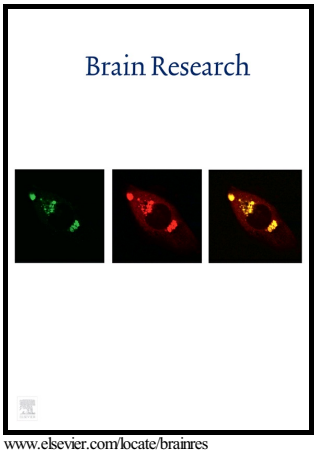

Short-term galvanic vestibular stimulation promotes functional recovery and neurogenesis in unilaterally labyrinthectomized rats

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Short-term galvanic vestibular stimulation promotes functional recovery and neurogenesis in unilaterally labyrinthectomized rats

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Abstract

Current experimental research on the therapeutic effects of galvanic vestibular stimulation (GVS) has mainly focused on neurodegenerative disorders. However, it primarily stimulates the vestibular nuclei and could be potentially effective in modulating imbalance between them in the case of unilateral labyrinthectomy (UL).

Fifty male Wistar rats (180-220 g) were used in 5 groups of 10: intact, sham, right-UL (RUL; without intervention), and two other right-UL groups with GVS intervention [one group treated with low rate GVS (GVS.LF; 6-7 Hz), and the other treated with high rate GVS (GVS.HF; 17-18 Hz)]. The UL models were prepared by intratympanic injection of sodium arsanilate. GVS protocols were implemented 30 min/day and continued for 14 days via ring-shaped copper electrodes inserted subcutaneously over each mastoid. Functional recovery was assessed by several postural tests including support surface area, landing and air-righting reflexes, and rotarod procedure. Immunohistochemical investigations were performed on ipsi- and contra-lesional medial vestibular nuclei (MVN) using bromodeoxyuridine (BrdU) and Ki67, as markers of cell proliferation.

Behavioral evaluations showed significant functional recovery of GVS-treated groups compared to RUL group. The percent of marked cells with BrdU and Ki67 were significantly higher in the ipsilesional MVN of both GVS-treated groups compared with other groups.

Our findings confirmed the effectiveness of GVS-intervention in accelerating static and dynamic vestibular compensation. This could be explained by the cell proliferation in ipsilesional MVN cells and rapid rebalancing of the VNs and the modulation of their motor outputs. Therefore, GVS could be promising for rehabilitating patients with unilateral vestibular weakness.

Abbreviations

GEHM, galvanic-evoked head movement; GVS, galvanic vestibular stimulation; GVS.HF, right labyrinthectomized rat stimulated by high-rate GVS; GVS.LF, right labyrinthectomized rat stimulated by low-rate GVS; RUL, right UL; UL: unilaterally labyrinthectomized; VC, vestibular compensation; VN, vestibular nucleus; VNs, vestibular nuclei

Keywords

Vestibular; galvanic; labyrinthectomy; BrdU; neurogenesis; rat

1 Introduction

Passing a brief electrical current between two electrodes on both mastoids or in both ears using bilateral bipolar galvanic vestibular stimulation (GVS) resulted in a simultaneous increase and decrease in the vestibular afferents' firing rate on the cathode-side and anode-side, respectively [1- 2].

GVS acted upon the spike trigger zone, which is situated between the vestibular sensory epithelium and the afferent terminals [3-4]. Therefore, post-synaptic mechanisms should be considered in explanations of GVS-evoked phenomena [3, 5].

GVS activates all primary vestibular afferents [3, 6], but the irregularly discharging (phasic) vestibular afferents have a higher galvanic sensitivity and a lower stimulation threshold compared to the regular ones [1, 3, 7-8]. However, non-vestibular inputs are not significantly affected by GVS [9]. Furthermore, GVS-induced modulation projects to the vestibular nuclei (VNs), secondary projection neurons from the VNs [10], and the multisensory vestibular centers, including the temporo-insular and temporo-parietal cortical areas [11]. Therefore, GVS could be considered a powerful and relatively pure vestibular stimulator impacting output motor actions of the vestibular system (the vestibulo-ocular and vestibulo-spinal reflexes) [12] and therapeutically impacting the widespread central vestibular network in various neurological and cognitive disorders [13-17].

Unilateral labyrinthectomy (UL) resulted in several static and dynamic balance symptoms in animals. Static symptoms are generated at rest, while dynamic symptoms are generated during motion [18]. Previous studies indicated that intratympanic injection of sodium arsanilate is very effective in inducing unilateral or bilateral chemical labyrinthectomy in rats [19-22]. The

researchers also noted that the damage was more extensive in the region of type I hair cells [22], which mainly connected to irregular discharge afferents known to be the main neural facilitators of GVS [3, 1]. Moreover, static and dynamic symptoms induced by unilaterally injected sodium arsanilate do not recover until about 36 and 42 days after injection, respectively [23]. Therefore, sodium arsanilate injections provide a long-term UL model for evaluating the potential therapeutic effects of GVS on UL.

The central nervous system (CNS), through a plasticity process known as vestibular compensation (VC), attempts to resolve UL-induced symptoms [24]. Of the structures involved in VC, including the VN, spinal cord, cerebellum, and cortical areas [24-27], the VNs play the most important role in VC [25] via the vestibular commissural inhibitory system that reciprocally connected bilateral VNs [24]. Thus, through a controlled excitatory-inhibitory stimulation like as GVS [9], it may be possible to constructively modulate the vestibular commissural inhibitory system to facilitate the recovery of static and dynamic vestibular symptoms.

The natural VC phenomenon could be effective in relieving static symptoms of UL [26-27]. However, evidence suggests that the modulation of phasic input gain to the contralesional VN is vital in compensating for dynamic symptoms following UL [28-29] because of the fact that after UL, the neuronal behavior of the contralesional and ipsilesional VN become more phasic and tonic, respectively [28-29]. Accordingly, it is interesting whether GVS, as a potential modulator of phasic inputs into VN, could be really constructive in rebalancing the phasic-tonic imbalance between VNs and consequently in accelerating static and dynamic post-UL VC. In addition, it is unclear whether any sign of plasticity could be traced in VNs, as a main area for VC, following GVS intervention.

To evaluate these assumptions, we explored the effectiveness of a short-term GVS intervention on functional behavioral recovery (using static and dynamic postural tests) and neurogenesis (using BrdU and Ki67 markers) in rats with chemical UL induced by intratympanic injections of sodium arsanilate.

1 Results

1.1 Behavioral observations

Behavioral observations of the UL rats clearly showed an apparent head-tilt (in the planes of roll and yaw), circling and falling toward the labyrinthectomized side. When the UL rats were lifted

by the tail, they spin around the long axis of their body for about 20–30 seconds. The limbs on the lesioned side were in flexion and adduction, while limbs on the intact side were extended and abducted. Although the circling and falling toward the lesioned side were predominantly observed during the first 3 days after TT injection, other signs were detectable until the final evaluations. None of these signs (i.e. classical postural and locomotor deficits [23]) were seen in the intact or sham groups.

During the procedure of GVS threshold detection in the intervention groups, we observed that the GEHM is almost always a leftward (i.e., contralesional) head rotation in the roll plane. Interestingly, the spatial plane and direction of the induced-GEHM was the same for both right-anodal and left-anodal stimulation (for review, see [DIB]).

1.2 Outcomes of the support surface measurement

Evaluation of the support surface area (SSA; in terms of cm^2) was performed with 4 groups at three time points (I, II, and III). As shown in **Fig. 1**, the mean SSA was about 35 cm^2 in intact and sham groups at all evaluations (note: SD of the SSA mean was nearly similar in all groups). Therefore, there was no significant difference ($p>0.9999$) between their SSA at evaluations I, II, or III. The mean SSA was about 68, 64, and 61 cm^2 at three time points respectively in RUL group. Moreover, there was no significant difference ($p>0.9999$) between SSA of intact and sham groups at each evaluation. A slight decrease in SSA occurred between evaluations I–III in the intact ($p=0.9708$), sham ($p=0.9283$) and RUL groups ($p=0.2128$), which was not statistically significant. Furthermore, it was about 70, 60, and 47 cm^2 at three time points respectively in both intervention groups. Thus, SSA significantly decreased in both intervention groups (GVS.LF, GVS.HF) from time points I–III ($p<0.0001$). At time points I and II, there was no significant difference ($p>0.9999$) between the RUL-GVS.LF and RUL-GVS.HF groups. However, a significant difference was seen in the RUL-GVS.LF ($p=0.0055$) and RUL-GVS.HF ($p=0.0080$) groups in evaluation III. Sham-RUL differences were statistically significant for each evaluation ($p<0.0001$). Sham-GVS.LF and sham-GVS.HF differences were statistically significant ($p<0.0001$) in evaluations I and II. The sham-GVS.LF and sham-GVS.HF differences were also significant ($p=0.0089$, $p=0.0062$) for evaluation III. The almost similar results were obtained for comparison of intact group with intervention groups.

1.3 Global reflex score outcomes

Evaluation of the GRS was performed for the 4 groups on three occasions (I, II, and III). The decrease in GRS was statistically significant ($p<0.0001$) between evaluations I and II, and between evaluations II and III for both intervention groups (**Fig. 2**). Differences between GVS.LF-GVS.HF scores were not statistically significant for any of the three evaluations (I and III: $p>0.9999$; II: $p=0.1490$). There was no statistically significant difference between GRS of intact and sham groups in each evaluation ($p>0.9999$). A slight GRS decrease over evaluations I–III was observed in RUL group, but it was not statistically significant ($p=0.0632$). At time point I, there was no significant difference ($p>0.9999$) between GRS for the RUL-GVS.LF and RUL-GVS.HF comparisons. However, a significant difference ($p<0.0001$) was obtained between the RUL-GVS.LF groups and RUL-GVS.HF groups in evaluations II and III. Sham-GVS.LF, and sham-GVS.HF score differences were statistically significant ($p<0.0001$) for the first and second evaluations. But at the third one, they were significant at the level of $p=0.0002$ and $p=0.0012$, respectively. The exactly similar results as sham group were obtained for comparison of intact group with intervention groups.

1.4 Rotarod performance test outcomes

Evaluation of the ST was performed for all 4 groups at three time points. As shown in **Fig. 3**, a significant ST increase occurred in the GVS.LF and GVS.HF groups over evaluations I-II ($p=0.0154$, $p=0.0002$), II-III ($p=0.0003$, $p=0.0002$) and I-III ($p<0.0001$, $p<0.0001$), respectively. There was no significant difference ($p>0.9999$) between GVS.LF and GVS.HF for any of the three evaluations. The same results ($p>0.9999$) were obtained for comparison of intact and sham groups. Slight ST increase over evaluations I–III were obtained for the intact, sham and RUL groups which were not statistically significant ($p>0.9999$). For evaluation I, there was no significant differences ($p>0.9999$) between ST for the RUL-GVS.LF and RUL-GVS.HF group comparisons. However, a significant difference in ST was obtained when comparing the RUL-GVS.LF groups in evaluation III ($p< 0.0001$) and between the RUL-GVS.HF groups for evaluations II ($p=0.0037$) and III ($p< 0.0001$). Sham-RUL, sham-GVS.LF, and sham-GVS.HF ST comparisons were statistically significant ($p< 0.0001$) for the first and second occasions. But at the third one, sham-GVS.LF comparison was statistically significant at $p=0.0005$ and sham-

GVS.HF comparison was not statistically significant (p=0.0650). The almost similar results as sham group were obtained for comparison of intact group with intervention groups.

1.5 Immunohistochemistry outcomes

1.1.1 BrdU

Figure 4 shows the percent of BrdU+ cells (**Fig. 5**) in the ipsilesional (right) and contralesional (left) MVNs. On the right-side, all pairwise comparisons between groups were statistically significant (p=0.0396 for GVS.LF versus GVS.HF and p< 0.0001 for all other comparisons) except of intact-sham comparison ((p>0.9999). Conversely on the left MVN, all pairwise comparisons resulted in statistically insignificant findings (p>0.9999 for all comparisons). Moreover, the maximum cell proliferation on the right-MVN was occurred for GVS.HF group followed by GVS.LF, and RUL groups. Cell proliferation was negligible in the right-MVN of sham group and in the left-MVN of all groups.

1.1.2 Ki67

All pairwise comparisons between groups in terms of the percent of ki67+ cells were statistically significant on the ipsilesional MVN (p=0.0005 for sham versus RUL, p< 0.0001 for sham/RUL versus GVS.LF/GVS.HF, and p=0.0458 for GVS.LF versus GVS.HF) except of intact-sham comparison ((p>0.9999). On the left MVN, all pairwise comparisons resulted in statistically insignificant findings (p>0.9999 for all comparisons). Therefore, the overall results for Ki67 was exactly the same with that of BrdU on both right- and left-MVNs.

2 Discussion

The main concern of the paper was to survey the effect of a short-term (14-day) low-rate and high-rate GVS intervention on vestibular rebalancing in right UL rats prepared with intratympanic injection of sodium arsanilate. Particular attention is paid to the functional behavioral recovery which assessed by the changes in support surface area (SSA), global reflex score (GRS: the sum of scores obtained in landing and air-righting reflex tests), and rotarod performance test results. The evaluations confirmed the significantly faster recovery of static and dynamic balance symptoms in both intervention groups (GVS-LF and GVS-HF) compared with

untreated group (RUL). We have also considered immunohistochemical study using BrdU and Ki67 markers to examine neurogenesis induced by GVS intervention in the medial vestibular nuclei. Remarkably, we were able to trace the $BrdU^+$ and $Ki67^+$ cells in the right medial vestibular nuclei of both intervention groups which was not observed in the other groups (i.e. intact, sham and RUL groups). To our knowledge, this is the first study to deal with GVS intervention in UL models.

2.1 Specifications of our GVS method

To deliver GVS intervention in unrestrained rats in order to avoid repeated anesthesia, we used ring-shaped mastoidal electrodes. The ring-shaped electrode provides a glue-free and screw-free but a secure implantation technique (based on our follow-up of its stability within 2 months). Moreover, the animals could not break down the electrodes which is possible even in the deeper implantation. This is important because attaching connectors to the short electrodes may hurt the animal's skin particularly during long-term stimulation. Furthermore, we recorded polarity-dependent GEHMs in the intact and sham groups which confirms the accuracy of the method [12].

2.2 GVS-intervention promotes the recovery of static and dynamic symptoms

During our intervention period, the mean SSA of normal and sham groups was nearly stable at about 35 cm^2 while a nonsignificant reduction in SSA from about 68 to 61 cm^2 observed in the RUL group and a significant SSA reduction from about 70 to 47 cm^2 recorded in both intervention groups (note: SD of the SSA mean was nearly similar in all groups). So, significant decrease in the mean SSA of the intervention groups confirmed significant static postural recovery which is considered as a good estimator of the behavioral vestibular adaptation to the induced vestibulospinal deficit [30]. Consequently, our GVS intervention was really effective in restoring static postural function via modulating the vestibulospinal pathways. Furthermore, as mentioned above, the arsanilate-induced damage was more extensive in the region of type I hair cells which mainly connected with the irregular discharge afferents [22]. These fibers are the most sensitive vestibular afferents to GVS [1, 3, 7-8] and the main input source for the vestibulospinal projection [31] that underlies the postural asymmetries after UL [32-33]. Accordingly, GVS intervention can compensate the loss of irregular phasic signal and in this

way, accelerate the vestibular adaptation to UL. However, electrophysiological recording in the vestibular nuclei is required to confirm the issue.

The same general pattern of SSA modification during the intervention period was also observed for the GRS (i.e. stable GRS of about 0 in the intact and sham groups; abnormal GRS of about 4 in the RUL group at all evaluations; and increasingly improved GRS from 4 to nearly 0 in the intervention groups). The score of air-righting reflex which attributed to the neural connections between otolithic organs and pontine reticular formation (PRF) [34] could be considered as an estimator of labyrinthine integrity [35]. The score of landing reflex which resulted from otolithic-ocular responses reflects the rat's response to vertical linear acceleration [23, 36]. Both scores have significantly improved by our GVS intervention. Consequently, GVS intervention can modulate the GRS, as a dynamic balance indicator, through influencing otolithic-PRF and otolithic-ocular neural networks.

The time of walking on the rotarod beam during our intervention period was also revealed the same stable pattern in the intact and sham groups, falling and trying to just stand at the first and the next evaluations, respectively, in the RUL group, and considerable progress from falling to walking in both intervention groups just over 7 days of GVS intervention. The rotarod performance test considered to be a reliable assessment of vestibulo-motor coordination [37-38] so that even vestibular dysfunction due to vitamin D deficiency in a mutant mice model [39] and also balance recovery using gene-therapy in a mice model of bilateral vestibular weakness [40] have been studied via this test. Moreover, studies on Parkinson's disease indicated that the improvement in locomotion and postural control after GVS intervention which attributed to a kind of neuromodulation mechanism resulted from its facilitatory effect on the vestibulo-spinal and other non-dopaminergic pathways [41]. As well, the improved rotarod performance in a hemiparkinsonian rat model was attributed to the distinct inhibitory role of noisy-GVS on the ipsilesional substantia nigra [41]. Therefore, with regard to our findings, it could be claimed that GVS intervention can significantly increase vestibulo-motor coordination in UL models through modulating vestibulo-spinal pathway which probably resulted from its facilitatory effect on vestibular nuclei.

Altogether, according to the usual recovery process of static (postural) and dynamic (locomotor) symptoms induced by intratympanic arsanilate injection, which takes 36 and 42 days at least [23], our findings in the static and dynamic tests showed significant symptom recovery in the

GVS.LF and GVS.HF groups within 7–14 days of the GVS intervention which didn't observe in the RUL group. These findings support the fact that GVS–induced modulation can result in significantly accelerated static and dynamic vestibular recovery.

2.3 The recovery dilemma in the arsanilate-induced UL

The protocol of arsanilate-induced UL used in our study, resulted in classical postural (examined by SSA) and locomotor (examined by GRS and rotarod) deficits indicated as post-UL deficits in the literature [19-23]. However, the recovery of the arsanilate-induced symptoms is much longer than that reported after tetrodotoxin-induced or mechanical-induced UL in rats [42] and cats [43]. Moreover, the delayed compensation of spontaneous nystagmus and head tilt in the arsanilate-induced UL models compared with that in the surgically-induced UL models was already reported [44]. Interestingly, Besnard et al. [21] considered a 3-week recovery period between two separate arsanilate injections in the right and left tympanum for preparing a bilateral vestibular lesioned model in rats. Therefore, the arsanilate-induced labyrinthectomy should be considered as a unique chemical labyrinthectomy which has similar consequences and compensation time course (more than 30 days at least) reported for vestibular neurectomy [for review, see 43].

Despite earlier indications about arsanilate-induced degeneration in the eighth cranial nerve [45-46], a recent study demonstrated that arsanilate-induced toxicity does not involve the Scarpa's ganglion at least within 3 months after intratympanic injection [22]. But the authors indicated that arsanilate-induced toxicity may damage synaptic contacts on calyx and bouton units [22]. As a result, the damage is not only irreversible as opposed to the tetrodotoxin-induced UL [22, 43] but also it probably distorts the electrophysiological input to the ipsilesional vestibular nuclei in contrast to the mechanical UL and vestibular neurectomy which completely disconnected that input [43, 47]. Consequently, any intervention (like as GVS intervention) that improves the recovery time course after arsanilate-induced UL will have to overcome this distortion. We can hypothesize that because GVS will potentially activates all primary vestibular afferents [3, 6] through excitable synaptic contacts in the arsanilate-UL model [22], it can overcome the distorted vestibular signal through the creation of more effective temporal synchrony [48] especially for the irregular phasic neural signal. Recently, the modulatory effect of noisy-GVS

on beta and gamma brain rhythms is reported [49]. Clearly, further study is needed to verify the issue.

2.4 GVS-intervention: its facilitatory and therapeutic roles

In addition to the facilitatory effects of GVS on the neurologic disorders like as hemi-spatial neglect [13-17] and Parkinson disease [41, 50], recent studies revealed that GVS-intervention could significantly increase the willingness of a normal person for dealing with novel vestibular situations probably through reweighting of balance inputs at the cerebellar level [51-52]. Moreover, GVS-intervention can play the same adaptive role for patients with Meniere's disease who are scheduled to undergo gentamicin-therapy. This means that such patients that experienced "pre-habilitation" will experience a less severe imbalance after UL [53]. On the other hand, the arsanilate-induced UL could be considered as a model of common vestibular disorders like as vestibular neuritis, or Ménière's disease [22], as opposed to that mentioned for tetrodotoxin-induced UL [43], thus, with regard to our findings, the GVS intervention could be promising for rehabilitating such patients. Our findings would be of interest when we consider that the main aim of a vestibular rehabilitation therapy should be focused on restoring the dynamic balance function [54].

2.5 GVS-intervention and contralesional head movements in UL models

An interesting point that should be noted is that, in addition to the potential facilitatory-inhibitory or tonic-phasic effect of GVS, the contralesional head movements probably contribute to the ultimate therapeutic role of our GVS intervention (for review, see [DIB]). Previous research on rotational testing has demonstrated that contralesional head rotations can be considered a stimulator for a more rapid recovery of UL symptoms [55-56]. Therefore, one question still unanswered is whether GVS presentation at a lower intensity level, which does not provoke head movement, could have the same beneficial effects on balance recovery.

2.6 GVS-intervention and neurogenesis

The reorganization of neural connections underlies behavioral recovery [57] and whenever this behavioral recovery is more pronounced, neurogenesis is more likely [58]. Therefore, in light of

the significant behavioral improvements observed in the present study, the obtained neurogenesis is not unreasonable.

Unlike the dual effect of GVS on cell proliferation in the hippocampus and on spatial memory that has been recently reported [59], our study has shown that cell proliferation in the ipsilesional MVN is associated with behavioral recovery in GVS.LF and GVS.HF groups. This finding is consistent with the results of previous studies, which found that exercise promotes cell proliferation in the mouse hippocampus [60-61].

Similar results observed with both markers (BrdU, as a marker of DNA replication, and Ki-67, as a reliable marker for mitosis [62-63]) may indicate that the labeled-cells are still in the cell cycle [59]. On the other hand, it should be noted that "even a small number of neurons can influence behavior [64]." Moreover, several studies indicated that following UL in cats, reactive neurogenesis is expected [65-66] which is more likely in the models prepared by vestibular neurectomy [43]. Thus, it could be expected that GVS–induced functional recovery in the present study is rooted in the cell proliferation within the ipsilesional MVN which could be considered an intervention-related neurogenesis. Clearly, further research using specific neuronal or glial markers will be required to more convincingly prove the phenotype of marked cells as neuronal or glial ones.

2.7 GVS.LF group versus GVS.HF group

Adaptive regulation of GABA receptors following UL potentiated the firing rates and increased the sensitivity of vestibular neurons in response to head movements, regardless of head movement frequency [67]. Thus, the impact of high-rate GVS on behavioral recovery should not be significantly different from that of low-rate GVS. Our behavioral findings in the GVS.LF and GVS.HF groups are in agreement with these expectations.

However, cell proliferation was significantly higher in the GVS.HF group. This could be attributed to the fact that high-rate GVS has a more phasic characteristic that could be more successful in inducing the required inhibition for vestibular plasticity. Moreover, it preferentially activates otolithic afferents and the reticulo-spinal pathway, which is a stronger input for adjusting standing posture, while low-rate GVS preferentially activates semicircular canal

afferents and the vestibulo-spinal pathway [68]. However, further research of the issue would be of interest.

2.8 Conclusions

Our findings confirmed the effectiveness of short-term GVS intervention in accelerating static and dynamic vestibular compensation. This could be explained by the cell proliferation in ipsilesional MVN cells and rapid rebalancing of the VNs and the modulation of their motor outputs. Therefore, GVS could be promising for rehabilitating patients with unilateral vestibular weakness. The findings also suggest that this approach could be useful when designing vestibular prostheses.

3 Experimental Procedure

All procedures and experiments described below have been audited and endorsed by the Animal Research Ethics Committee of the Tehran University of Medical Sciences furthermore by the Animal Care Committee of the University of Social Welfare and Rehabilitation Sciences. Financial support was provided by the University of Social Welfare and Rehabilitation Sciences.

3.1 Animals

Fifty male Wistar rats (180-220 g) were used in 5 groups of 10: 1- intact group; 2- sham group (rats with intratympanic injection of saline solution); 2- RUL group (right labyrinthectomized rats without GVS intervention); 3- GVS.LF (right labyrinthectomized rats that underwent a low-rate GVS intervention); and 4- GVS.HF (right labyrinthectomized rats that underwent a high-rate GVS intervention). Thirty rats including RUL, GVS.LF, and GVS.HF groups were modeled as right-UL by intratympanic injection of sodium arsanilate.

Animals were housed 3-4 per cage under constant temperature (22±1°C) with 12 h light /dark cycle. Food and water were freely accessible.

3.2 Chemical Labyrinthectomy and intratympanic saline injection

Unilateral labyrinthectomy was carried out by intratympanic injection of sodium arsanilate following the approaches of Horn et al. [19] and Besnard et al. [21].

All treated rats were anesthetized by an intraperitoneal (IP) injection of a solution of Ketamine (100 mg/kg, Alfasan, Netherlands) and Xylazine (10 mg/kg, Alfasan, Netherlands). Arsanilate solution was prepared by dissolving 300 mg of sodium arsanilate (Sigma-Aldrich) in 1 ml of 0.9% saline solution. Each UL rat was received a single-dose (0.1 ml) of the arsanilate solution transtympanically (TT) while underwent a 30-45 min anesthesia.

Intratympanic injection of sodium arsanilate was made by advancing injection needle (1 ml insulin syringe with 0.8 mm diameter) through anterior part of the right tympanic membrane until resistance of the middle-ear ossicles was met. Then the needle pulled slightly back and injection was completed during 4-5 seconds. Subsequently the ear canal was firmly packed with small pieces of absorbable hemostat (Ethicon, Australia). After the rats become fully alert (about 45-70 min later), we put them back to their cages. For preventing otitis media, all rats received an IP injection of 50 mg/Kg of amoxicillin (Sigma-Aldrich) twice a day for 3 days.

In the sham group, intratympanic saline injection (0.1 ml of 0.9% saline solution) was exactly made by the same procedure as arsanilate injection in UL rats.

3.3 Electrode preparation and implantation

For preparing our GVS electrodes, short lengths of uninsulated copper wires (with diameter of about 1 mm and length of about 50 mm) were selected. Each wire piece was placed in the tail of a gray angiocath needle (with diameter of 1.7 mm and length of 45 mm) just like threading a needle. Then the copper wires were inserted subcutaneously behind each ear by using the angiocath needle. The exact place of electrodes was about 1 cm of each pinna and parallel to the angle of them. Finally, free tails of wire twisted to form a "ring-shaped" electrode for attaching alligator-connectors of our GVS device. The length of subcutaneous part was about 5 mm (**Fig. 6**). Electrode implantation was performed under the same anesthesia protocol mentioned above and conducted within 24 hours after TT injection for RUL, GVS.LF, and GVS.HF groups. For preventing skin infections due to electrode embedding, Tetracycline-NAJO 3% ointment used twice a day for 3 days.

3.4 GVS intervention

3.4.1 GVS-threshold

Before initiating the GVS intervention that should be done in the GVS threshold level, we determined that level through delivering bilateral GVS (rectangular pulses, 1-2 Hz random

frequency, and 5-ms pulse duration) in the GVS.LF and the GVS.HF groups. The left mastoid was selected for anodal stimulation. The GVS-intensity was gradually increased from zero until an obvious repeatable 1-2 Hz GEHM was clearly observed. The lowest intensity that induced an apparent GEHM considered as the GVS-threshold. Consequently, the GVS-threshold was obtained in the range of 80-85 μA for different rats and kept specifically constant during GVS intervention.

3.4.3 GVS intervention protocol

GVS intervention was done in freely-moving rats, and at threshold level with randomized presentation rate of 6-7 and 17-18 Hz on the GVS.LF and the GVS.HF groups, respectively. The former rate (6-7 Hz) considered as a low-rate stimulator for mostly exciting the semicircular canals' afferents and the latter rate (17-18 Hz) as a high-rate stimulator for mostly stimulating otoliths' afferents [46]. Furthermore, randomized frequency was selected to reduce any potential effect of adaptation. The 14-day period of intervention was initiated within 30-36 hours after TT injection in both groups. For each rat, GVS intervention comprised 30-min continuous stimulation per day. Moreover, the left mastoid was chosen for presenting anodal stimulation.

3.5 Behavioral evaluation

Behavioral evaluations comprised one type of static balance test and three types of dynamic balance tests, each administered at three time points (I, II, and III): I) before the GVS intervention (26–30 hours after TT injection); II) 1 week after initiating the GVS intervention; and III) 2 weeks after the initiation of the GVS intervention. The static balance test consisted of calculating the support surface area. The dynamic balance tests included assessments of the landing reflex and the air-righting reflex, as well as the rotarod performance test.

3.5.1 Support surface area

A plexiglas box (16 × 28 × 25 cm) with a laser-engraved floor, comprised 5 × 5 mm squares, was prepared for evaluating the support surface area (**Fig. 7**). During each evaluation the rats moved freely and their motions were recorded by a video recorder camera (Canon, IXUS 100 IS, 12.1 megapixels, 30 fps) focused on the box floor. For each rat at each time point, at least one-minute video clip was obtained. Five intermittent pictures, taken at moments when all four paws were

touching the box floor, were selected from each video clip. The selected pictures were prepared by slowing down video clips, cutting, and extracting the pictures using Corel VideoStudio software (ver. Pro X5). Then, the support surface area in each selected picture were precisely determined by connecting the measurement lines fitted to the middle of each rat's paw using Dinolite Digital Microscope software (ver. 3.2.0.5) (**Fig. 7**). Afterward, the calculated support areas for the 5 pictures were averaged to estimate the mean support surface area (in cm^2) for each rat at each time point.

3.5.2 Landing reflex

To assess the landing reflex, rats were held by the tail and lowered toward the surface of a table. The proper response involves neck flexion and forelimb extension when approaching the table, prior to either body part contacting the surface. A 0 score was given for the proper response, a score of 1 for an imperfect response (minor body contact before correct response) and a score of 2 for a complete failure (or no reflex) [23].

3.5.3 Air-righting reflex

To test the air-righting reflex, each rat was kept in a supine position by the examiner's hands about 50 cm above a cushion. The examiner detached his hands quickly to induce a sudden drop and observed the rat's body as it contacted the cushion. A 0 score was given for a proper response (landing on four limbs), a score of 1 for an imperfect response (landing on the body side), and a score of 2 for a complete failure (landing on the back) [23].

3.5.4 The global reflex score

The global reflex score (GRS) for each rat was obtained by adding the scores of both reflex tests for a given time point (I, II, or III) to obtain an integer score of 0–4. This global score was used for statistical analysis instead of considering individual reflex test scores [23].

3.5.5 The rotarod performance test

The rotarod performance test was used to evaluate vestibular balance recovery after UL [69-72]. The test was conducted using Rotorod system (IITC Life Science, Series 8, USA). The test consisted of 3 trials at each time point: 2 training trials and 1 evaluation trial [48]. The initial

speed and the ramp were set to 5 and 1.17 round-per-minute, respectively. The parameter of interest was the latency of fall, or stop time (ST). The ST was defined as the duration (in seconds) the rat could walk on the rod before falling.

3.6 BrdU labeling

To mark proliferating cells, all animals were intraperitoneally injected with BrdU (50 mg/kg; Sigma-Aldrich) once daily from days 11–15 following UL surgery.

3.7 Perfusion, fixation, and tissue preparation

Three hours after the final BrdU injection, rats were anesthetized by intraperitoneal injection of a ketamine (100 mg/kg, Alfasan, Netherlands) and xylazine (10 mg/kg, Alfasan, Netherlands). The depth of anesthesia was confirmed by the pinch-response method. At that point, rats were thoracotomised and perfused with 4% paraformaldehyde solution via a transcardial perfusion procedure. Subsequently, the brain was removed and immersed in a same fixative solution overnight at 4°C. Then, the fixed brains were divided into 3 segments, where the caudal segment was comprised the cerebellum and medulla oblongata [73]. The specimens were dehydrated using a graded concentration of ethanol embedded in paraffin blocks. Then, the specimens were serially sectioned into 5 μm–thick coronal sections that enclosed the entire VN. Afterward, the sections were mounted on polylysine-coated slice glasses, stained with hematoxylin–eosin, and immunohistochemical visualizations were performed to enable microscopic examination.

3.8 Immunohistochemistry

Immunohistochemical investigations were performed on the prepared sections of ipsi- and contra-lateral VN. These investigations used a single labeling of BrdU and a single labeling of Ki-67.

After deparaffinization and rehydration of coronal brain sections, the slides were rinsed 3 × 5 min in PBS. Then the blocking buffer (0.3% Triton-X100 and 10% goat normal serum in PBS) was applied for 1 h at 37°C. At that time, 2M HCl was used for antigen retrieval, and PBS washing was performed again. Then, rabbit monoclonal anti-BrdU antibody (Roche) at a 1:200 dilution and rabbit polyclonal Ki-67antibody (Abcam Ltd) at a 1:100 dilution were separately applied to 1 slide each. The slides were incubated overnight at 4°C. Afterward, they were washed

with PBS (3 × 5 min) and incubated in 0.3% H_2O_2 solution for 30 min, followed by washing with PBS for 3 × 5 min.

The tissue slides were incubated with avidin-biotin complex secondary antibody (Abcam Ltd) for 1 h at room temperature. Then samples were immersed in a freshly prepared chromogen substrate reagent solution for 10 min. Finally, the sections were mounted and prepared for microscopic evaluation. For counterstaining, hematoxylin staining was performed for about 1 min.

3.8.1 Microscopy and quantification method

A light microscope (Nikon, Japan) equipped with a digital video camera was used to prepare the representative images (1000x). For each rat, 10 random sections were selected from the rostral (-10.56 mm from Bregma) to the caudal (-12.60 mm from Bregma) regions of the brainstem based on Paxinos and Watson's stereotaxic atlas of the rat [74]. To count the number (and calculate the percentage) of BrdU-labeled or Ki-67–labeled cells, we selected 5 random areas of each selected section at 1000x, with an overall area of about 4860 μm^2 for each slide. It should be noted that these areas were mainly confined to the medial VN (MVN), without any differentiation between magnocellular and parvocellular sections (**Fig. 8**). The results acquired from the areas of all sections were pooled for each rat and their overall mean was considered to be n=1. The percent of $BrdU^+$ cells (or Ki-67^+ cells) was calculated using the number of BrdU-labeled cells (or Ki-67–labeled cells) divided by the total cells in the inspected area. Neuronal cells were identified by these criteria: a central nucleolus, a typical nucleus, observable cytoplasm, existence of dendritic processes, and larger cell body size. The following criteria were considered as characteristic for glial cells: sparse cytoplasm, and smaller cell body size [75] (**Fig. 9**). It should be noted that only neuronal labelled cells were considered in the final analysis.

3.9 Statistical analysis

For analyzing "support surface area" (3 times among 4 groups), "the global balance scores" (3 times among 4 groups), scores of "latency of fall" (3 times among 4 groups), percent of "BrdU-labelled cells" (right-MVN versus left-MVN among 4 groups), and percent of "Ki67-labelled cells" (right-MVN versus left-MVN among 4 groups), mixed between-within groups ANOVA were used. $P< 0.05$ was considered as statistically significant and "Bonferroni correction" was

used for multiple comparisons. GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) was used for statistical analysis and preparing graphs.

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Figure legends

Fig 1. Evaluation of the support surface area (SSA). The measurement (cm^2; mean ±SD; n=10) was performed on three occasions (I, II, and III). The histograms reveal significant SSA-decrease in both intervention groups from I-to-III experiments (*p < 0.0141, **p = 0.0183, ***p < 0.0001, ****p = 0.0003). There was not any significant difference between them in either of three evaluations (p>0.9999). A slight SSA-decrease (i.e. I-III difference) is observable for intact (9708), sham (p>0.9283) and RUL (p=0.2128) groups which is not statistically significant. I: before GVS intervention; II: one week after GVS intervention; and III: two weeks after GVS intervention.

Fig 2. Evaluation of the global reflex score (GRS). The measurement (predefined scores; mean ±SD; n=10) was performed on three occasions (I, II, and III). The histograms reveal significant GRS-decrease in both intervention groups from I-to-II, and II-to-III experiments (*p < 0.0001). There was not any significant difference between them in either of three evaluations (I and III: p>0.9999; II: p=0.1490). A slight GRS-decrease (i.e. I-III difference) is observable for RUL group which is not statistically significant (p=0.0632). I: before GVS intervention; II: one week after GVS intervention; and III: two weeks after GVS intervention.

Fig 3. Evaluation of the stop time (ST). The measurement (seconds; mean ±SD; n=10) was performed on three occasions (I, II, and III). The histograms reveal significant ST-increase in both intervention groups from I-to-II, and II-to-III experiments (*p= 0.0154, **p=0.0003, ***p<0.0001, ****p = 0.0002). There was not any significant difference between them in either of three evaluations (p>0.9999). A slight ST-increase (i.e. I-III difference) is observable for intact, sham and RUL groups which is not statistically significant (p<0.9999). I: before GVS intervention; II: one week after GVS intervention; and III: two weeks after GVS intervention.

Fig 4. Evaluation of the BrdU-labelled cells ($BrdU^+$). The histograms reveal significant BrdU+ cells in the ipsilesional MVN of both intervention groups. All pairwise comparisons in the right-MVN showed statistically significant difference (p=0.0396 for GVS.LF versus GVS.HF and p<0.0001 for all other comparisons) except of intact-sham comparison (p>0.9999). MVN: medial vestibular nucleus.

Fig 5. BrdU-labelled cells ($BrdU^+$) in the right medial vestibular nucleus. The above pictures reveal no $BrdU^+$ Cells in the sham and RUL groups. The lower pictures show the $BrdU^+$ Cells (marked with arrows) in the GVS.HF and the GVS.LF groups. Rod length=10µm.

Fig 6. Ring-shaped electrode. This electrode type was made by a short length of flexible copper wire (with diameter of about 1mm). The wire piece was placed in the tail of a gray angiocath needle. Then the copper wire was inserted subcutaneously behind each ear (paralleled to the

angle of pinna) by using the angiocath needle just like as threading a needle and stitching. Finally, free tails of wire twisted to form a "ring-shaped" electrode for attaching alligator-connectors of our GVS device.

Fig 7. The plexiglas box and evaluation of support surface area (SSA). This box with laser-engraved floor used for SSA evaluation. The left picture shows a rat of GVS.LF group (No. II) during the first evaluation (i.e. before GVS-intervention). The right picture shows the same rat on the second evaluation (i.e. 2 weeks after initiation of GVS-intervention). For calculation of the SSA, the measurement lines are connected the middle part of paws to each other in order to determine the distance between them (in mm) as well as the circumference (in mm) and area of the resulting rectangle (in mm^2).

Fig 8. Medial vestibular nuclei (MVNs). The upper picture shows a slice encompassed the floor of fourth ventricle and approximate locations of medial vestibular nuclei (rod length: 100μm). The lower picture shows the estimated borders of MVNs where our study was conducted (rod length: 100μm). 4V: 4th ventricle; chp: choroid plexus; mlf: medial longitudinal fasciculus; MVeMC: medial vestibular nucleus magnocellular part; MVePC: medial vestibular nucleus parvocellular part; Pr: prepositus nucleus; SpVe: Spinal vestibular nucleus.

Fig 9. Neuronal, glial and capillary cells in MVNs. Neuronal cells (N) were identified by these criteria: a central nucleolus, a typical nucleus, observable cytoplasm, existence of dendritic processes, and larger cell body size. The following criteria were considered as characteristic for glial (G) cells: sparse cytoplasm, and smaller cell body size. Capillaries (C) usually appear as a hole or cylinder in the tissue in which cells (and everything else) is conspicuously absent. Here, endothelial cells lining the walls of the capillary.

Highlights

- GVS intervention is proposed for rehabilitating the UL rats.
- GVS intervention promoted the recovery of static balance symptoms following UL.
- GVS intervention promoted the recovery of dynamic balance symptoms following UL.
- GVS intervention resulted in neurogenesis in the ipsilesional MVN.
- High-rate GVS is relatively more efficient in provoking neurogenesis.

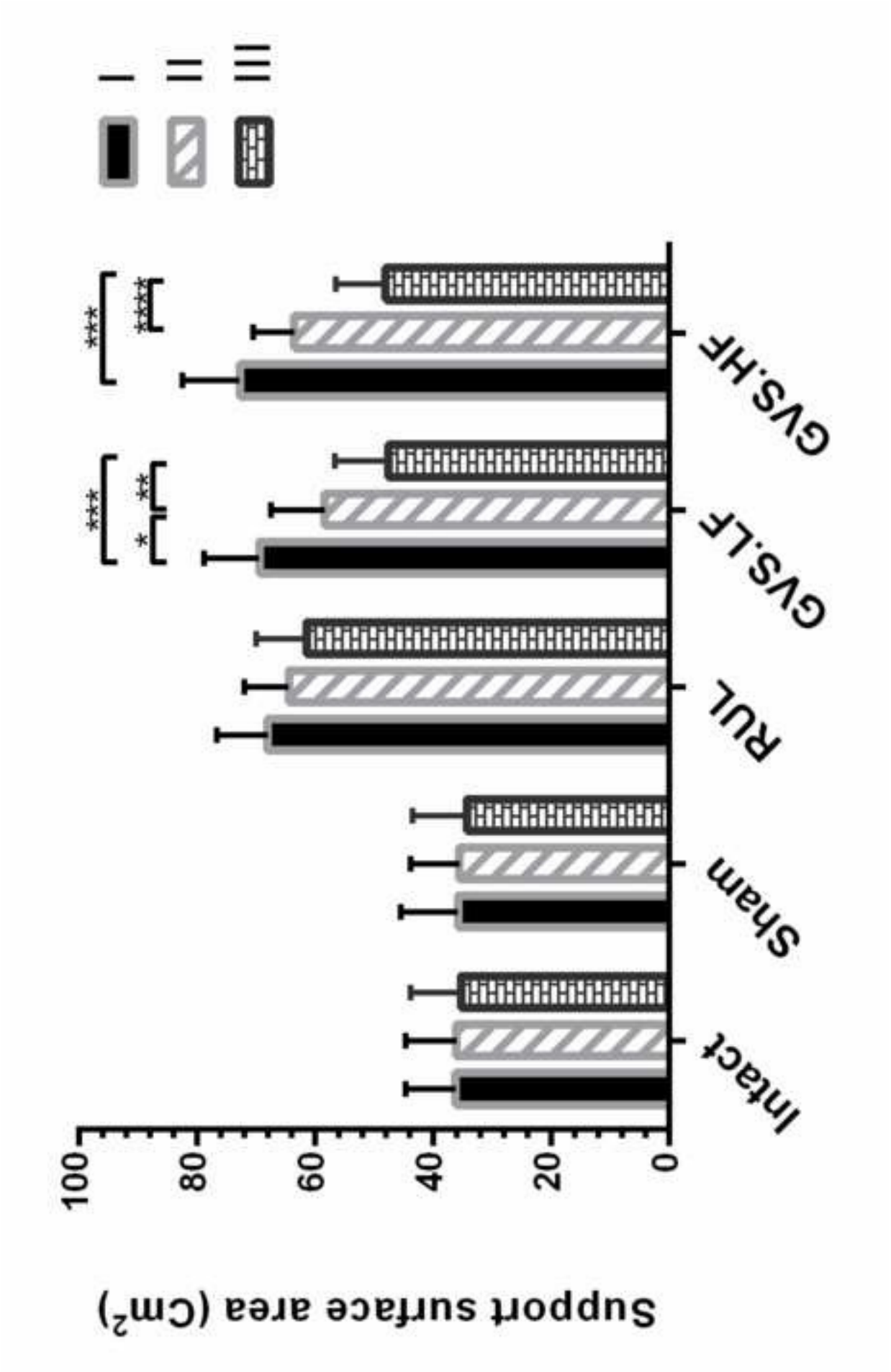
Support surface area (Cm²)
0
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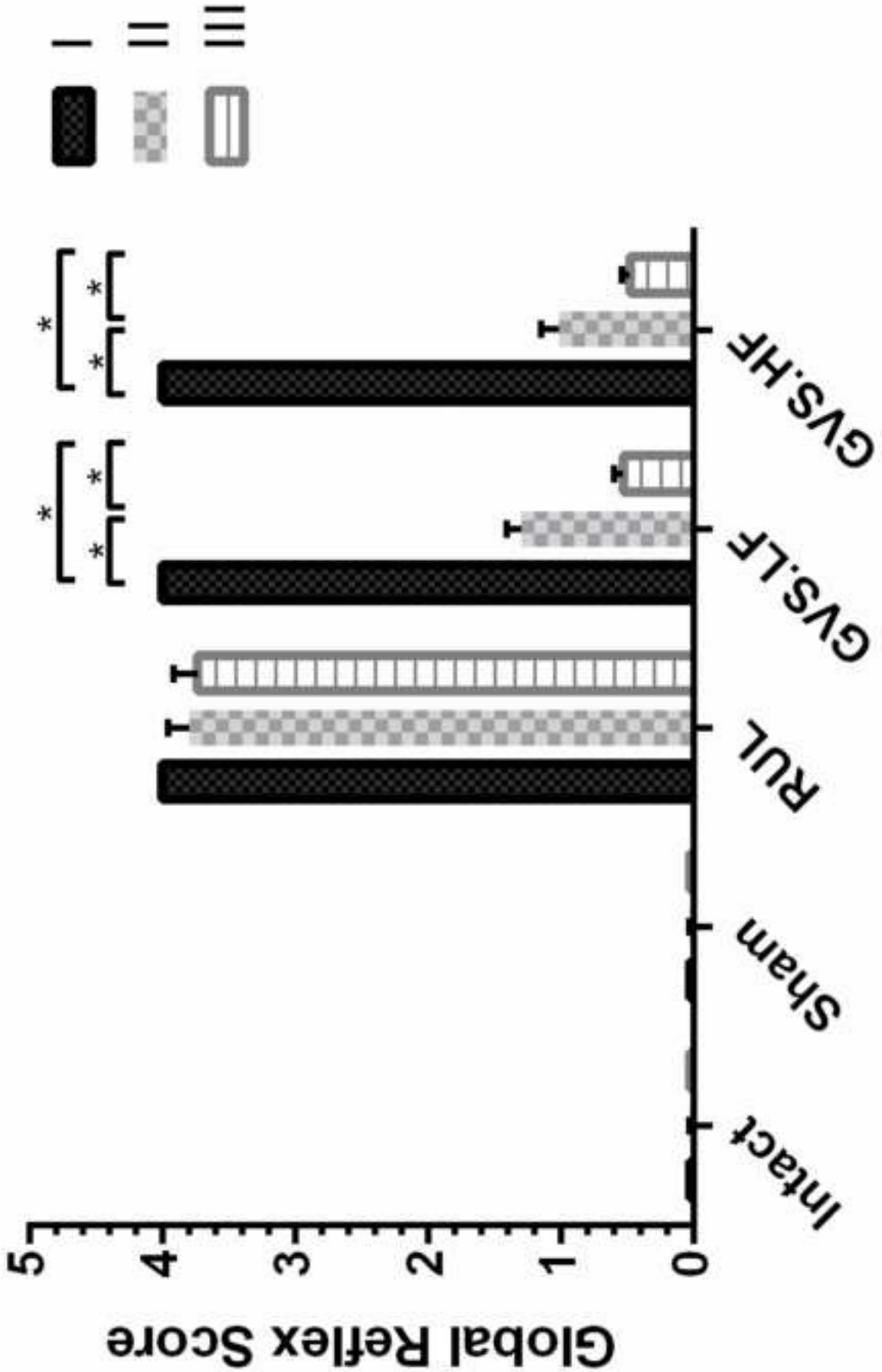
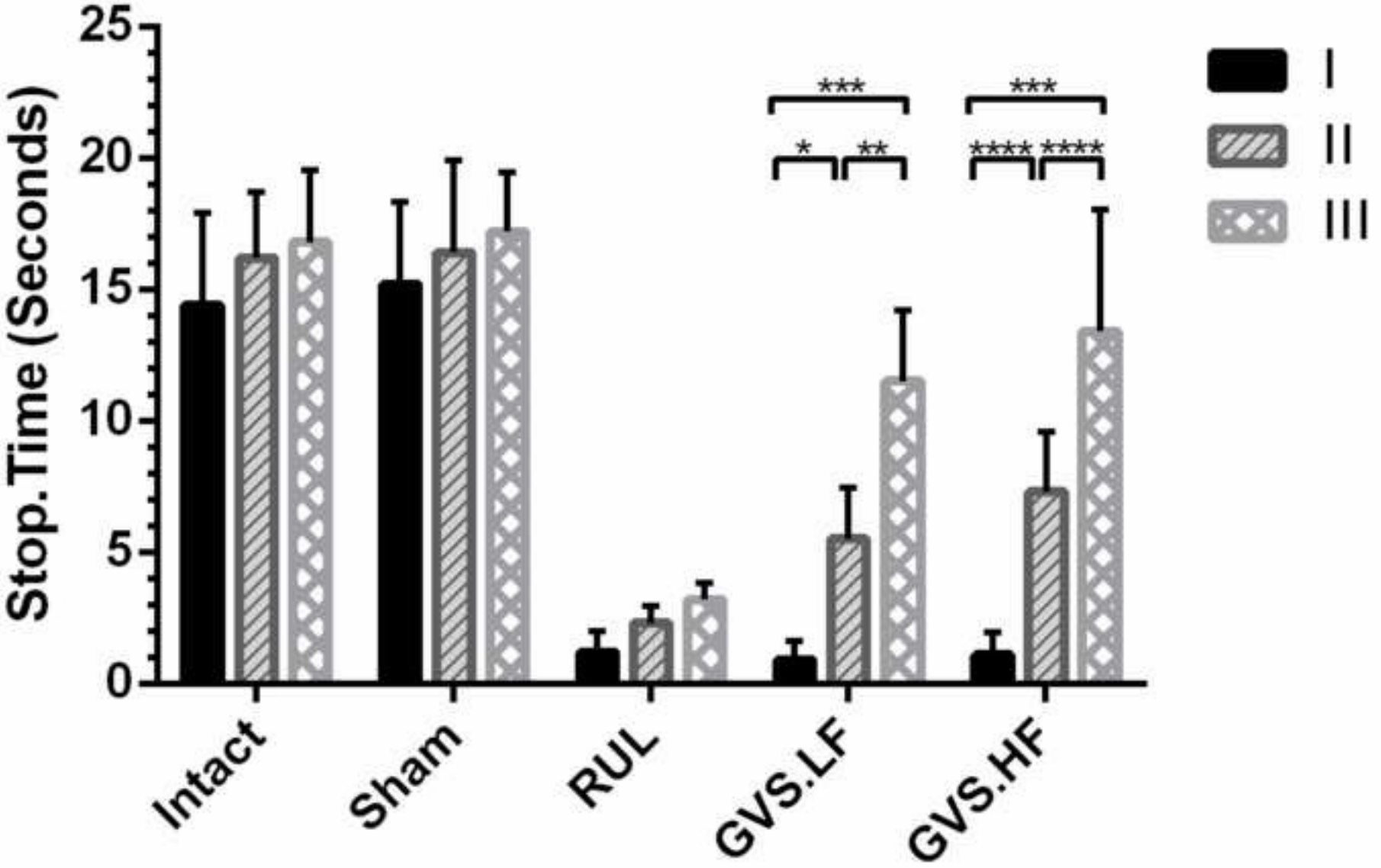
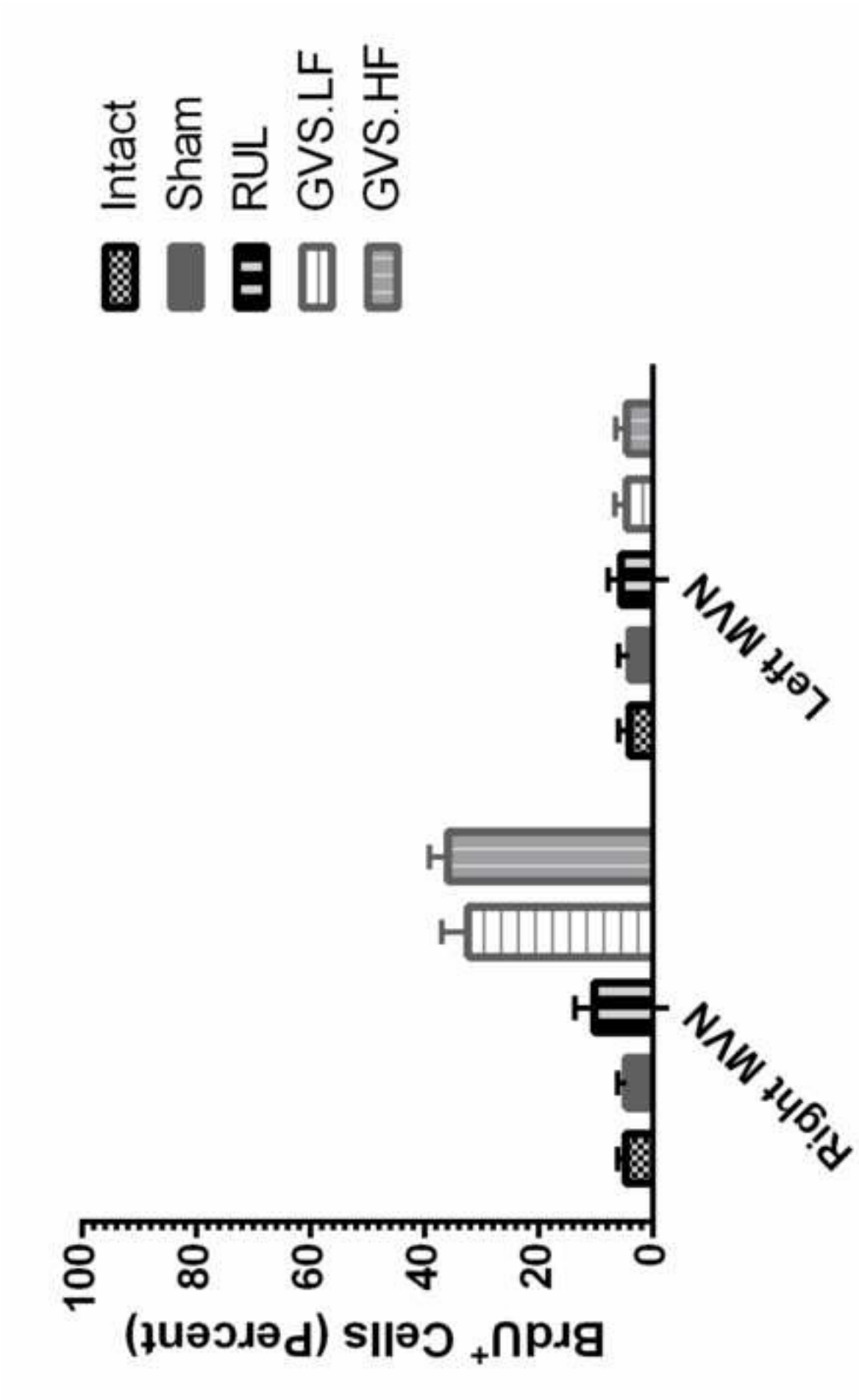
Figure


Global Reflex Score
0
1
2
3
4
5
Intact
Sham
RUL
GVS.LF
GVS.HF
*
*
*
*
*
*
I
II
III

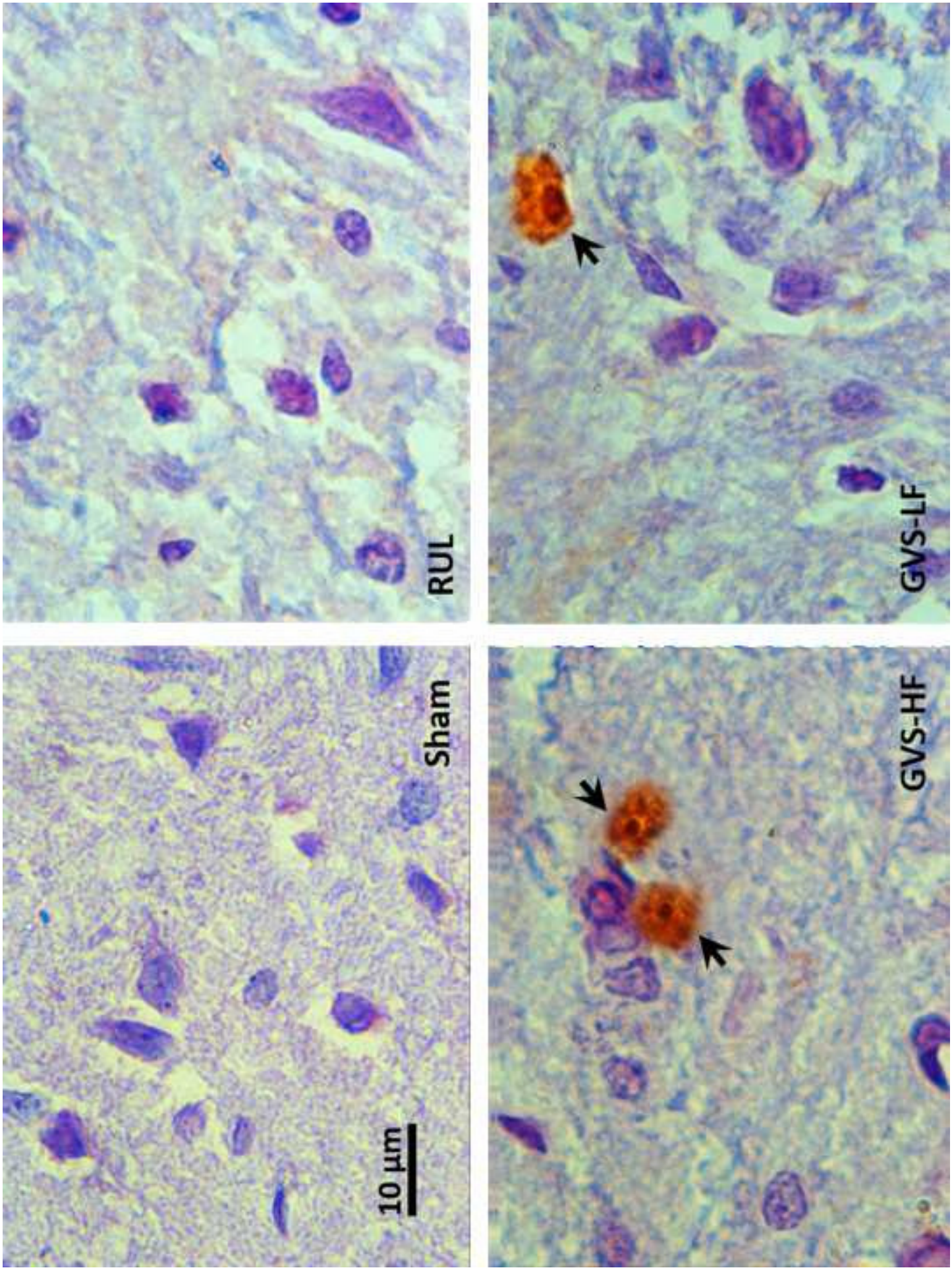
Figure

Figure



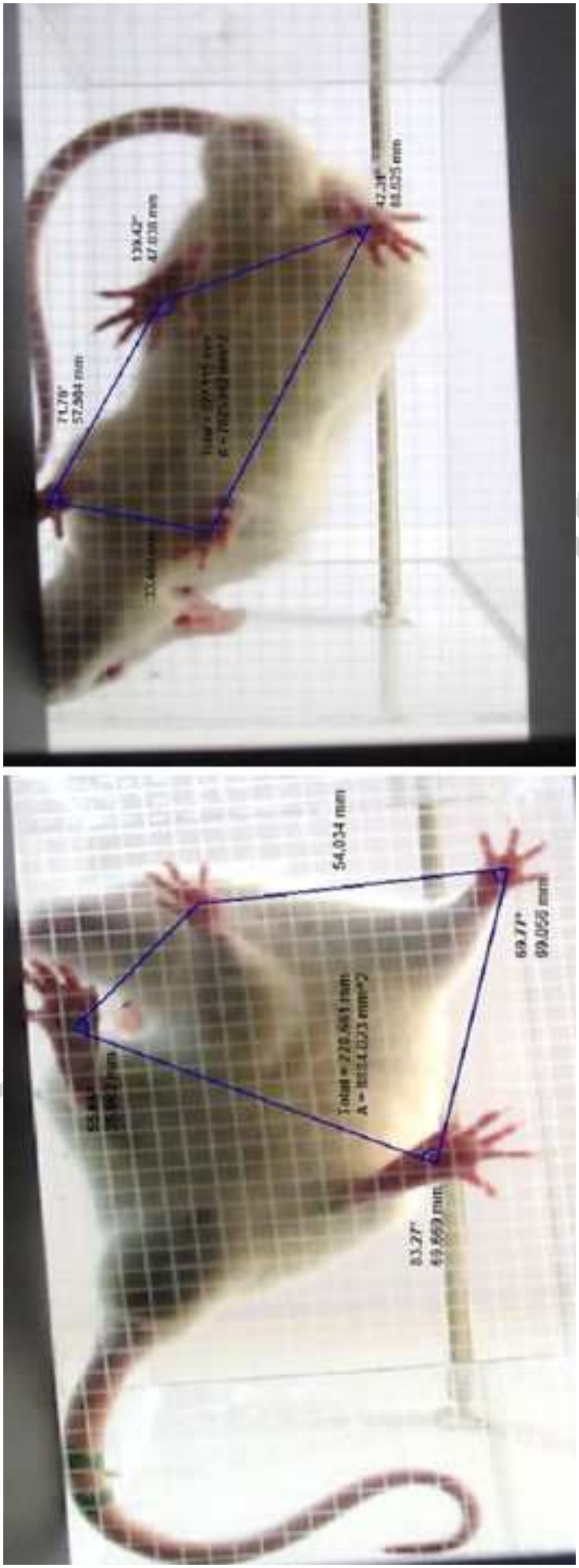

Intact
Sham
RUL
GVS.LF
GVS.HF
BrdU+ Cells (Percent)
0
20
40
60
80
100
Right MVN
Left MVN

Figure

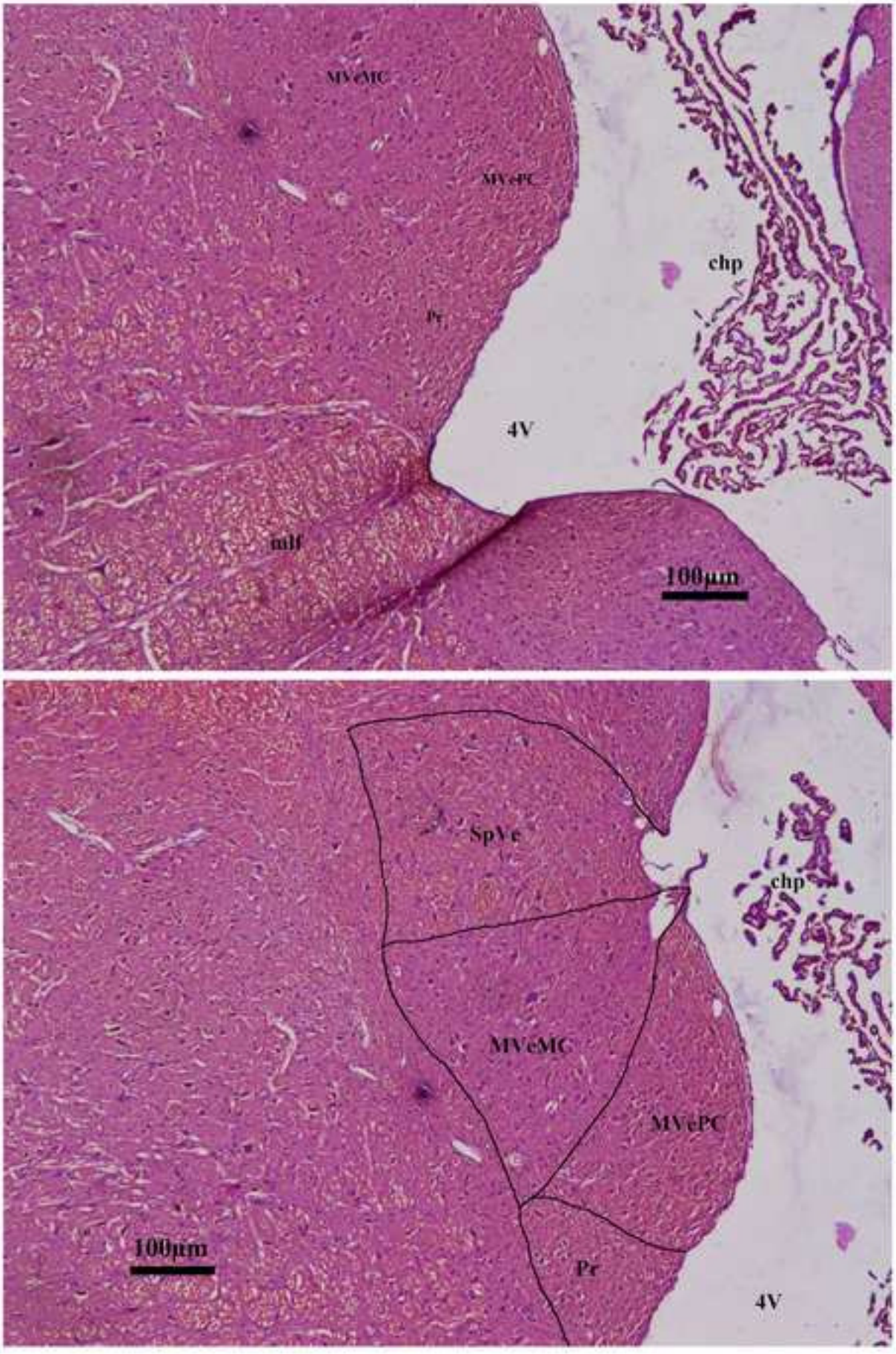


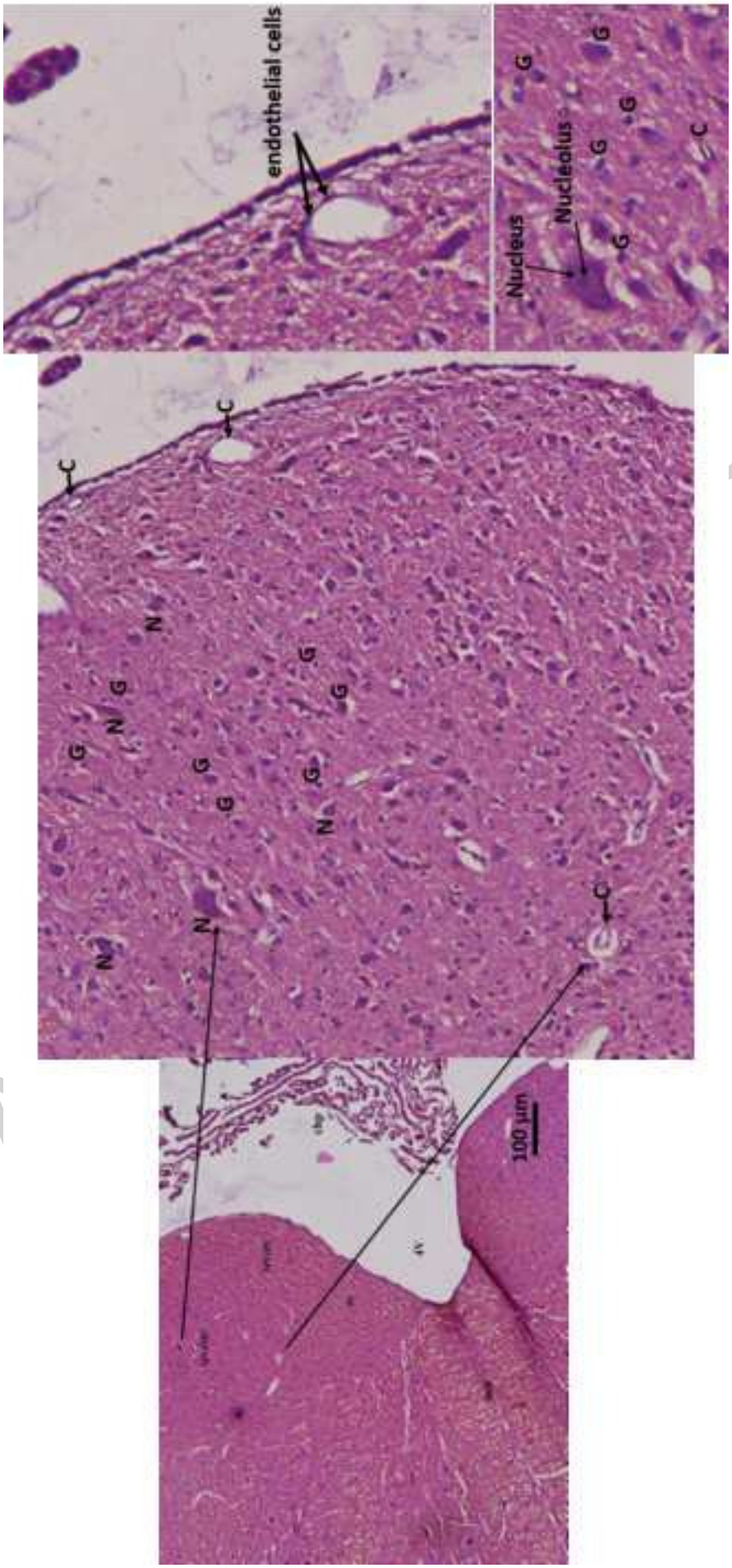
Figure

Figure



Figure





Figure