathetic activity of the thyroid gland.[11] Sympathetic innervation of the thyroid gland on the other hand is provided by the superior cervical ganglion,[12] which contributes to enlargement of the gland and might modulate tissue and hormone (TSH) interactions.[13] Unilateral superior cervical ganglion section leads to a decrease in the size of thyroid gland. The superior cervical ganglion projects to the pineal and other glands such as the salivary glands. Postganglionic nerve transection relies on various histomorphological abnormalities in the related glands.[14] Acute superior cervical ganglionectomy leads to significant depression of the thyroid economy.[15] The middle and/or inferior cervical ganglia also send their axons through external carotid nerve to the thyroid.[16] Hypothalamo-hypophyseal and pineal gland injuries may result in decreased thyroid hormone levels secondary to follicular atrophy.[17] Since the somatic innervation should have major roles on the formation and continuation of thyroid gland morphology, functions and the prevention of thyroid gland pathologies, in the current study, we aimed to investigate the alterations in the somatosensory innervation of the thyroid gland following subarachnoidal hemorrhage in rabbits.

## Materials and Methods

The study protocol was approved by the Ethics Committee for Animal Experiments, Atatürk University School of Medicine, Erzurum, Turkey. The care of the animals and the experiments were conducted according to the guidelines set forth by the same ethics committee. The animals were kept in individual metal cages at room temperature with 12 h of light per day and 50% relative humidity under veterinary supervision. They were fed with standard laboratory diet and water *ad libitum*. The animals were randomly assigned into three groups. After anesthesia isoflurane applied using a face mask, 0.2 ml/kg of the anesthetic combination (Ketamine HCL, 150 mg/1.5 ml; Xylazine HCl, 30 mg/1.5 ml in distilled water) was injected subcutaneously before surgery. A balanced, injectable analgesic was used to reduce pain and mortality.

Five rabbits were selected as the control group (Group 1, n=5). In the sham group, 1 ml saline was injected into the cisterna magna (Group 2, n=5). To induce experimental subarachnoid hemorrhage, autologous blood (1 ml) was taken from the auricular artery. While the head of the animal was held in a hyperflexed position, the posterior notch of the foramen magnum was identified and CSF was aspirated from the cisterna magna. After the confirmation of the cisterna magna, 1 ml of autologous blood was injected by using a 22-gauge needle for over one minute.

Following the injection, all animals were observed for one month without any medical treatment. Animals that developed severe ischemia were chosen for the SAH group (Group 3, n=13), and their vital findings were monitored for ten minute-periods, two times a week. Their hormone levels were examined weekly during the experiments. After formalin perfusion under the general anesthesia, the thyroid glands, cervical spinal cords and C4 DRGs were carefully removed for histologic examination. The extracted tissues were passed through a graded alcohol series, and then embedded in liquid paraffin. Tissue sections from each block were collected on glass slides for haematoxylin-eosin (H&E) and TUNEL stainings to examine SAH-related damage under light microscopy.

The thyroid glands were sectioned at 5 μm thickness with 30 μm intervals. Every 30th and 31st sections were sampled for quantification of the follicles. The total number of follicles in the thyroid gland was estimated by the fractionator method. For the analysis of C4 DRG sections, the spinal cord specimens together with their extensions were longitudinally embedded in paraffin blocks in order to observe all the roots during the histologic examination. For the stereological analysis, the first pair of sampled sections was selected randomly from a starting point within the first 30 section interval. Thereafter, every 30th section and its neighbor was sampled. The section sampling fraction (f1) was therefore f1=1/30. Section pairs not containing the thyroid tissue and vagal nuclei were discarded. This

sampling fraction yielded on average 10 to 11 section pairs. Area of sampling fraction, f2, was 1/1. We preferred to use physical dissector method to evaluate the numbers of thyroid follicles; because, this method is intuitively simple, free from assumptions about particle shape, size and orientation, and the particle number can be readily estimated and unaffected overprotection and truncation. Two consecutive sections (dissector pairs) obtained from tissue samples were mounted on each slide. Reference and look-up sections were reversed in order to double number of dissector pairs without taking new sections. The number of counted follicles was designated ΣQ-. The total numbers of thyroid follicles (N), in thyroid glands were estimated from the equation:

$N=\Sigma Q^{-} \times 1/f1 \times 1/f2.$

After counting procedure, total follicle volume (TFV) was estimated via summation of each vesicle volumes. Since the shape of thyroid follicles resembled as ellipsoid, their volumes were estimated by using the following formula: $Vn=4/3\pi r_n^3$.

The TFV was estimated as: $TFV = \sum_{N=1}^{N=N} N \times Vn.$

$$\sum_{1}^{n} Vf = \sum_{f=1}^{n} n\left[\frac{4}{3}\pi < \frac{a+b+c}{3} > 3\right]^{*}$$

The physical dissector method was used to evaluate the numbers of living and degenerated neurons in C4 DRG. Two consecutive sections (dissector pairs) obtained from reference tissue samples were mounted on each slide. The paired reference sections were reversed in order to double the number of dissector pairs without having to cut new sections.

The mean numerical density of the C4 DRG (Nv/Gv) per $mm^3$ was estimated using the following formula.

$Nv/Gv=\Sigma Q^{-}/\Sigma Axd$

Where ΣQ-N is the total number of counted neurons appearing only in the reference sections, d is the section thickness, and A is the area of the counting frame. The most effective way of estimating ΣA for the set of dissector is via ΣA=ΣP.a. ΣP is the total number of counting set frames points and a is a constant area associated set point. The total volume of each specimen were estimated by the Cavalieri volume estimation method. Then, the total number of neurons was calculated by multiplication of the volume ($mm^3$) and the numerical density of neurons in each ganglion.

The differences between the thyroid follicle densities per unit volume of the thyroid gland and degenerated neuron densities in the C4 DRG were analyzed using a commercially available statistics software package (SPSS® for Windows, version 12.0, Chicago, IL, USA). In all the groups, the numbers of follicles did not show normal distribution according to Kolmogorov-Smirnov and Shapiro-Wilk test. Therefore, data analysis consisted of the Kruskal-Wallis and Mann-Whitney U tests. Differences were considered to be significant at $p<0.05$. The p value used for multiple comparisons were calculated by dividing 0.05 with six for Bonferroni correction and considered as statistically significant for ≤0.0083.

## Results

In control animals, the average heart rate was 280±15/min, the respiratory rate was 35±9/min and the blood oxygen concentration was 95±7%. In experimental animals, soon after inducing SAH, the heart rate decreased to 150±30/min, the mean respiratory rate to 18±5/min, and the mean blood oxygen concentration to 78±10%. Considerable electrocardiographic changes were observed such as ventricular extra systoles, ST depression, QRS separation, bigeminal or trigeminal extra systoles and fibrillations. Four animals in the SAH group and one animal in the SHAM group were dead within the seven days of surgery. Neck stiffness, unconsciousness, convulsive attacks, fever, apnea, cardiac arrhythmia, and breathing disturbances were observed in all hypothermic animals. However, in the late phase of fatality-inducing SAH, the heart rate increased to 350±40/min. When analyzing respiratory parameters, both decreased respiration frequency (bradypnea) (14±3) and increased respiratory amplitudes were observed in the first hours following SAH. Conversely, at longer intervals following SAH, increased respiration frequency (tachypnea) and decreased respiration amplitude were observed, resulting in shortened inspiration, a longer expiration time, apnea-tachypnea attacks, diaphragmatic breath and respiratory arrest. Massive subarachnoid hemorrhage was observed in the basal cisterns of GIII animals. They showed meningeal irritation signs, cardiorespiratory dysrhythmia.

Average thyroid hormone levels of animals are shown in **Table 1** and statistical comparisons of groups in **Table 2**. T3 and T4 hormone levels of the SAH group (T3= 61±8 μg/dl; T4= 1.01±0.12 μg/dl) were significantly lower than those of control (T3= 103±6 μg/dl; T4= 1.37±0.36 μg/dl) and sham (T3= 94±10 μg/dl; T4= 1.24±0.87 μg/dl) groups. On the other hand, TSH levels were higher than 0.5 ng/dl in sham and SAH groups (**Table 1**).

While mean TFV was 41%/$mm^3$ in control groups, it was significantly decreased in sham (35%/$mm^3$) and SAH (23%/$mm^3$) groups (**Tables 1** and **2**). The significance level was more prominent between control and SAH) groups ($p<0.00005$) than those of control and sham groups ($p<0.001$) as displayed in **Table 2**.

Similarly, density of degenerated neurons in C4 DRG was significantly higher in SAH group (253±49/mm$^3$) than those of control (6±2/mm$^3$) and sham (22±7/mm$^3$) operated groups, respectively (**Table 1**). Comparisons of the groups that displayed a significance level was more prominent between control and SAH groups (p<0.00001) than those of control and sham groups (p<0.001; **Table 2**).

Postmortem gross morphological and histopathological investigations of the tissues in the central nervous system of the rabbits showed brain edema, stiffness and enhanced leptomeningeal thickness in animals with SAH. The brain swelling and increased brain weight were seen in all animals who developed hyperthermia. In general, the basal cisterns and rarely the fourth and the lateral ventricles were filled with blood. Arachnoid membranes of the lower cranial parts were sticked to the lower cranial nerve roots.

Histology of the normal thyroid gland, follicles (F1-n) and the volume estimation method of follicles are shown in **Figure 1**. In sham rabbits, partially atrophic and lessened thyroid follicles were evident in the thyroid gland under light microscopy (**Figure 2**). On the other hand, size of the thyroid glands decreased dramatically in SAH animals. Acinar cell loosing, decreased number of hormone filled vesicles, total volume reduction of thyroid follicles, ductal epithelial cell injury related ductal closing, degenerative changes of extra thyroidal parasympathetic ganglia neurons and apoptotic changes in the acinar cells, tubular cells and supporting cells were detected in the histological sections of their thyroid glands stained with H&E (**Figure 3**).

**Table 1**

Thyroid hormone levels, quantitative analyses of thyroid follicles and DRG neurons for control (Group 1), sham (Group 2) and SAH (Group 3).

| | Group 1/ Group 2 | Group 1/ Group 3 | Group 2/ Group 3 |
|---|---|---|---|
| T4 | p<0.001 | p<0.0001 | p<0.0005 |
| TFV | p<0.001 | p<0.00005 | p<0.0001 |
| DND in C4 DRG | p<0.001 | p<0.00001 | p<0.0001 |

**DND:** degenerated neuron density; **DRG:** dorsal root ganglion; **TFV:** thyroid follicule volume.

**Table 2**

Statistical comparisons for control (Group 1), sham (Group 2) and SAH (Group 3) groups.

| | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| T3 (µg/dl) | 103±6 | 94±1 | 61±8 |
| T4 (µg/dl) | 1.37±0.36 | 1.24±0.87 | 1.01±0.12 |
| TSH I (ng/dl) | 0.5 | >0.5 | >0.5 |
| Mean TFV /mm$^3$ | 41% | 35% | 23% |
| DND of C4 DRG/mm$^3$ | 6±2 | 22±7 | 253±49 |

**DND:** degenerated neuron density; **DRG:** dorsal root ganglion; **TFV:** thyroid follicule volume.

Histology of the cervical spinal cord with C4 DRG is shown in **Figure 4**. Cytoplasmic condensation, nuclear shrinking, cellular angulations and peri-cytoplasmic halo formation were accepted as neuronal degeneration crite-

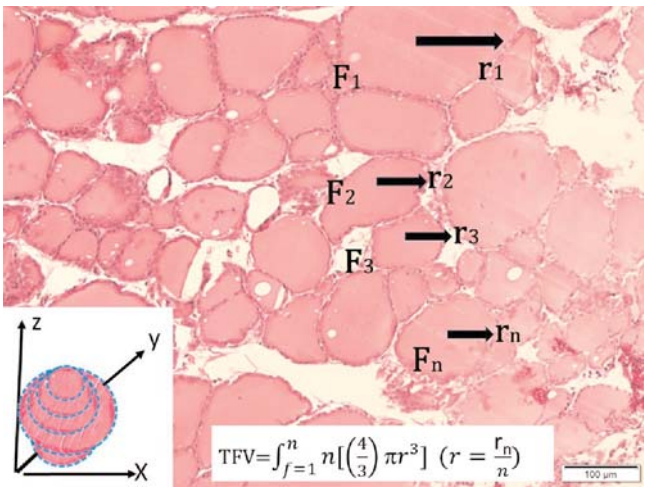


**Figure 1.** Thyroid gland follicles (F1-n) in control animals and stereological method used for follicle volume estimation (H&E stain; scale bar=100 µm). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

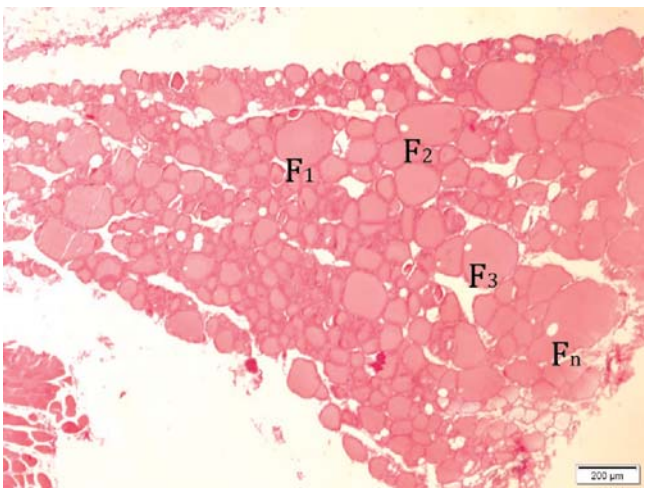


**Figure 2.** Thyroid gland and normal follicles in sham operated rabbit (H&E stain; scale bar=200 µm). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

ria. Degenerating neuronal profiles and apoptotic neuronal changes were apparently more abundant in DRG of the animal with SAH. Especially, the arteries supplying nerve roots and ganglia were more vasospastic in SAH rabbits. Therefore, the ratio of arterial wall surface to lumen surface accepted as vasospasm index (VSI), which can be calculated by using the ($R^2$-$r^2$/$r^2$) formula, displayed alterations in animals following SAH (**Figure 4**) in comparison to control animals (**Figure 5**).

## Discussion

In this study, we used a SAH model in rabbits to investigate alterations in the thyroid gland cytoarchitecture and somatosensory neural network. Our results displayed significant changes in the total volume of thyroid follicles and enhanced degeneration in the cervical DRG neurons innervating the thyroid gland. SAH accounts for approximately 5% of all strokes, with an incidence of 5–10 per 100,000 in most populations.[18] This life-threatening condition interrupts the productivity of an individual by causing major disabilities and a severe socio-economic impact with the estimated life time costs more than double that of ischemic stroke.[19] Small laboratory animals, such as rats and mice can be used as model to induce SAH. In these animal models, different surgical methods are used to create an effective degree of vasospasm. However, many traditional SAH animal models do not adequately mimic the acute pathophysiological changes seen in human and have been criticized for missing delayed cerebral ischemia.[20]

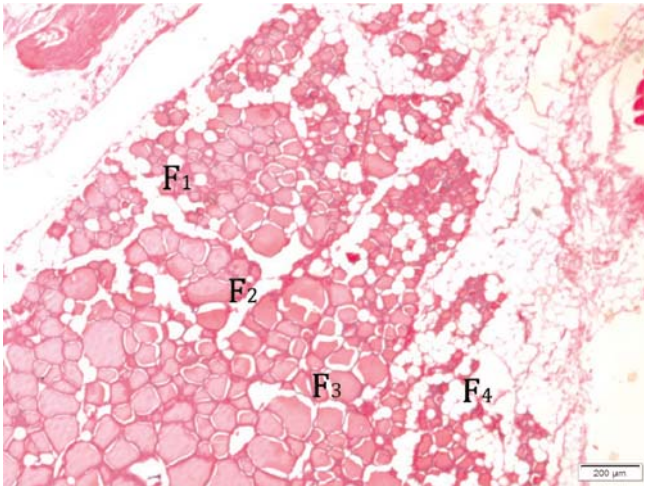


**Figure 3.** Thyroid gland and partially atrophic follicles in a rabbit with SAH (H&E stain; scale bar=200 µm). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

Although more fitting animal models for this purpose seem like bigger experimental animals such as dogs and primates, it is very difficult to use these animal models due to ethical and financial problems. Instead, injection of a single standard volume of blood (1 ml) into the cisterna magna of a rabbit has been shown as a useful and reliable animal model of SAH. This model exerts biphasic pattern of vasospasm and vasoconstriction and morphological alterations in the arteries display similarities to changes observed in humans.[21]

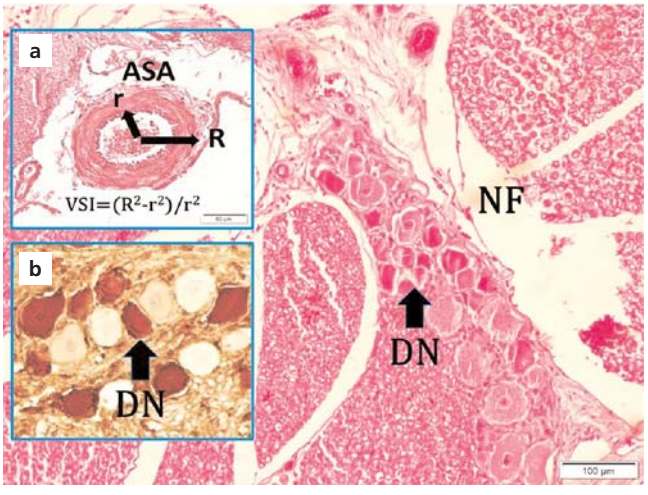


**Figure 4.** (**a**) Cervical spinal cord with constructed anterior spinal artery (**ASA**) and vasospasm index calculation method. (**b**) Degenerated apoptotic neurons (**DN**) in C4; TUNEL stain. Nerve fibers (**NF**) are shown with H&E staining (**black arrow**). Scale bar=100 µm. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

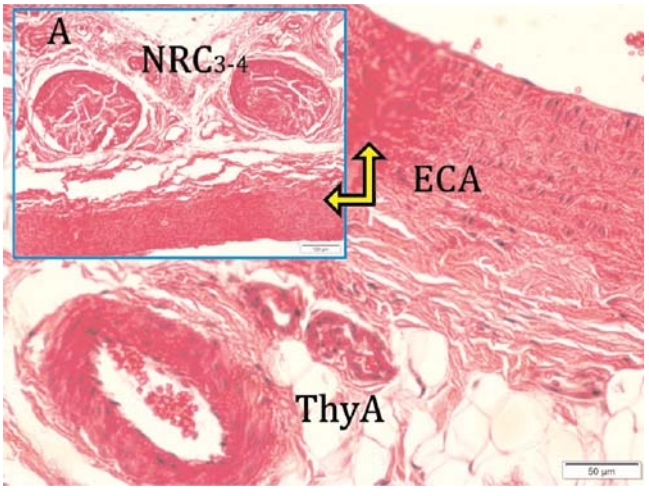


**Figure 5.** Thyroid gland with constructed thyroidal artery (**ThyA**) and a branch of external carotid artery (**ECA**). (**A**): Cervical 3-4 nerve roots (NRC3-4) stained with H&E. Scale bar=50 µm. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

In humans, although about 70% of patients may develop focal arterial vasospasm, and only 30% will manifest neurological deficits. Vasospasm of the major cerebral arteries is usually focal, but it also might be diffuse usually with the onset on day 3 after SAH, maximal at days 6–8, and lasting for 2–3 weeks.[22] Symptomatic vasospasm is characterized by the insidious onset of confusion and decreased level of consciousness, followed by focal motor and/or speech impairments. In the surviving patients, secondary insults caused by the cerebral vasospasm results in various complications. In this study, we detected alterations in the ratio of arterial wall surface to lumen surface of the thyroidal arteries in animals following SAH. We thought that changes in the perivascular nerves might be responsible for the observed outcomes in the thyroid gland.

The distribution and origin of the nerve fibers innervating the thyroid gland were studied comprehensively in rats by immunohistochemistry, retrograde tracing and denervation experiments. Different subpopulations of nerve fibers containing noradrenaline (NA), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), galanin (GAL), substance P (SP), and calcitonin gene-related peptide (CGRP) have been shown around the blood vessels and thyroid follicles. Injection of a retrograde tracer, True Blue into the thyroid gland labelled cell bodies in the thyroid, laryngeal, superior cervical, the jugular-nodose, C2-C5 DRG and trigeminal ganglia.[2] In the light of the number of retrogradely labelled cell bodies, it seems like the most of the innervation of the gland is supported by the superior cervical and thyroid ganglia while the contribution of the laryngeal and trigeminal ganglia is the least. Denervation studies showed that neurons located in the superior cervical ganglion provide all NA-containing and the majority of NPY-containing nerve fibers to the thyroid gland. On the other hand, all VIP- and a minor population of NPY- and GAL-containing fibers in the thyroid gland originate from the thyroid ganglion. Whereas, SP and/or CGRP containing nerve fibers derive from the jugular, cervical dorsal root and/or trigeminal ganglia. These results emphasize the importance of multiple sources of innervation arriving to the thyroid gland and their different neuropeptide expression in regulation of the activity of thyroid follicles.[2]

Innervation of the thyroid gland and control of thyroid activity occur mostly via the superior cervical ganglia, intrathyroidal ganglia of vagal nerves as well as the trigeminal ganglia and cervical DRG.[2] Extirpation of the nodose ganglion decreases of parasympathetic activity on thyroid gland.[11] Bilateral inferior laryngeal nerve section causes reduction in circulating T4 for up to four weeks after surgery, while unilateral section causes a transient T4 decrease one week after surgery. In contrast, electrical stimulation of parasympathetic activity in the superior laryngeal nerve increases the thyroid blood flow via dilatation of the blood vessels. Thus, parasympathetic vasodilation has a supplementary role in regulating both the secretion of thyroid hormones and alteration in the thyroid blood flow.[9]

Sympathetic innervation of thyroid gland is provided by the superior cervical ganglion[12] which contributes to the enlargement of gland and might modulate the tissue and hormone (TSH) interactions.[13,16] In addition, middle and/or inferior cervical ganglia send their axons through the external carotid nerve to the thyroid gland.[16] Neuron numbers especially in the superior cervical ganglion are important in regulating sympathetic activity of the thyroid gland.[14] The sympathoadrenal system also closely interacts with thyroid hormone levels. Exaggerated responses to catecholamines or exposure to cold trigger thyroidal sympathetic activity and dominate the manifestations of thyrotoxicosis.[23] In contrast, unilateral transection of the superior cervical ganglion leads to decrease in the size of thyroid gland and reduction of follicle volume.[24] Acute superior cervical ganglionectomy causes significant depression in the thyroid economy.[15] It is important to highlight that superior cervical ganglion also project to the pineal and other salivary glands in the head.[25] Therefore, postganglionic nerve transection causes various histomorphological abnormalities on the related glands.

Beside the sympathetic and parasympathetic innervation, several studies draw special attention to the role of the serotonergic system in the modulation of the thyroid gland functions. It has been shown that serotonin might inhibit the secretion of thyrotropin by the pituitary gland, but has a direct stimulatory effect on thyrocytes mediated by the serotonin 5-HT2 receptors.[26] In hypothyroidism, synthesis and metabolism of serotonin in the brain are slowed down. In depression, reduction in the concentration of serotonin is accompanied by inhibition of the enzyme activity deiodinase type 2. Activation of 5-HTI receptor lead to increased levels of intracellular calcium, causing inhibition of the promoter of CGRP.

CGRP has a special importance among neuropeptides, because it is one of the most potent microvascular vasodilators identified to date. Its vascular relaxation effects are mediated via activation of a G protein-coupled receptor, called as calcitonin receptor-like receptor.

Vasoconstriction has been shown to be associated with a decrease in CGRP levels in nerves and an increase in CGRP levels in draining blood, suggesting that CGRP is released from nerves to oppose the vasoconstriction.[27] This might be an important mechanism in the pathogenesis of vasospasm after SAH; because, similar to the thyroid gland, the cerebral arteries also have sympathetic, parasympathetic, and sensory innervation. It is quite possible that SAH causes an imbalance in the neuronal regulatory mechanisms, which in turn leads to vascular smooth muscle contraction.[28] In humans, reduced levels of CGRP in the cerebral perivascular nerves have been associated with vasoconstriction and an enhancement in CGRP levels in blood draining from the external jugular vein, suggesting that CGRP is released antidromically from trigeminal sensory perivascular nerves to oppose the vasoconstriction.[29]

## Conclusion

In combination with the knowledge from the literature and findings of the current study, we suggest that control of thyroid follicle growth and its secretory activity is under the control of quite complex, multi-originated, yet incompletely understood innervation pattern. We postulated that underestimated role of an interganglionary link among superior cervical, thyroid, laryngeal, nodose, trigeminal and dorsal root ganglia might be important in the etiopathogenesis and/or treatment of SAH-associated vasospasm. Further immunohistochemical labeling of somatosensitive fibers in the thyroid gland following SAH will shed light into these issues.

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