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Original Article

Effect of Neonatal Hypoxia on the Development of Intraspinal Serotonergic Fibers in Relation to Spinal Motoneurons

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) -containing neurons trophically affect target neurons and modulate central nervous system neuronal activity. We studied effects of neonatal hypoxia on postnatal development of intraspinal 5-HT fibers in spinal motoneuron pools. Postnatal day (PND) 0 Sprague-Dawley rats received a hypoxic load and survivors were used for histological analyses on PNDs 1, 7, and 14. Spinal motoneurons were labeled using cholera toxin B subunit as a retrograde neurotracer, and 5-HT fibers were detected immunohistochemically. On PND 1, 5-HT fibers were present in the lateral portion of the ventral horn at the cervical level, but were sparsely distributed at the lumbar level. On PND 14, cervical and lumbar level distributions were nearly identical. The 5-HT fibers and varicosities in close apposition to motoneurons increased from PNDs 1 to 14, however, the close apposition of cervical motoneurons was significantly different from lumbar motoneurons only on PND 1. Density of 5-HT fibers in control and hypoxic rats was not different on PND 1, while those in hypoxic rats were significantly reduced on PND 14. Close appositions of lumbar motoneurons were reduced more than cervical MNs after neonatal hypoxia. Neurodevelopmental deficit after neonatal hypoxia with a rostro-caudal gradient is associated with significant changes in the 5-HT system.

1. INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) -containing neurons are among the earliest to be detected in the brainstem during the embryonic period, and their axons project widely to the central nervous system (CNS) [1-3]. 5-HT has been implicated in multiple processes in the CNS, including trophic effects on target neurons [4] and modulation of neuronal activity [5]. Injury of the 5-HT system during early developmental

periods can therefore result in nervous system deficits.

Perinatal hypoxic brain injury is a major cause of psychomotor disturbance. The effects of hypoxia and/or ischemia on the central nervous system have been well studied from several perspectives [6], including histological changes [7,8], biochemical changes [9], and apoptotic mechanisms [10]. Neonatal asphyxia induces permanent changes in brainstem 5-HT neurons with regional differences [11], and blockade of central 5-HT biosynthesis by *p*-chlorophenylalanine retards cerebral maturation [12] and locomotor activity [13]. These observations are consistent with the idea that injury of the 5-HT system plays an important role in the sequelae of neonatal hypoxia.

In the present study, we estimated the effect of neonatal hypoxia on postnatal development of intraspinal 5-HT fibers in relation to spinal motoneurons (MNs) using neurotracing techniques and immunohistochemistry. Here we compare the findings of the cervical cord to those of the lumbar cord, and discuss their significance in postnatal motor development with a rostro-caudal gradient and clinical findings of neonatal hypoxic insult.

2. MATERIALS AND METHODS

All experimental procedures were approved by the Animal Studies Committee of Asahikawa Medical College in accordance with the Guide for the Care and Use of Laboratory Animals (NIH

Guide, revised 1996). Every attempt was made to minimize animal suffering and reduce the number of animals used.

2.1 Animals

Sprague-Dawley rats aged 1, 7, and 14 postnatal days (PND) were used. Histological analysis was performed for four rats at each age and in each group.

2.2 Hypoxic load

We subjected 52 PND 0 Sprague-Dawley rats (4 litters) to hypoxia as previously described [8]. The rats were placed in a plastic chamber warmed to 37°C and supplied with humidified 5% oxygen (balance nitrogen) for 3 h. Twenty-five control rats (2 litters) were placed in an identical plastic chamber with room air for an equivalent time. The control rats and the 18 rats that survived hypoxia were used for histological analyses described below. Although an objective behavioral study was not conducted, we noticed that hypoxia rats were less active during locomotor activities than control rats on PND 14.

2.3 Histological procedures

For labeling of spinal MNs, fluorescein isothiocyanate labeled cholera toxin B subunit (CTb) (List Biologicals, Campbell, CA) was used as a retrograde neurotracer. On PNDs 0, 6, and 13, the animals were anesthetized with sodium pentobarbital (18mg/kg body weight, i.p.) and the skin of the fore- and hindlimbs was incised to expose the triceps brachii and quadriceps femoris muscles. Using a 5- μ l Hamilton syringe, 3-5 μ l of 0.5% CTb solution was injected into both muscles. Care was taken to avoid diffusion of the injected solution into adjacent muscles.

Post-injection survival time was kept constant at a minimum of 24 h. On PNDs 1, 7, and 14, the CTb-treated rats were re-anesthetized with sodium pentobarbital (50mg/kg body weight, i.p.) and perfused transcardially with 0.01M phosphate buffered saline (pH7.4). This was followed by administration of 4% paraformaldehyde, 0.2% picrate, and 0.35% glutaraldehyde in 0.1M phosphate buffer (pH7.4) at 4°C. The spinal cord of each rat was removed and post-fixed overnight with 4% paraformaldehyde and 0.2% picrate in 0.1M phosphate buffer at 4°C, followed by immersion for at least 4 h in 0.1M phosphate buffer containing 15% sucrose at 4°C. Coronal sections, 14µm thick, were cut using a cryostat and incubated overnight at 4°C with a 1:10000 dilution of rabbit 5-HT antiserum whose specificity was confirmed by a preabsorption test (courtesy of Dr. H Kimura, Shiga Medical College, Shiga, Japan), incubated for 2 h at room temperature with a 1:40 dilution of tetramethylrhodamine isothiocyanate labeled goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and treated with Vectashield (Vector Laboratories). Analyses were conducted as described below.

2.4 Quantitative and semiquantitative analyses

Quantitative analysis of the density of 5-HT fibers in the MN pools of the cervical and lumbar spinal cord segments was performed. The area of 5-HT fibers was measured using NIH Image (<http://rsb.info.nih.gov/nih-image/>), and densities were calculated by dividing the area of 5-HT fibers by the total measured area. The measured portion was set within Rexed's

lamina IX where the CTb-labeled MNs were located. In addition, 5-HT projection to the spinal MNs was analyzed by merged photographs at a high magnification (x400). According to previously established criteria [14,15], when the gap between a 5-HT varicosity and a MN is 1 μ m or less, it is designated as "close apposition (CA)". The occurrence of CA was counted in each CTb-labeled MN with visible nuclei. The count gives a relative frequency of the occurrence of CA in each MN, and hence, this analysis is semiquantitative.

3. RESULTS

3.1 Development of intraspinal 5-HT system

The distribution pattern of 5-HT fibers was evident on various PNDs in the cervical and lumbar spinal cord segments using camera lucida tracings (Fig.1). At the cervical level, 5-HT fibers were already evident on PND 1 in the lateral portion of the ventral horn including the triceps brachii MN (T-MN) pools. In contrast, they were sparsely distributed on PND 1 in the quadriceps femoris MN (Q-MN) pools at the lumbar level. By PND 7, the 5-HT fibers had increased markedly in the ventral horn, and further extended to the intermediate gray matter and dorsal horn at both levels. On PND 14, the distribution pattern of 5-HT fibers in the lumbar level was almost identical to that of the cervical level. On both levels, numerous 5-HT fibers were distributed in the ventral horn corresponding to Rexed's lamina IX.

Representative photomicrographs of sections that had been double stained for CTb-labeled spinal MNs and 5-HT fibers on each PND are shown in Fig.2. The 5-HT fibers and varicosities were sparsely distributed in both T-MN (Fig.2A) and Q-MN (Fig.2B) pools, and from PNDs 1 to 14 the density of 5-HT fibers and varicosities in both MN pools had markedly increased.

Developmental changes in the density of 5-HT fibers and the number of CA are summarized in Table 1. The number of CA per MN increased continuously between PNDs 1 and 14 at both cervical and lumbar levels. Only on PND 1 did, T-MNs display significantly more CA than Q-MNs ($P < 0.05$).

3.2 Effect of hypoxia on intraspinal 5-HT system

There is no macroscopic change on cervical and lumbar cord in all hypoxia rats on PND 14 in comparison with control rats. Figure 3 shows 5-HT fibers in T-MN (Fig.3A) and Q-MN (Fig.3B) pools in the control and hypoxia rats on PND 14. Although there was no obvious difference between the control and hypoxic rats on PND 1, a marked reduction in the density of 5-HT fibers was seen in hypoxic rats on PND 14; these changes were statistically significant in both cervical and lumbar levels (Table 2). Double-stained photomicrographs of spinal MNs and 5-HT fibers on PND 14 are shown in Fig.4. The 5-HT fibers and varicosities that were in close apposition to T-MNs (Fig.4A) and Q-MNs (Fig.4B) were less distributed in hypoxic rats than in control rats. Q-MN CA numbers were more potently reduced after neonatal hypoxia than those of T-MNs on PND 14 (Table 3), suggesting that development of 5-HT fibers was more severely affected by neonatal

hypoxia in the lumbar cord than in the cervical cord.

4. DISCUSSION

Our previous report [15] showed that the noradrenergic (NA) fibers reach cervical MNs rather before lumbar MNs in early developmental periods. This rostro-caudal gradient in the intraspinal development of NA fibers may be related to the fact that all NA fibers originate from the locus ceruleus in the brainstem [16]. Because all 5-HT fibers also originate from the raphe nuclei in the brainstem [17], a similar rostro-caudal gradient in development of 5-HT fibers found in the present study is conceivable.

Hypoxic effect on the 5-HT system

We previously studied hypoxic changes in medulla-spinal cord descending neurons labeled by CTb injected into the cord's lumbar enlargement [18]. In control rats, CTb-labeled cells were observed in some medulla nuclei including the nuclei raphe magnus (B3), raphe obscurus (B2), and raphe pallidus (B1), where 5-HT-containing neurons are known to be located. In hypoxic rats, there was a notable decrease in the number of CTb-labeled cells in the medulla nuclei including B1. Neonatal asphyxia induces permanent changes in brainstem 5-HT neurons with regional differences [10]. In this report, while a marked reduction of 5-HT neurons was observed in B1, no significant reduction was found in B2 and B3. The authors speculated that the reduction of 5-HT neurons was the results of axonal

degeneration and/or hypofunctioning of the 5-HT neurons. Since 5-HT system develops earlier than other neural system, development of 5-HT system may not be affected by injury of other system. Thus, we also think the change of intraspinal 5-HT system in our study is not some kind of secondary effects but a primary change by hypoxia.

A previous study on brainstem 5-HT neurons projecting to spinal cord [17] classified the descending 5-HT system as the dorsal pathway originating mainly from B3 and terminating in the dorsal horn of the spinal cord; the intermediate pathway originating mainly from the arcuate cell group, B1 and B2, and terminating in the intermediate grey; and the ventral pathway originating mainly in B1 and B2 and terminating in the ventral horn of the spinal cord. These previous results are compatible with our findings that the marked reduction of 5-HT fibers at spinal MN pools possibly originated from B1 after hypoxic insult. The hypoxic effect on the 5-HT neurons also occurs in humans; a decrease in tryptophan hydroxylase-positive 5-HT neurons in the periaqueductal gray matter was reported in sudden infant death syndrome infants, which may be the result of chronic or repeated hypoxia [18].

Trophic and neuroprotective effects of 5-HT system

Depending on the target, 5-HT stimulates the development of the neuropile, myelinization of axons, and differentiation of the synaptic contacts. It can also indirectly influence differentiation of 5-HT neurons through the intermediary of astrocytes [19]. The morpho-functional development of the

visual cortex in newborn rats and the stimulating effects of 5-HT on the visual cortex has been demonstrated in tissue culture [20]. A study on the regulatory effects of monoamines including 5-HT on the synthesis of brain-derived neurotrophic factors in astrocytes led to the suggestion that there is a positive reciprocal interaction between monoaminergic neuronal activity and astrocytic neurotrophic support [21]. Another study demonstrated that cervical dorsal rhizotomy enhances 5-HT terminal density near phrenic MNs and 5-HT-dependent long-term facilitation of phrenic motor output [22], prompting the suggestion that upregulation of the 5-HT system in the cervical spinal cord enhances phrenic MN excitability, thereby compensating for the functional deficits in respiratory motor control caused cervical dorsal rhizotomy.

Functional and pathophysiological implications

The present data shows that development of 5-HT fibers is more severely affected by neonatal hypoxia in the lumbar cord than in the cervical cord. In our previous study concerning the effects of neonatal hypoxia on spinal MNs in rats [8], we noted that the dendrites of lumbar MNs on PND 14 tend to be shorter and less extensive in rats with hypoxic insult at birth than in rats without such an insult, although no significant difference was observed in cervical MNs between hypoxic and non-hypoxic rats. From these similarities in rostro-caudal gradients, our present results strongly suggest that hypoxic changes of the spinal 5-HT system are involved in the developmental deficit only in lumbar spinal MN after neonatal

hypoxia. Neonatal injury of the 5-HT system might not only pose a problem to the 5-HT system itself but also may affect the postnatal development of other neural systems benefiting from the trophic effects of 5-HT. There are the muscle tone inhibitory system and locomotion executing system in the spinal cord, and their activities can be steadily balanced by a net excitatory cortical input and a net inhibitory basal ganglia input [23]. These two systems consist of spinal motoneurons, interneurons, primary afferents, and so on. If the injuries of 5-HT system influence the development of these two systems, it may induce spastic motor disturbance. Furthermore, our findings may account for one of the pathogenetic mechanisms of spastic diplegia, in which symptoms are more apparent in the lower limbs than in upper limbs. Future studies must address the prevention of hypoxic effects on the 5-HT system and confirm whether the prevention of hypoxic effects on the 5-HT system reduces impairment of other neural systems such as neocortex and spinal MNs.

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Figure legends

Fig. 1 Distribution patterns of 5-HT fibers. A: cervical cord, B: lumbar cord. Note that the 5-HT fibers were already evident in the ventral horn at the cervical level on PND 1, but they were sparsely distributed in the quadriceps femoris motoneuron (MN) pools at the lumbar level. At later PNDs, the distribution pattern of 5-HT fibers in the lumbar level was almost identical to that of the cervical level.

Fig. 2 Representative photomicrographs of sections double stained for CTb-labeled spinal MNs and 5-HT fibers at each PND. The green and red ones visualize the MNs and 5-HT fibers, respectively. Arrows in PND 14 (B) indicate the examples of

close appositions. This MN has three close appositions. A: cervical cord, B: lumbar cord. From PNDs 1 to 14, the density of 5-HT fibers and varicosities in both cervical and lumbar MN pools markedly increased. Bar indicates 50 μ m.

Fig. 3 5-HT fibers in the MN pools of control and hypoxic rats on PND 14. A: cervical cord, B: lumbar cord. Marked reduction in the density of 5-HT fibers was seen in hypoxic rats. Bar indicates 50 μ m.

Fig. 4 Double stained photomicrographs of spinal MNs and 5-HT fibers on PND 14. The green and red ones visualize the MNs and 5-HT fibers, respectively. A: cervical cord, B: lumbar cord. The 5-HT fibers and varicosities that were in close apposition to MNs were more sparsely distributed in hypoxic rats than control rats. Bar indicates 50 μ m.

Table 1 Postnatal changes in the density of 5HT fibers at MN pool

		Density of 5HT fibers at MN pool		Close Appositions	
PND 1	Cervical cord	1.7±0.4] *	0.52±0.72] *
	Lumbar cord	0.8±0.5		0.25±0.53	
PND 7	Cervical cord	4.9±2.0] n.s.	4.91±1.48] n.s.
	Lumbar cord	4.2±0.7		4.31±1.36	
PND 14	Cervical cord	6.5±2.7] n.s.	5.25±1.92] n.s.
	Lumbar cord	7.0±2.5		5.35±2.08	

Density of fibers (%) = (Area of the fibers / Total measured area) x 100

Five specimens were measured in each groups for statistical analysis.

Mann-Whitney U-test, *; p<0.05

Table 2 Density of 5HT fibers after Hypoxic Load

	PND 1		PND 14	
	cervical	lumbar	cervical	lumbar
Control	1.7±0.4	0.8±0.5	6.5±2.7	7.0±2.5
Hypoxia	1.6±1.1	1.0±0.5	2.9±0.6	1.5±0.7

Density of fibers (%) = (Area of the fibers / Total measured area) x 100

Five specimens were measured in each groups for statistical analysis.

Mann-Whitney U-test, *; p<0.05 **; p<0.01

Table 3 Number of Close Appositions after Hypoxic Load at PND14

	cervical		lumbar
Control	5.25±1.92] *	5.35±2.08
Hypoxia	3.65±2.03		1.95±1.61

Mann-Whitney U-test, *; p<0.05 **; p<0.01

Figure 1
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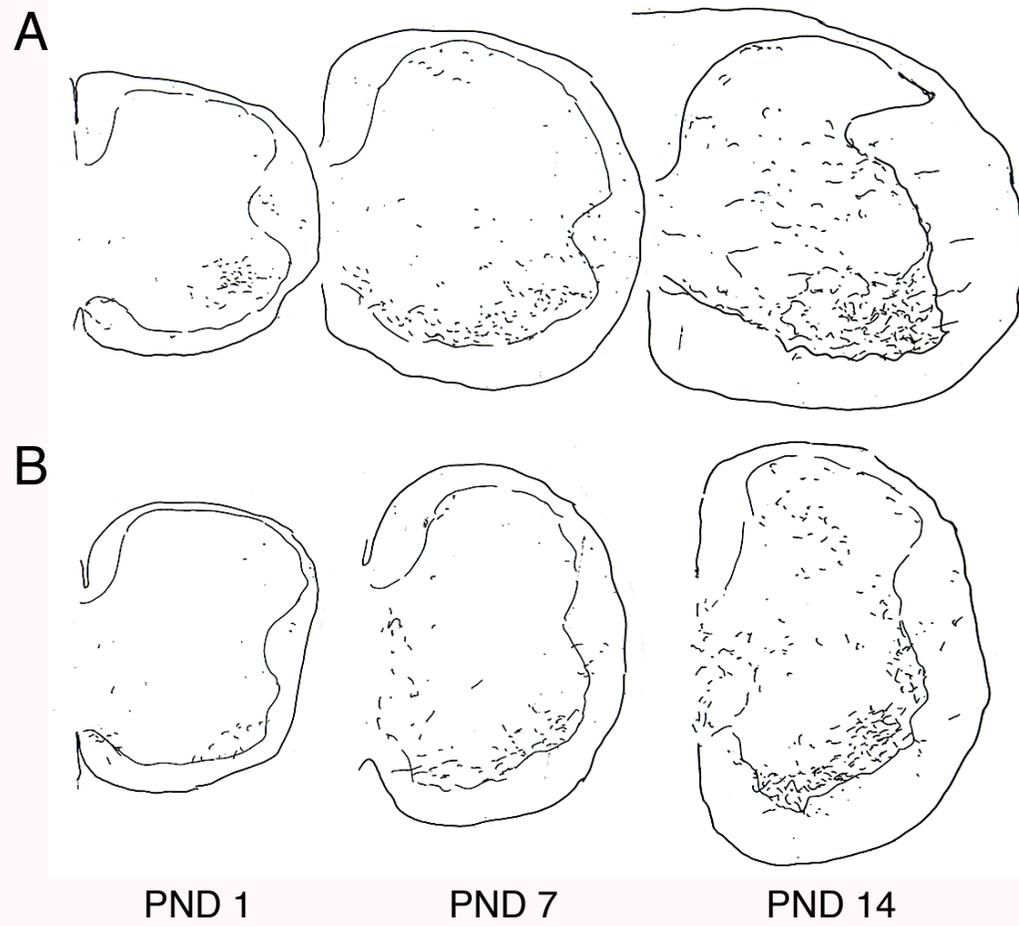


Figure 2
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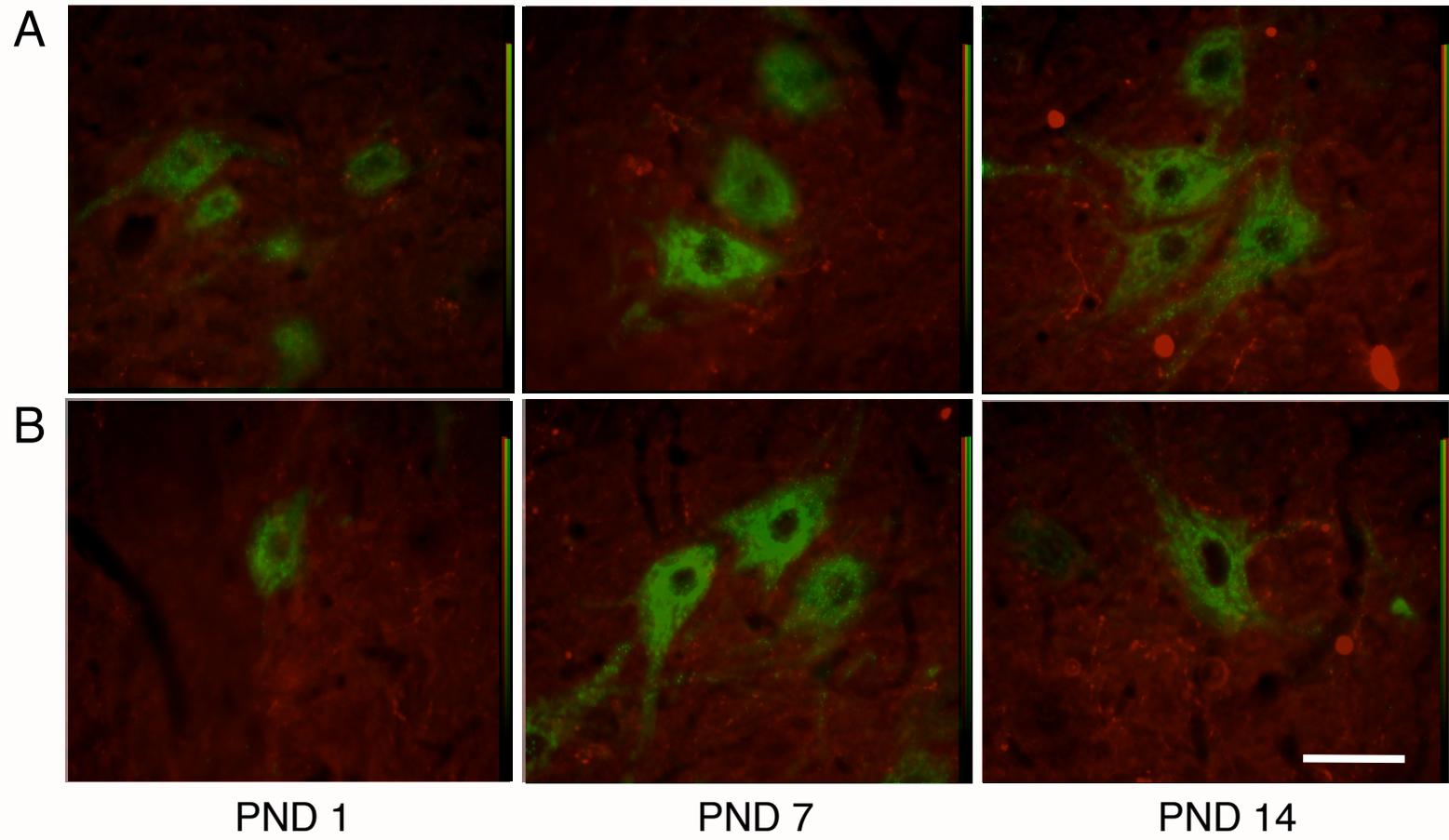


Figure 3
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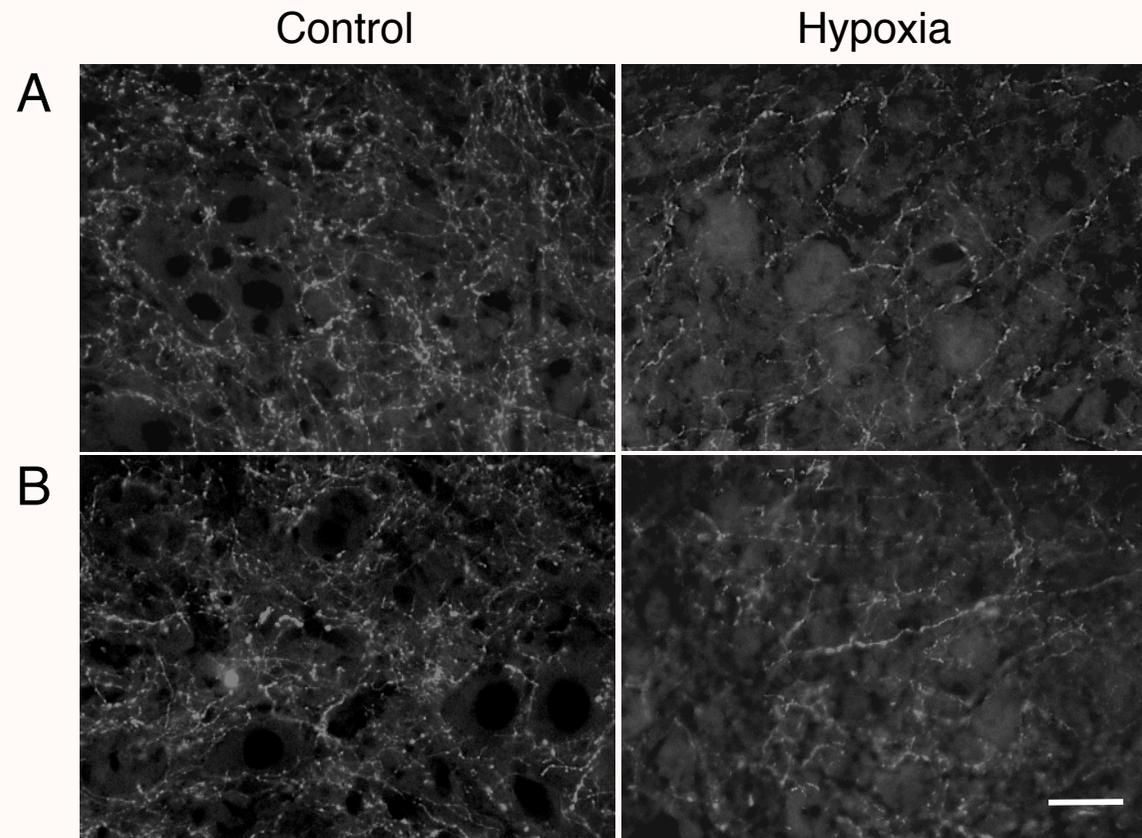


Figure 4
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