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The effects of unilateral cochlear ablation on the expression of vesicular glutamate transporter 1 in the lower auditory pathway of neonatal rats

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Abstract

Objectives: Unilateral cochlear damage has profound effects on the central auditory pathways in the brain. Methods: We examined the effects of unilateral cochlear ablation on VGLUT1 expression in the cochlear nucleus (CN) and the superior olivary complex (SOC) in neonatal rats. Results: VGLUT1 expression in the CN subdivisions (the AVCN, the PVCN and the DCN-deep layers) and the SOC (the MnTB, the LSO and the MSO) ipsilateral to the ablated side was significantly suppressed by unilateral cochlear ablation. Interestingly, VGLUT1 expression in the PVCN and the DCN-deep layers contralateral to the ablated side was also reduced. Conclusion: Our findings indicate that unilateral cochlear ablation affects VGLUT1 expression in the central auditory pathways not only ipsilateral but also contralateral to the ablated side.

Key words: vesicular glutamate transporter 1, cochlear ablation, auditory pathway

Introduction

Cochlear damage causes morphological and functional changes in the central auditory pathways of the brain such as the cochlear nucleus (CN) and the superior olivary complex (SOC) [1-4]. Because, in addition to changes in cochlear function, changes in the central auditory pathways affect hearing ability, these changes have been intensively investigated [5, 6]. It was reported that unilateral cochlear lesions induced abnormal axonal connections between the ventral CN (VCN) and the SOC, including the medial nucleus of the trapezoid body (MnTB), the medial superior olive (MSO) and the lateral superior olive (LSO) [7]. Although numerous morphological changes caused by unilateral cochlear damage in the central auditory pathways have been uncovered [8], the neurochemical changes caused by unilateral cochlear damage still remain largely unclear. Neurochemical changes caused by unilateral cochlear damage, especially those related to neurotransmission, are of great interest because they may indicate functional changes in the central auditory pathways.

Vesicular glutamate transporters (VGLUTs), such as VGLUT1 and VGLUT2, are responsible for the active transport of glutamate and play crucial roles in glutamatergic transmission in the central nervous system [9-11]. Because of the importance of VGLUTs in glutamatergic transmission, we examined the changes in VGLUT expression in the rat central auditory pathways in response to unilateral cochlear ablation. Among VGLUTs, it has been reported that VGLUT1 is widely expressed in the central auditory pathways [12-16]. Immunohistochemical studies showed that VGLUT1 was expressed in the MSO and the LSO, as well as in the VCNs [17]. We therefore examined the expression of VGLUT1 in the rat central auditory

pathways.

Because it was demonstrated that earlier cochlear damage during development led to stronger morphological changes in the central auditory pathways [8], we performed unilateral cochlear ablation soon after the birth of rat pups, and examined VGLUT1 expression using immunohistochemistry. We found that the expression of VGLUT1 in the rat central auditory pathways was markedly affected. This study therefore provides important insights regarding the neurochemical changes in the central auditory pathways in response to cochlear damage. It would be important to uncover the entire picture of the changes in the central auditory pathways in response to cochlear damage.

Materials and Methods

Animals

All procedures were performed in accordance with protocols approved by the Animal Research Committee of Kanazawa University Graduate School of Medical Sciences. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories, Japan, and were reared on a normal 12 hr light/dark schedule. The day of birth was counted as postnatal day 0 (P0). In total, twelve P60 rats were included this study.

Surgical procedures

Unilateral cochlear ablation was performed on newborn rat pups as described previously, with modifications [8]. Briefly, newborn rats (P3-5) were anesthetized with hypothermia,

and a skin incision was made inferior to the left pinna. Using a post-auricular approach in microsurgery, the lateral semicircular canal and middle ear soft tissues were exposed by blunt dissection with a coagulator, with care taken not to damage the facial motor nerve and blood vessels. After removing the stapes, the cochlea was identified and ablated mechanically by crushing it with a forceps and aspirating the remaining tissues. The cochlear damage was confirmed by visual inspection at the end of the surgery. The skin incision was closed with glue. The rats were warmed under a heating lamp, and returned to their mothers after recovery. Brain samples were obtained at P60. In addition to these seven experimental animals, five non-ablated, age-matched naive control animals were used in this study. Naive control animals and ablated animals were used simultaneously along the entire procedure.

Immunohistochemistry

Immunohistochemistry was performed as described previously, with slight modifications [18-20]. Both ablated animals and naive control animals at P60 were deeply anesthetized and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS at the same time. Brains were dissected, post-fixed in 4% PFA/PBS overnight, cryoprotected by two-day immersion in 30% sucrose, and embedded in Optimal Cutting Temperature compound (Tissue-Tek OCT; Sakura, USA). To distinguish sides of the brain ipsilateral and contralateral to the cochlear ablation, the thin needle was used to make a tiny whole which indicated the right cortex or the right brainstem. Coronal sections of 40 μ m thickness were made using a cryostat (CM 1850, Leica). After being treated with 2% skim milk and 0.5% Triton X-100 in PBS, the sections were incubated overnight with rabbit anti-VGLUT1

antibody (Synaptic Systems) at 4°C. The sections were washed, and incubated with Alexa 488-conjugated secondary antibody (Molecular Probe) and red-fluorescent Nissl stain (Invitrogen) for 2 hours. The sections were then washed and mounted. Epifluorescence microscopy was performed with an AxioImager A1 microscope (Carl Zeiss).

Quantification of the sizes of the CN subdivisions

Coronal sections were stained with red-fluorescent Nissl stain (Invitrogen), and sections that contained the largest size of each CN subdivision were used for quantification. The outlines of each subdivision were traced, and the areas within the outlines were measured using ImageJ software (NIH) for the analysis.

Quantification of VGLUT1 immunoreactivity

VGLUT1 signal intensities in the subdivisions of the CN (the AVCN, the PVCN and the DCN) and the SOC (the LSO, the MSO and the MnTB) were measured. Coronal sections containing the largest size of each subdivision were used for quantification. After background signal intensities were subtracted, the mean signal intensity in each subdivision was measured using ImageJ software as follows. First, the outline of each subdivision was traced manually. After the "Mean gray value" option was selected, the "Measure" command was performed. To minimize the variation of the signal intensities among different sections, the mean signal intensities in each subdivision were divided by those of the ventrolateral principal trigeminal nucleus (Pr5VL) or the oral part of the spinal trigeminal nucleus (Sp5O), which we used as reference nuclei, in the same sections. In this study, we analyzed VGLUT1 immunoreactivities ipsilateral and

contralateral to the ablated side compared with those of naive control animals.

Statistical analysis

Statistical significances were analyzed with Student's *t*-test and were determined with a confidence limit of $p < 0.05$.

Histological analysis of the cochlea

To confirm the effects of cochlear ablation, the temporal bone ipsilateral and contralateral to the ablated side were isolated and treated with a decalcification solution overnight (K-CX, Falma, Japan). After being embedded in paraffin, sections of 3 μm thickness were made using a microtome (SM2000R, Leica) and were stained with haematoxylin and eosin. The sections were washed, mounted and observed with a microscope (Eclipse E1000, Nikon).

Results

The reduction of the sizes of subdivisions of the CN after unilateral cochlear ablation

Our previous report showed that unilateral cochlear ablation soon after birth resulted in the reduction of the size of the CN ipsilateral to the ablated side [8]. We therefore performed unilateral cochlear ablation using rat neonates between postnatal days 3-5 (P3-5) and confirmed histochemically that the cochlea was clearly disrupted (Fig. 1). We then examined the sizes of the subdivisions of the CN such as the anterior ventral

CN (AVCN) and the posterior ventral CN (PVCN). Coronal sections of the brainstem were prepared at P60 and subjected to fluorescent Nissl staining (Fig. 2). Consistent with our previous report [8], the sizes of the AVCN and the PVCN ipsilateral to the ablated side were markedly smaller than those contralateral to the ablated side (Fig. 2).

To quantify the sizes of the AVCN and the PVCN, we used coronal sections containing the largest areas of these nuclei and measured the areas using ImageJ software. Consistent with our observations (Fig. 2), the areas of the AVCN and the PVCN ipsilateral to the ablation side were significantly smaller than those contralateral to the ablated side. The areas of the AVCN and the PVCN ipsilateral to the ablated side were $56.8 \pm 6.4\%$ ($p=0.00000038$) and $64 \pm 8.4\%$ ($p=0.000057$) of those contralateral to the ablated side. These results are consistent with our previous report showing the effects of cochlear ablation on the size of the CN [4, 8]. These results therefore indicate that the cochlea is disrupted by our surgical procedure.

We then compared the sizes of the AVCN and the PVCN in ablated animals with those in naive control animals. The areas of the AVCN and the PVCN ipsilateral to the ablated side were significantly smaller than those in naive control animals (AVCN, $53.9 \pm 2.3\%$, $p=0.00000059$; PVCN, $67.5 \pm 5.1\%$, $p=0.0000029$). In contrast, the areas of the AVCN and the PVCN contralateral to the ablated side did not show statistically significant changes compared with those in naive control animals (AVCN, $95.6 \pm 3.7\%$, $p=0.081$; PVCN, $95.1 \pm 4.9\%$, $p=0.096$). These results clearly indicate that unilateral cochlear ablation reduces the sizes of the ipsilateral AVCN and PVCN.

Changes in VGLUT1 expression in the ipsilateral CN induced by unilateral cochlear ablation

To examine the effects of unilateral cochlear ablation on VGLUT1 expression, we performed unilateral cochlear ablation using rat pups at P3-5. Coronal sections of the brainstem were prepared at P60, and the sections containing the largest size of each subdivision were stained with anti-VGLUT1 antibody. We examined VGLUT1 expression in the AVCN (Fig. 3A), the PVCN (Fig. 3B) and the fusiform cell and deep layers of the DCN (hereafter referred to as DCN-deep layers) (Fig. 3C, double arrows), which mainly receive auditory information [21, 22]. Within the DCN, we focused on the fusiform cell and deep layers (Fig. 3C, double arrows) because these layers receive auditory information, whereas the molecular layer receives non-auditory inputs [23, 24]. We found that VGLUT1 expression in the AVCN and the PVCN ipsilateral to the ablated side was markedly decreased compared with that of naive control animals (Fig. 3, A and B), while that in the DCN-deep layers was moderately suppressed (Fig. 3C, double arrows).

We next quantified the expression levels of VGLUT1 in the AVCN, the PVCN and the DCN-deep layers. After background signal intensities were subtracted, VGLUT1 mean signal intensities in each subdivision of the CN were measured. Consistent with our observations (Fig. 3, A-C), the expression levels of VGLUT1 in the AVCN, the PVCN and the DCN-deep layers ipsilateral to the ablation side were significantly lower than those in naive control animals (AVCN, ablation 1.041 ± 0.066 , control 1.449 ± 0.073 , $p=0.000017$; PVCN, ablation 1.039 ± 0.160 , control 1.994 ± 0.195 , $p=0.000036$; DCN-deep layers, ablation 1.044 ± 0.198 , control 1.546 ± 0.088 , $p=0.0034$) (Fig. 3D). These results suggest that VGLUT1 expression in the ipsilateral CN was reduced by unilateral cochlear ablation. Our findings may indicate that glutamatergic transmission in the central auditory pathways is suppressed in

cochlea-ablated animals.

It should be noted that certain levels of VGLUT1 expression remained in the CN even after cochlear ablation. Previous studies have reported that the CN receives inputs from multiple sources besides the auditory nerve, including descending, intrinsic and somatosensory pathways [14, 25]. The residual VGLUT1 expression could be due to inputs from these pathways.

Changes in VGLUT1 expression in the ipsilateral SOC induced by unilateral cochlear ablation

In addition to passing through the CN, auditory information derived from the cochlea is also transferred to the SOC. Therefore, we next examined VGLUT1 expression in nuclei of the SOC such as the LSO, the MSO and the MnTB. As in the case of the CN, VGLUT1 expression in the LSO, the MSO and the MnTB ipsilateral to the ablated side was markedly suppressed compared to that of naive control animals (Fig. 4, A and B). Consistently, our quantification showed that the expression levels of VGLUT1 in the LSO, the MSO and the MnTB ipsilateral to the ablated side were significantly lower than those of naive control animals (LSO, ablation 0.714 ± 0.307 , control 1.316 ± 0.214 , $p=0.0032$; MSO, ablation 1.009 ± 0.315 , control 1.657 ± 0.157 , $p=0.0016$; MnTB, ablation 0.659 ± 0.206 , control 1.079 ± 0.085 , $p=0.0016$) (Fig. 4C). These results indicate that VGLUT1 expression is suppressed by unilateral cochlear ablation not only in the CN, but also in the SOC ipsilateral to the ablated side.

Changes in VGLUT1 expression in the contralateral CN and SOC induced by unilateral cochlear ablation

We next examined if unilateral cochlear ablation affects VGLUT1 expression in the CN and the SOC contralateral to the ablated side. Unexpectedly, we found that VGLUT1 expression in the contralateral PVCN (Fig. 5B) and the DCN-deep layers (Fig. 5C, double arrows) was reduced by unilateral cochlear ablation, whereas those in the contralateral AVCN, LSO, MSO and MnTB were not apparently affected (Figs. 5A, 6A and 6B). Our quantification of VGLUT1 signal intensities was consistent with our observations (AVCN, ablation 1.736 ± 0.465 , control 1.464 ± 0.036 , $p=0.13$; PVCN, ablation 1.442 ± 0.363 , control 2.127 ± 0.196 , $p=0.0033$; DCN-deep layers, ablation 1.067 ± 0.082 , control 1.601 ± 0.140 , $p=0.000096$; LSO, ablation 1.597 ± 0.399 , control 1.298 ± 0.260 , $p=0.11$; MSO, ablation 1.429 ± 0.317 , control 1.714 ± 0.156 , $p=0.061$; MnTB, ablation 0.927 ± 0.319 , control 1.119 ± 0.286 , $p=0.18$) (Figs. 5D and 6C). These results suggest that unilateral cochlear ablation also reduced the expression levels of VGLUT1 in the PVCN and the DCN-deep layers contralateral to the ablated side. Our findings demonstrate that unilateral cochlear ablation reduces the expression levels of VGLUT1 not only in the ipsilateral central auditory pathways, but also in parts of the contralateral central auditory pathways, especially in the PVCN and the DCN-deep layers.

Discussion

Although a number of previous studies have revealed morphological and electrophysiological changes in the central auditory pathway following cochlear ablation, neurochemical changes still remain largely unclear [26]. Here, we have shown

that VGLUT1 expression in the central auditory pathways was strongly affected by unilateral cochlear ablation in neonatal rats. By the mechanical cochlear ablation, the effects on the central auditory pathways are stronger than that of clinical implications. Furthermore, to establish the animal model of absolute unilateral deafness is so quite difficult that we only use the animal model of mechanical cochlear ablation or injection of ototoxic drugs.

A previous report demonstrated that kanamycin-induced unilateral cochlear damage significantly decreased VGLUT1 expression in the ipsilateral AVCN and the ipsilateral PVCN in adult guinea pigs [12]. Taken together with our results using neonatal rats, these findings indicate two points. First, the changes in VGLUT1 expression in response to unilateral cochlear damage are conserved among different animal species, and therefore it seems reasonable to speculate that the changes in VGLUT1 expression can also be observed in humans. Second, because the previous study used adult guinea pigs and our study used neonatal rats, it seems likely that the changes of VGLUT1 expression can be induced by unilateral cochlear damage throughout the entire life of animals. On the other hand, using the visual system and other systems, it has been established that developmental plasticity is more strongly induced in younger animals [27]. Therefore, it would be intriguing to investigate whether the changes in VGLUT1 expression in the central auditory pathways are more strongly induced by unilateral cochlear damage in younger animals. In addition, previous studies have shown synaptic reorganization in the cochlear nucleus in response to cochlear damage in adult animals [28-32], but it is unclear whether the similar changes can be induced by cochlear damage in neonates. It would be intriguing to investigate whether stronger synaptic changes could be caused by cochlear ablation in

neonates than in adult animals.

In addition to demonstrating changes in VGLUT1 expression in the ipsilateral auditory pathways, we showed that VGLUT1 expression in the contralateral auditory pathways was also suppressed by unilateral cochlear ablation. Although a previous report showed that kanamycin-induced unilateral cochlear damage failed to affect VGLUT1 expression in the contralateral AVCN, PVCN and DCN [12], our unilateral cochlear ablation resulted in reduced expression of VGLUT1 in the contralateral PVCN and DCN. Our results may indicate that the PVCN and the DCN are affected by auditory inputs not only from the ipsilateral side but also from the contralateral side. It was reported that there was interaction between the two cochlear nuclei by way of the CN-commissural pathway [33-35]. It seemed possible that the CN-commissural pathway is involved in the change of VGLUT1 expression in the contralateral CNs induced by our unilateral cochlear ablation. Because we cannot exclude the possibility that neuronal circuits other than the CN-commissural pathway are responsible for the change, it would be intriguing to uncover the neuronal circuits responsible for the changes in VGLUT1 expression in the contralateral PVCN and DCN. On the other hand, it is unclear why VGLUT1 expression was not affected in the previous report [12]. There seem three possible reasons for this discrepancy. The first possible reason is the age of the animals used. We used neonatal rats, while the previous study used adults. Consistent with this idea, it has been shown in other parts of the brain that developmental plasticity is more strongly induced in younger animals [36, 37]. The second possibility is the difference in animal species. While we used rats, the previous report used guinea pigs for the experiments, and rats could be more sensitive to cochlear ablation. The third possible reason is the difference in the methods used to cause

cochlear damage. While we surgically disrupted the cochlea, the previous report used kanamycin injection. Because our method using mechanical cochlear ablation leads to profound deafness, it seems possible that it could have a stronger overall effect than kanamycin injection. This difference in strength could be the reason why our method resulted in apparent changes in VGLUT1 expression in the contralateral CN and the other did not. Future investigation would be necessary to address these possibilities.

In addition to the CN, the SOC also receives auditory inputs. The SOC is the initial site for the convergence of bilateral auditory inputs [38, 39] and plays important roles in interaural comparisons of intensities and timing of auditory inputs that reach both ears. Despite the importance of the SOC in auditory processing, it had been unclear whether VGLUT1 expression in the SOC was also affected by unilateral cochlear ablation. In the present study, we uncovered that VGLUT1 expression in the ipsilateral SOC was also reduced by unilateral cochlear ablation. Interestingly, the MnTB preferentially receives auditory inputs from the contralateral VCN [40-42]. Therefore, the possible reason for the reduction of VGLUT1 expression in the ipsilateral MnTB is a change in the glutamatergic projections from the contralateral VCN. This seemed plausible because, in addition to the ipsilateral VCN, the contralateral VCN was also affected by unilateral cochlear ablation, as discussed above. Consistently, Cramer et al have shown that contralateral VCN innervates MnTB on both sides in response to unilateral cochlear ablation when cochlear ablation is performed before hearing onset [43, 44]. Taken together with our findings, it is likely that the reduction of VGLUT1 expression in the ipsilateral MnTB results from a change in the glutamatergic projections from the contralateral VCN. Because the SOC is most likely involved in the localization of sounds [38], the change of VGLUT1 expression in the MnTB could

mediate the compensation of sound localization in response to unilateral cochlear ablation. On the other hand, our data demonstrated that VGLUT1 expression was not affected in the contralateral MnTB. There are two possible explanations for this. First, unilateral cochlear ablation indeed does not affect VGLUT1 expression in the contralateral MnTB. The other possibility would be that VGLUT1 expression in the contralateral MnTB was changed by unilateral cochlear ablation, but the change was masked by some compensatory mechanisms during development.

We have shown that VGLUT1 expression in the CN and the SOC is markedly affected by unilateral cochlear ablation in neonatal rats. Because previous pioneering studies have demonstrated that VGLUT1 immunoreactivity in the auditory pathways is located both in the axon terminals and in the soma [15], it would be intriguing to investigate whether the changes in VGLUT1 expression shown in this study involved VGLUT1 in the axon terminals or VGLUT1 in the soma. Unfortunately, because it was technically difficult to measure VGLUT1 signal intensities in the axon terminal and those in the soma, separately, our quantification included VGLUT1 signal intensities both in axon terminal and in the soma. In addition, because we have shown that the expression of VGLUT1 protein was reduced by unilateral cochlear ablation, it would be intriguing to examine if the expression of VGLUT1 mRNA is also reduced by unilateral cochlear ablation. *In situ* hybridization would be helpful to address this issue.

In this study, we demonstrated that VGLUT1 expression was reduced by unilateral cochlear ablation in the CN and the SOC. There are several possible mechanisms underlying the changes in VGLUT1 expression. First, it appeared plausible that plastic changes were induced by cochlear ablation. Second, because this study used neonatal rats, in which developmental changes in the pattern of neuronal connectivity

are still ongoing, it seemed possible that these developmental changes were modified by cochlear ablation. In addition to these primary effects of cochlear ablation, it seemed also possible that secondary compensatory/adaptive changes could be also involved in the changes in VGLUT1 expression. Future investigations would be required for obtaining a complete understanding of the mechanisms underlying the changes of VGLUT1 expression in response to unilateral cochlear ablation.

In the present study, we showed that the size of the ipsilateral CN was reduced by unilateral cochlear ablation. Although the mechanisms underlying this reduction are unclear, it seems plausible that neuronal loss induced by apoptosis is involved. This is because previous studies reported that apoptosis in the CN was induced by cochlear ablation [4, 37]. It would be intriguing to uncover the entire mechanism underlying the reduction of the size of the CN in response to cochlear ablation.

Conclusion

We examined the effects of unilateral cochlear ablation on VGLUT1 expression in the CN and the SOC in neonatal rats. VGLUT1 expression in the CN subdivisions and the SOC ipsilateral to the ablated side was significantly suppressed by unilateral cochlear ablation. VGLUT1 expression in the PVCN and the DCN-deep layers contralateral to the ablated side was also reduced. Most importantly, our findings indicate that unilateral cochlear ablation affects VGLUT1 expression in the central auditory pathways not only ipsilateral but also contralateral to the ablated side. These results suggest that neonatal cochlear damage leads to profound neurochemical changes in the central auditory pathway.

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Conflict of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure Legends

Fig. 1. Histological examination of an ablated cochlea. Unilateral cochlear ablation was performed mechanically with a forceps using P3-5 rats. The temporal bones ipsilateral and contralateral to the ablated side were isolated at P60, and sections of the temporal bone were subjected to haematoxylin and eosin staining. The ablated cochlea (right) and the intact cochlea contralateral to the ablated side (left) are shown. Scale bar = 1 mm.

Fig. 2. Nissl-stained coronal sections of the AVCN (**A**, dashed line) and the PVCN (**B**, dashed line) ipsilateral and contralateral to the ablated side. Unilateral cochlear ablation was performed using neonatal rats at P3-5. Coronal sections of the brainstem were prepared at P60 and were subjected to fluorescent Nissl staining. The sizes of the AVCN and the PVCN ipsilateral to the ablated side were smaller than those contralateral to the ablated side. Scale bars = 250 μm .

Fig. 3. VGLUT1 expression in the CN ipsilateral to the ablated side. Unilateral cochlear ablation was performed using neonatal rats at P3-5. Coronal sections of the brainstem were prepared at P60 and were subjected to VGLUT1 immunostaining. VGLUT1 expression in the AVCN (**A**), the PVCN (**B**) and the DCN-deep layers (**C**, double arrows) is shown. Scale bars = 250 μm . (**D**) Quantification of VGLUT1 signal intensities. VGLUT1 signal intensities in the AVCN, the PVCN and the DCN-deep layers ipsilateral to the ablated side were measured (N=7 deafened animals, N=5 control animals). VGLUT1 signals in the ipsilateral AVCN, the PVCN and the DCN-deep layers were significantly reduced by unilateral cochlear ablation (Student's *t*-test; *,

$p < 0.05$). Error bars represent mean \pm SD.

Fig. 4. VGLUT1 expression in the SOC ipsilateral to the ablated side. Unilateral cochlear ablation was performed using neonatal rats at P3-5. Coronal sections of the brainstem were prepared at P60 and were subjected to VGLUT1 immunostaining. VGLUT1 expression in the MnTB (**A**, arrows), the LSO and the MSO (**B**, dashed line) is shown. Scale bars = 250 μ m. (**C**) Quantification of VGLUT1 signal intensities. VGLUT1 signal intensities in the LSO, the MSO and the MnTB ipsilateral to the ablated side were measured (N=7 deafened animals, N=5 control animals). VGLUT1 signals in the LSO, the MSO and the MnTB were significantly reduced by unilateral cochlear ablation (Student's *t*-test; *, $p < 0.05$). Error bars represent mean \pm SD.

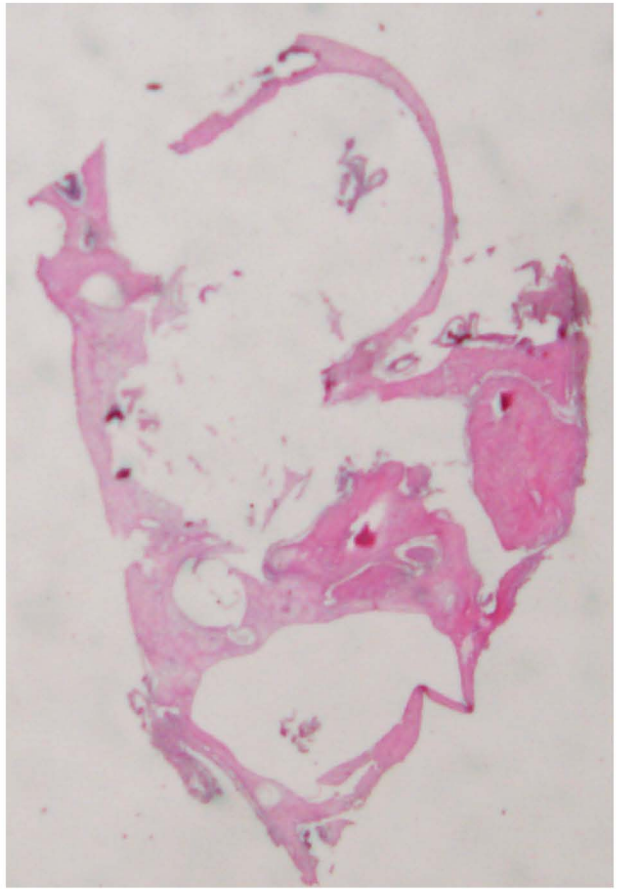
Fig. 5. VGLUT1 expression in the CN contralateral to the ablated side. Unilateral cochlear ablation was performed using neonatal rats at P3-5. Coronal sections of the brainstem were prepared at P60 and were subjected to VGLUT1 immunostaining. VGLUT1 expression in the AVCN (**A**), the PVCN (**B**) and the DCN-deep layers (**C**, double arrows) is shown. (**D**) Quantification of VGLUT1 signal intensities. VGLUT1 signal intensities in the AVCN, the PVCN and the DCN-deep layers contralateral to the ablated side were measured (N=7 deafened animals, N=5 control animals). VGLUT1 signals in the PVCN and the DCN-deep layers were significantly reduced by unilateral cochlear ablation (Student's *t*-test; *, $p < 0.05$). Error bars represent mean \pm SD.

Fig. 6. VGLUT1 expression in the SOC contralateral to the ablated side. Unilateral cochlear ablation was performed using neonatal rats at P3-5. Coronal sections of the

brainstem were prepared at P60 and were subjected to VGLUT1 immunostaining. VGLUT1 expression in the MnTB (**A**, arrows), the LSO and the MSO (**B**, dashed line) is shown. Scale bars = 250 μm . (**C**) Quantification of VGLUT1 signal intensities. VGLUT1 signal intensities in the LSO, the MSO and the MnTB contralateral to the ablation side were measured (N=7 deafened animals, N=5 control animals). VGLUT1 signals in the LSO, the MSO and the MnTB were not apparently affected by unilateral cochlear ablation (Student's *t*-test; *, $p < 0.05$). Error bars represent mean \pm SD.

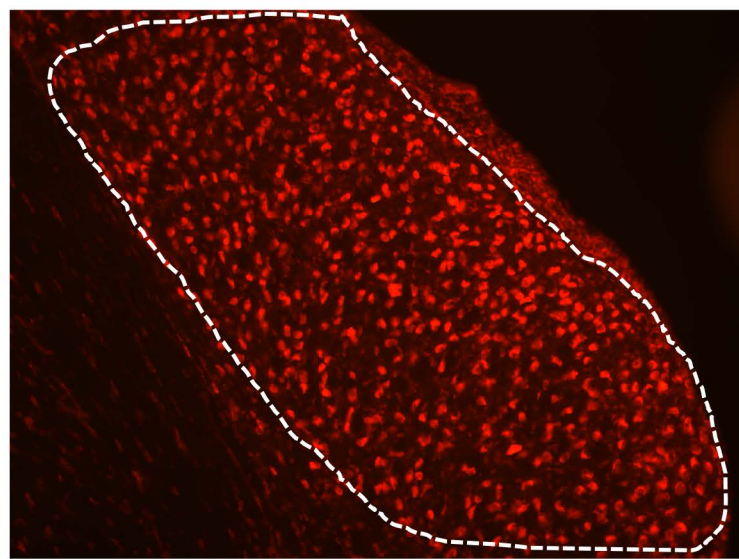
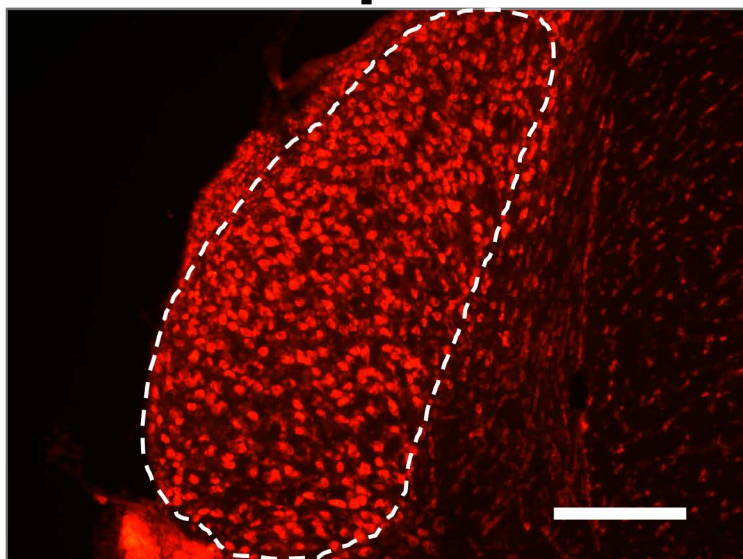
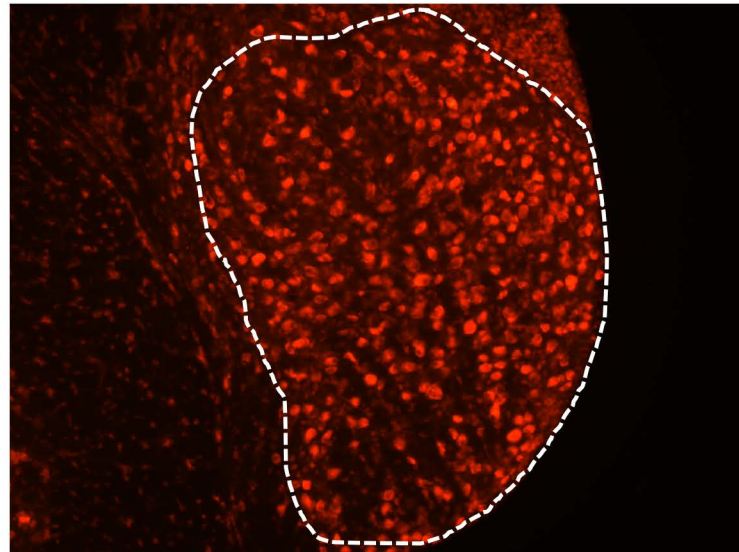
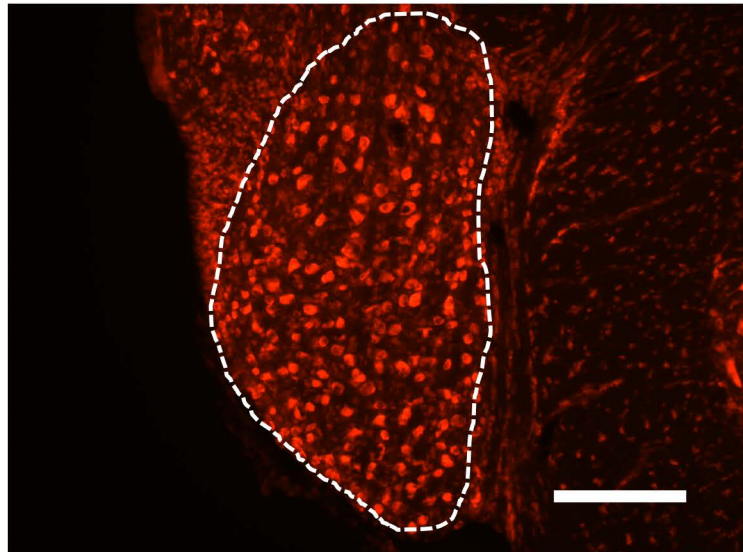


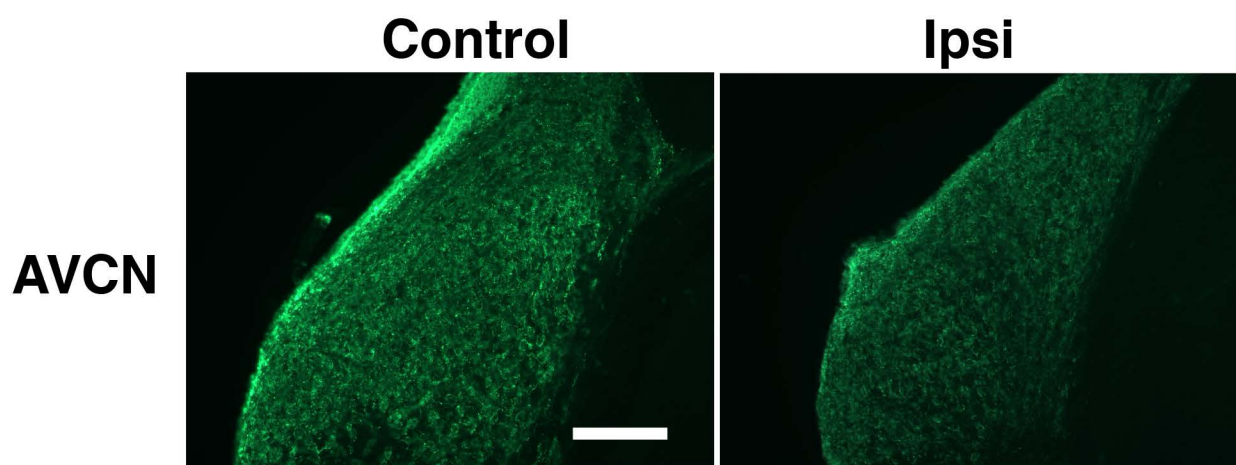
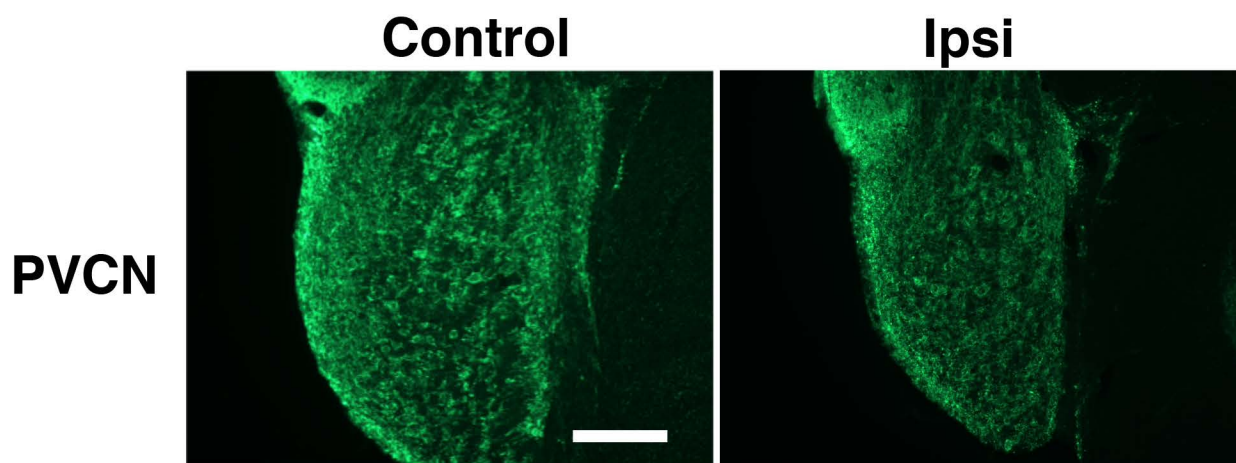
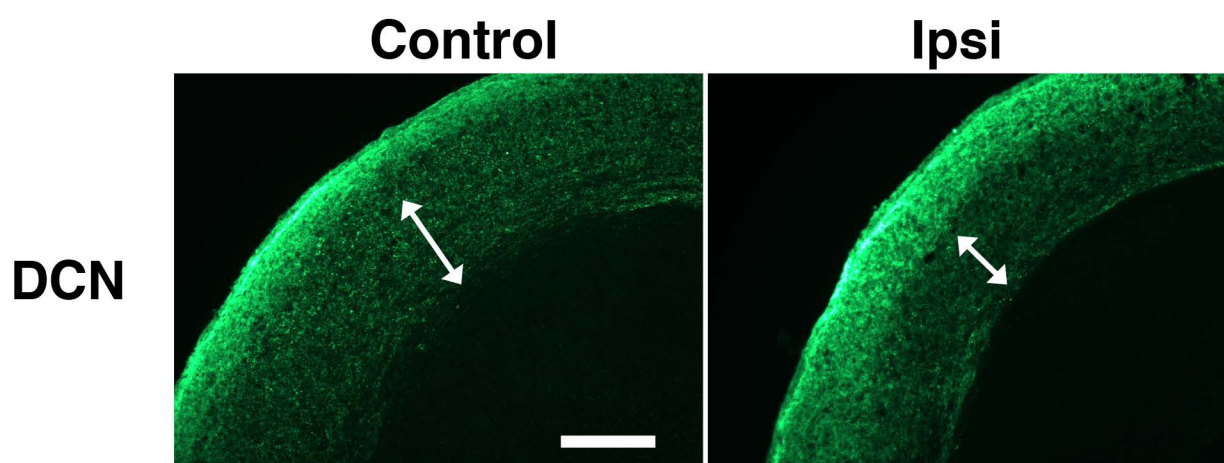
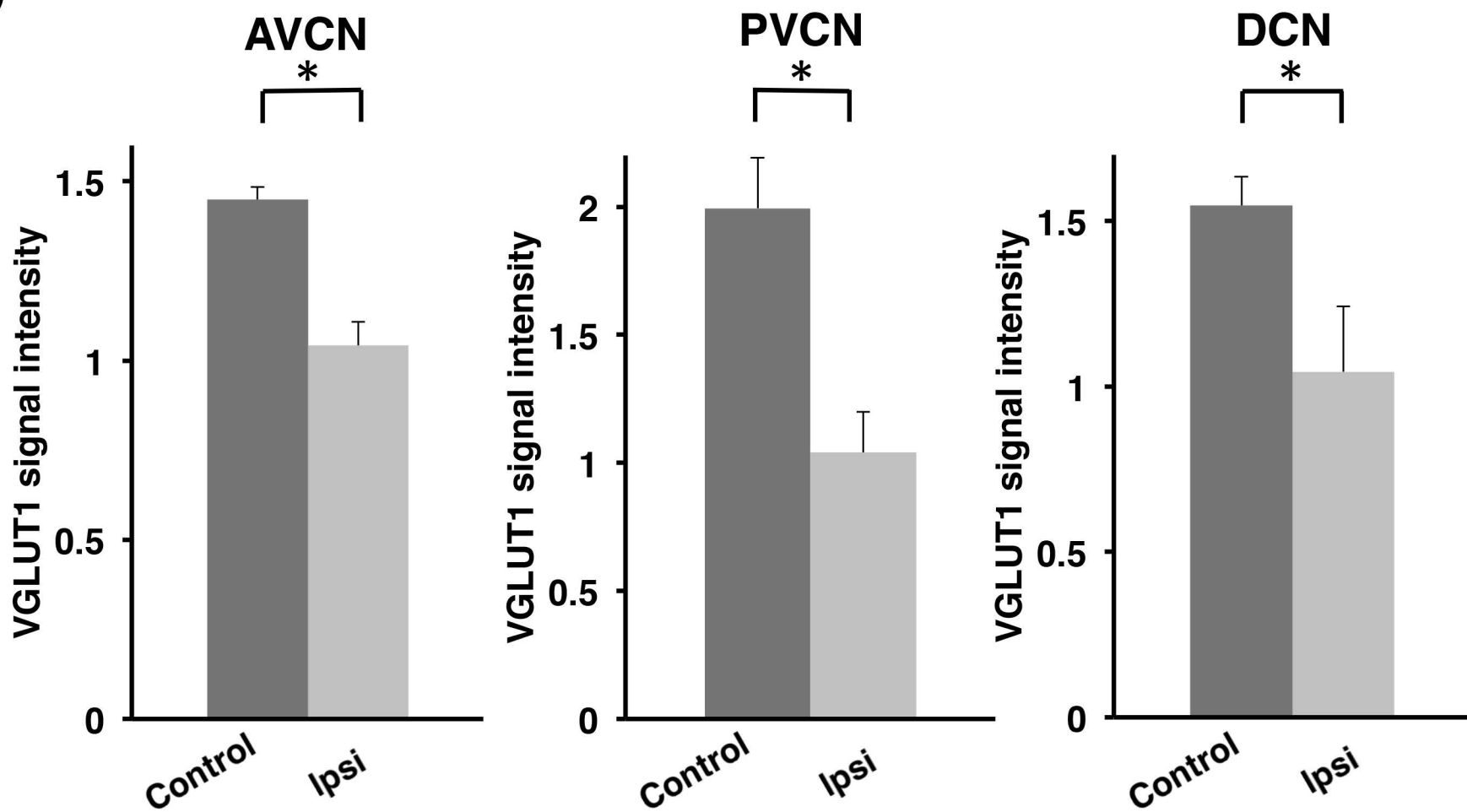
Control



Ablation

Figure 1

A**Ipsi****Contra****AVCN****B****Ipsi****Contra****PVCN****Figure 2**

A**B****C****D****Figure 3A,B,C,D**

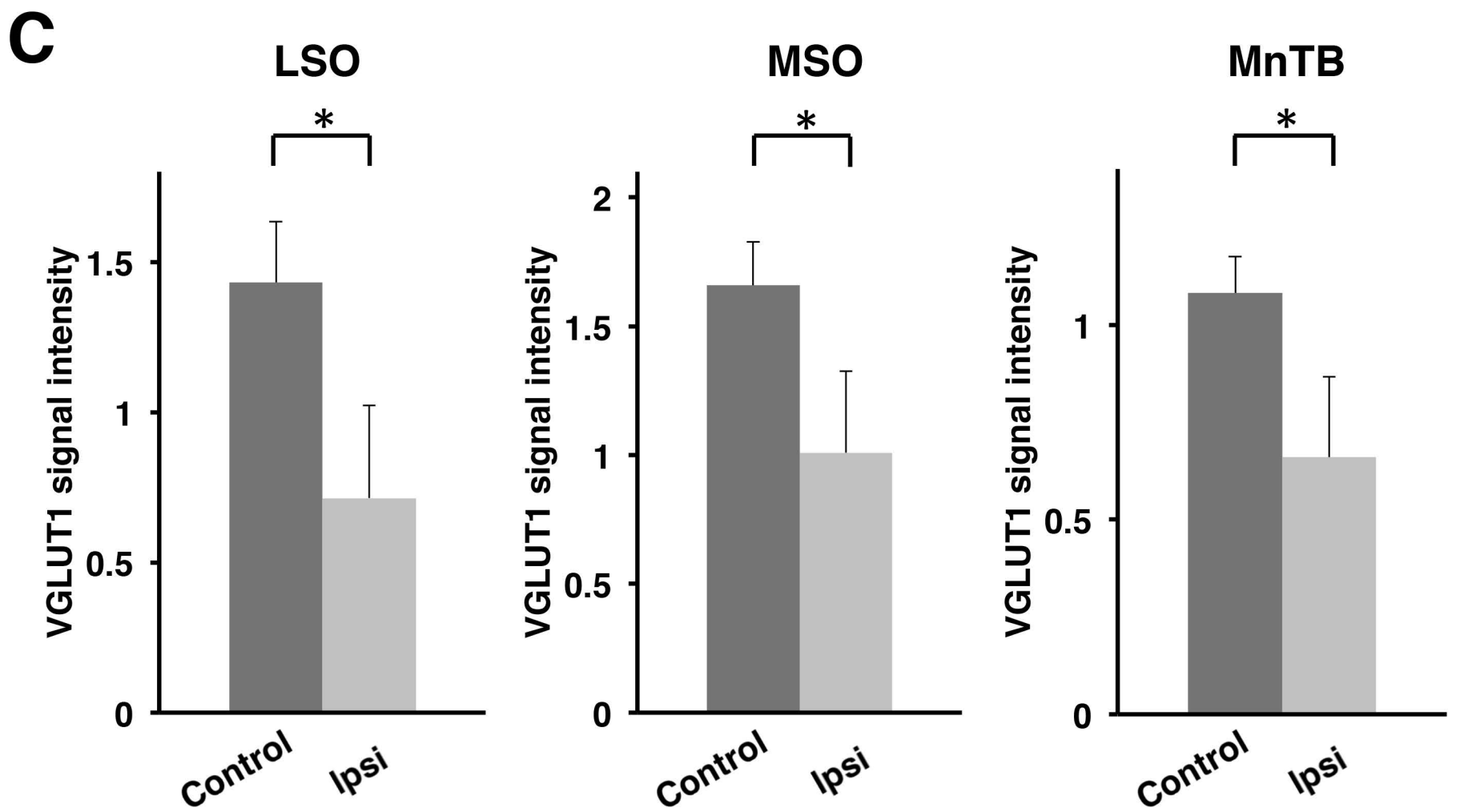
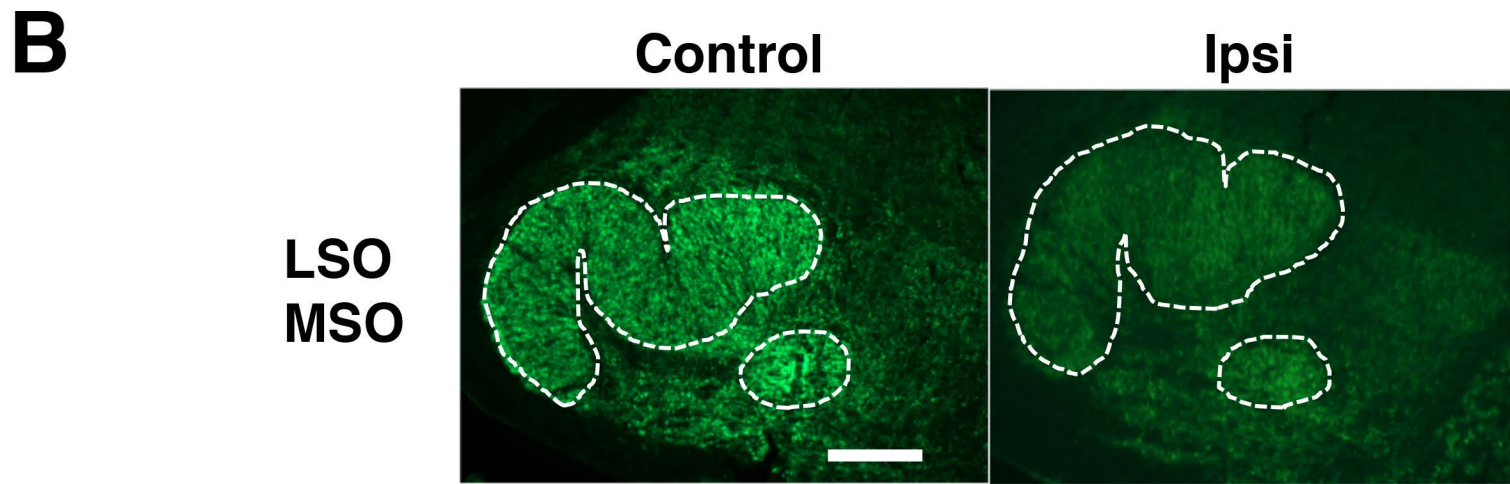
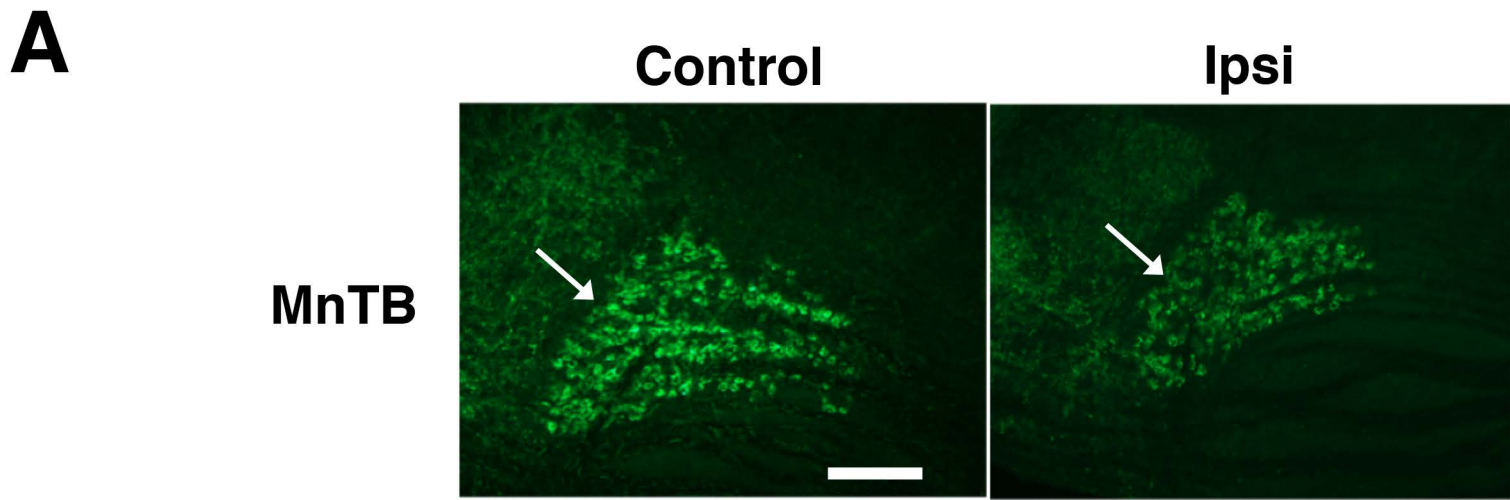
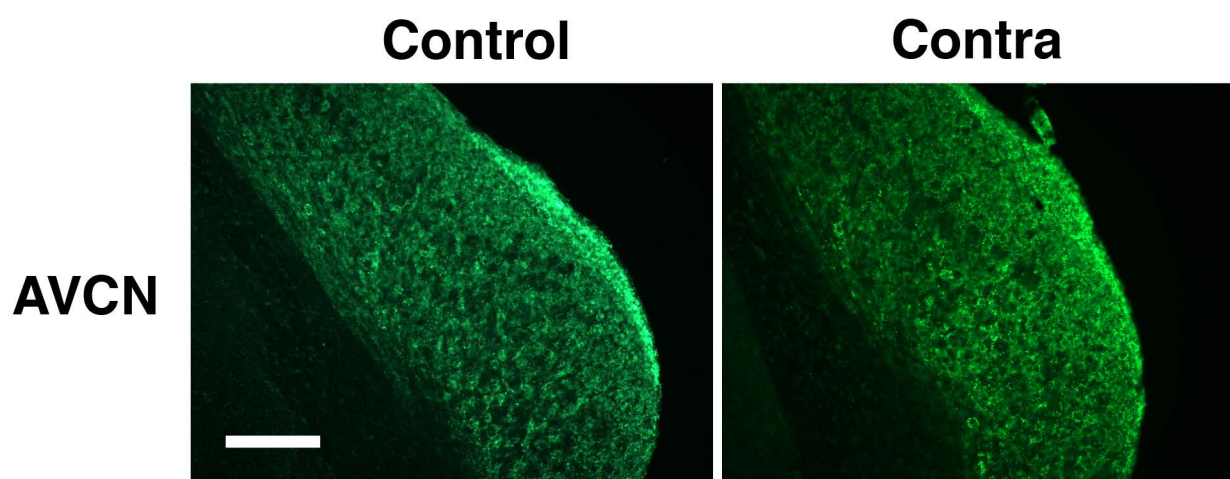
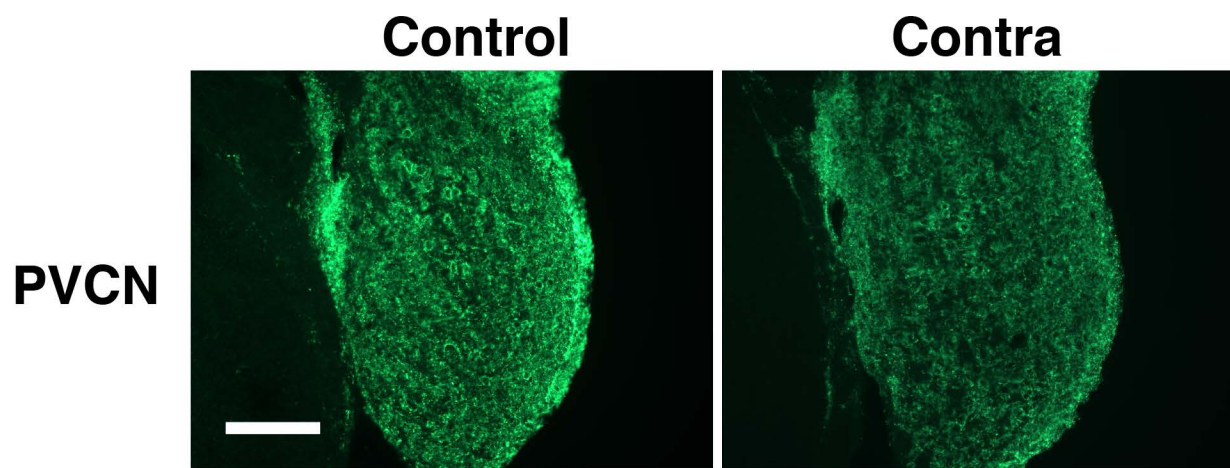
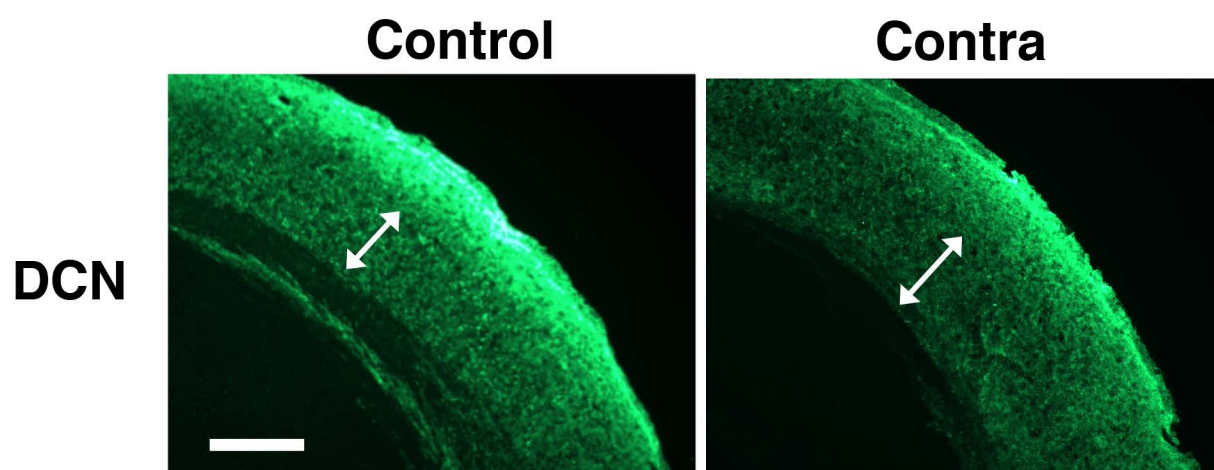
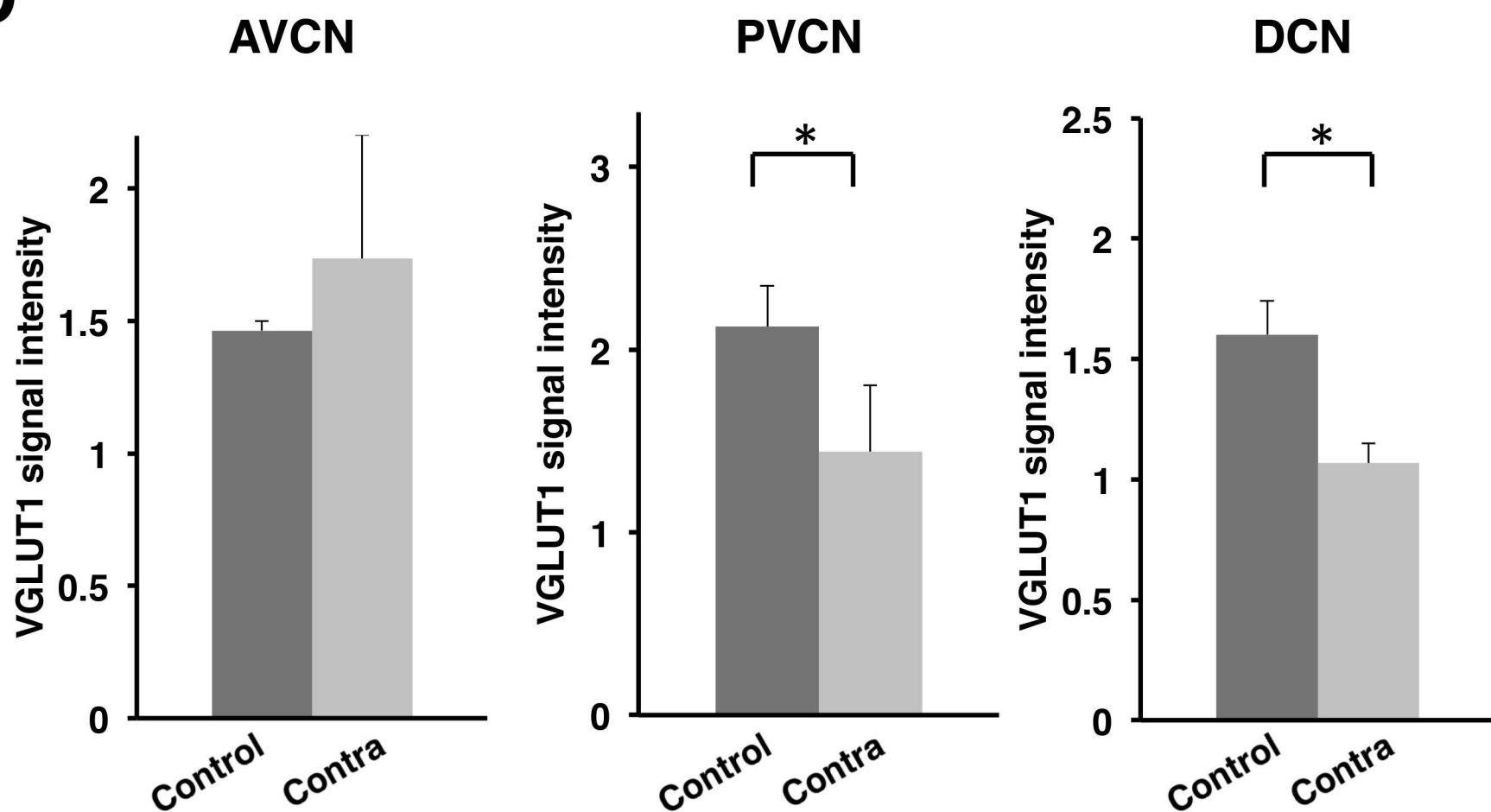
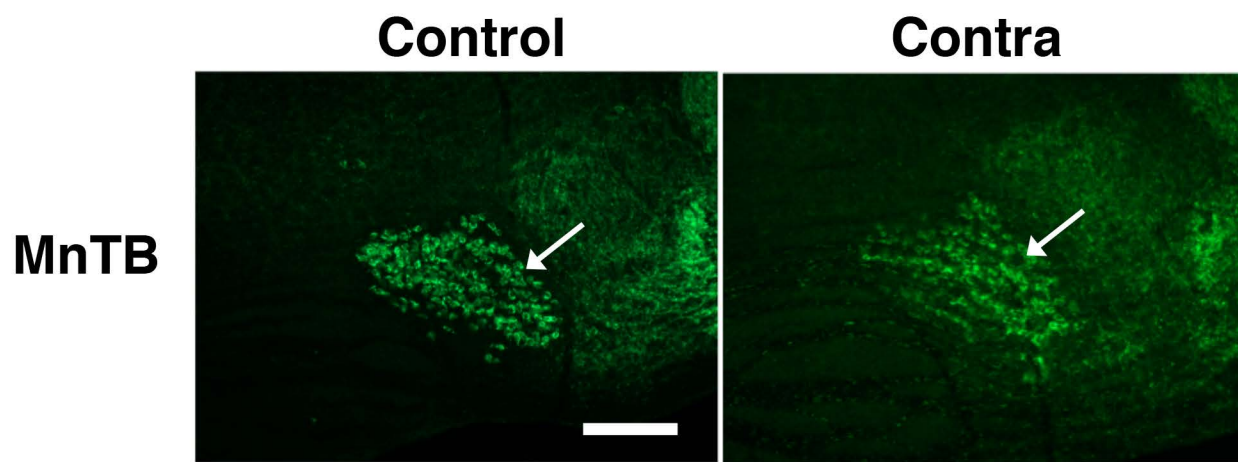
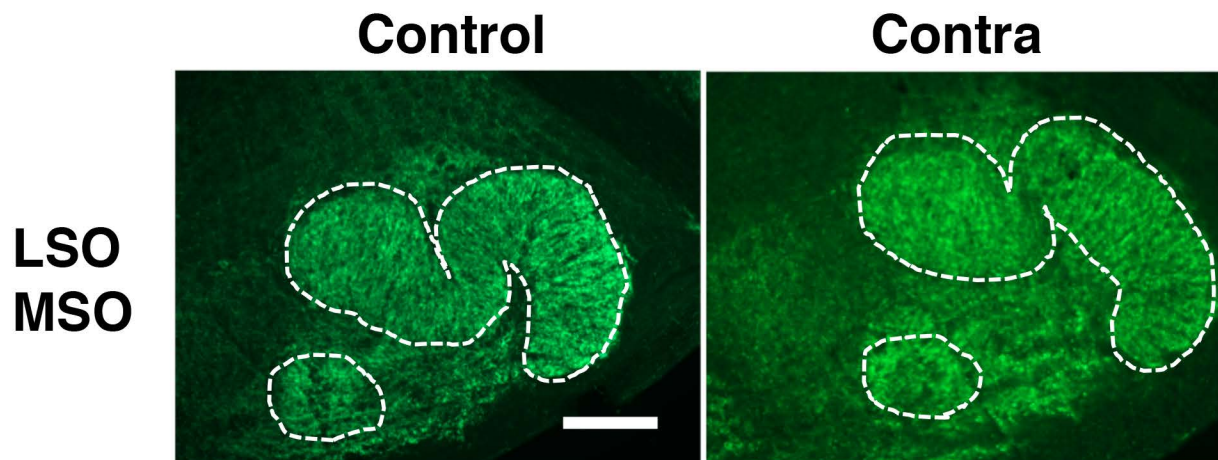
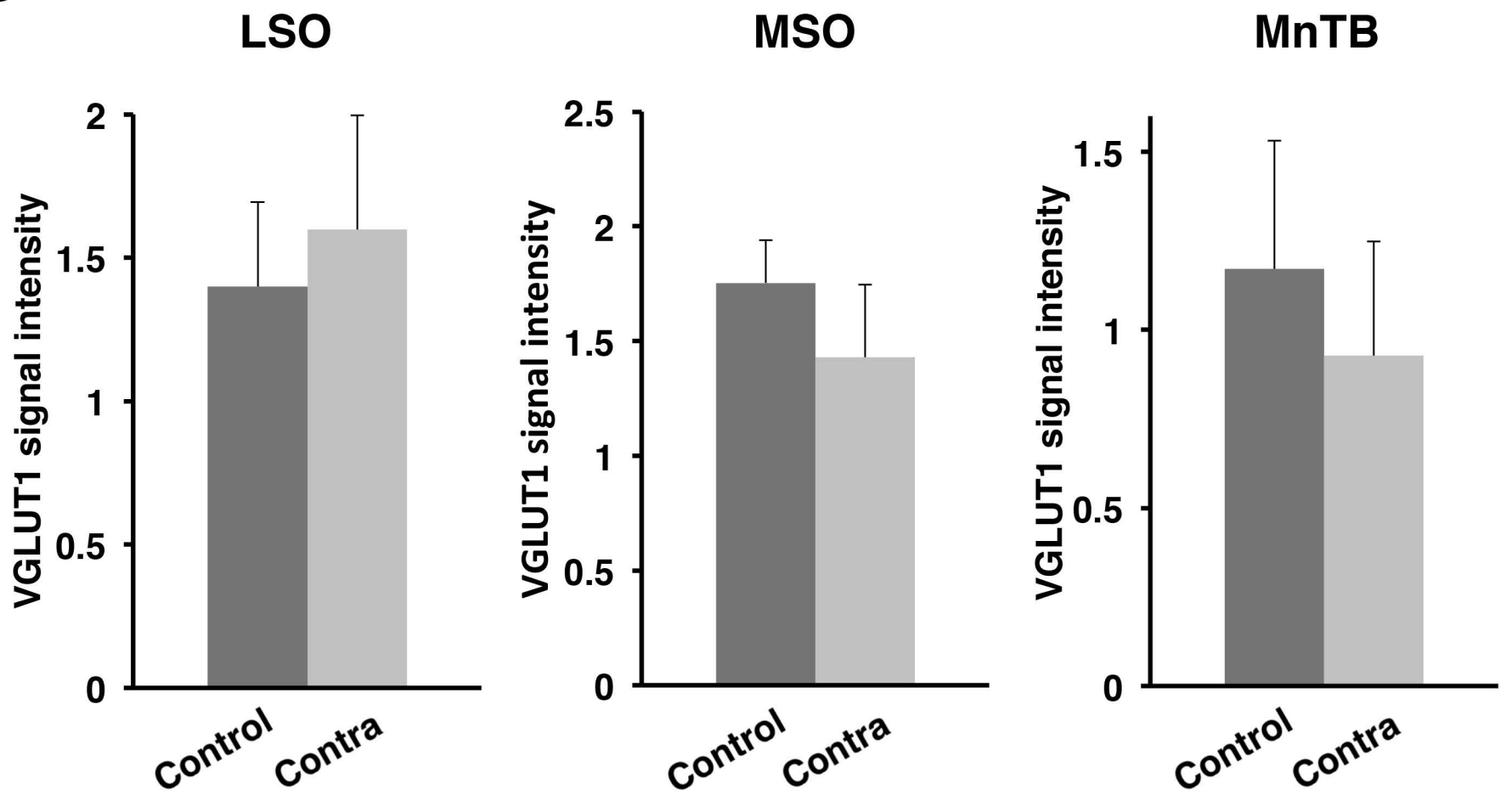


Figure 4A,B,C

A**B****C****D****Figure 5A,B,C,D**

A**B****C****Figure 6A,B,C**