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The mRNA Expression of Neurotrophins in Different Skeletal Muscles of Young Rats

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ABSTRACT

Skeletal muscles are a target for motoneurons and synthesize neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Both at the embryonic stage and the adult stage, the mRNA expression of neurotrophins in skeletal muscles of rats has been reported. However, little was known about the mRNA expression patterns of neurotrophins in skeletal muscles of rats at the young developmental stage. In this study, we investigated the mRNA expressions of BDNF and NT-3 in three different skeletal muscles in 4 - to 8 - week - old rats using the reverse transcriptional polymerase chain reaction (RT-PCR) method. The expression of BDNF mRNA in the soleus muscle gradually became higher with age from 5 to 8 weeks. But BDNF mRNA in the tibialis anterior and extensor digitorum longus muscles did not change with growth. The expression of NT-3 mRNA did not show a specific tendency during this period. The differences of muscle fiber types, recruitment patterns of the muscles, and roles of neurotrophins may cause these mRNA expression patterns.

Neurotrophins are target-derived, activity-dependent neurotrophic factors and are transported retrogradely. There is a possibility that the different expression patterns of neurotrophins in muscles may be involved in the maturation of neuromuscular function in different muscles during the young developmental period.

Key words: *Neurotrophin, mRNA, Skeletal muscle, Young rats*

Skeletal muscles are a target for motoneurons and these muscles express neurotrophins[4,7-9,12,15,24]. Neurotrophins are one of the families of neurotrophic factors and consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5)[10].

Lohof et al[14] reported that application of either BDNF or NT-3 rapidly enhanced synaptic transmission at the neuromuscular junction. Mantilla et al[17] reported that presynaptic activation of tyrosine kinase B (TrkB) receptors by BDNF or NT-4 reduces neuromuscular transmission failure causing muscle fatigue. As for NT-3, embryonic over-expression of NT-3 in developing limbs or muscles increases the proprioceptive neurons in number[27,29], while the lack of NT-3 results in a loss of sensory neurons in muscles[28]. Neurotrophins not only act at the site of expression but are transported retrogradely[2]. It can be said that skeletal muscles are the target organ of motor neurons, and, at the same time, the source of neurotrophins which play an important role in the development and function of synapses.

At both the embryonic and adult stages, the mRNA expression of neurotrophins in skeletal muscles of rats has been widely reported. Griesbeck et al[8] reported that the highest levels of mRNA expression of BDNF and NT-3 in the hind limb skeletal muscle of rats could be seen at embryonic day 15. They reported that the neurotrophin mRNA express abundantly in the hind limb skeletal muscle at the embryonic stage and after birth rapidly decrease[8]. Funakoshi et al[5] reported that in early postnatal development, BDNF and NT-3 mRNA in the gastrocnemius muscle of rats were maximal at age 1 to 3 weeks but decreased thereafter. Nagano et al[19] investi-

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gated the mRNA expression of neurotrophins in the gastrocnemius muscle and the soleus muscle of 6-, 15-day and 3-month-old rats. In the gastrocnemius muscle, the mRNA expression levels of BDNF and NT-3 did not change significantly. In the soleus muscle, BDNF mRNA increased at 3 months, while NT-3 mRNA showed no significant change across these stages. Meanwhile Gomez et al[6] reported that physical activity increases the mRNA expressions of BDNF and NT-3. These reports showed that the mRNA expression patterns of neurotrophins differ according to age, and the particular muscles, type of neurotrophins and physical activity in rats. At the embryonic stage, neurotrophin mRNA may mainly be expressed innately, while after birth innate expression may decrease and activity dependent expression increase. There may be a time point of change in the manner of neurotrophin mRNA expression at the young developmental stage. However, little is known about the mRNA expression patterns of neurotrophins in skeletal muscles at the young developmental stage.

In this study, we used the reverse transcriptional polymerase chain reaction (RT-PCR) method to investigate the mRNA expressions of BDNF and NT-3 in three different skeletal muscles in 4 - to 8 - week - old rats. This study provided the fundamental information to define the role of neurotrophins during the young developmental period.

MATERIALS AND METHODS

In this study, we used two types of skeletal muscles; one was the soleus muscle (Soleus), which mainly consists of slow muscle fibers, and the others were the tibialis anterior (TA) and extensor digitorum longus muscles (EDL), which mainly consist of fast muscle fibers. We investigated the mRNA expression of BDNF and NT-3 in Soleus, TA, and EDL of young rats by using the RT-PCR method.

This study was carried out with the permission of the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University, School of Medicine.

Animals

Thirty Wistar rats (Clea Japan, Inc., Ishibe, Japan) were used. The animals were randomly housed in cages and maintained under artificial conditions at 23 ± 1°C, with a constant humidity of 55 ± 5%, and a 12 - hour light / dark cycle. The animals had free access to food and water.

At ages 4, 5, 6, 7 and 8 weeks (n = 6 rats at each time-point), the rats were anesthetized by injection of sodium pentobarbital (50 mg / kg, i.p.), and weighed. Soleus, TA and EDL were harvested from each rat. All muscles were immediately frozen in liquid nitrogen and stored at - 80°C until processed. All samples were harvested in the evening.

Procedure

Isolation of total RNA

Total RNA was isolated from the tissues using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The homogenized sample was treated with chloroform, and the aqueous phase was separated by centrifuging the samples at 12000 rpm for 15 min at 4°C. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. After that, the pellet containing the RNA was washed with 75% ethanol. After drying, the pellet was dissolved in RNase- and DNase-free water. The RNA concentration was measured spectrophotometrically at 260 nm. The RNA sample was diluted by 0.05 μg / μl with RNase- and DNase-free water.

RT-PCR for the mRNA expression of neurotrophins

For semi-quantification of mRNA expression, One-step RT-PCR was performed using SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen, Carlsbad, CA, USA) with GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's indications. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for each sample to monitor the equal amounts of total RNA for all samples. The primer sequences and product length for each gene are shown in Table 1.

Table 1. Primer sequences used for one step RT-PCR

Gene name	Primer sequence: 5'-3'	Product length (bp)
GAPDH	5'-ACCACAGTCCATCAC-3'	450
	5'-ACCACAGTCCATCAC-3'	
rat_bdnf	5'-GGGCCCTTACTATGGATAGCAAA-3'	368
	5'-AACGGCAACAAACCACAACATTATCGAG-3'	
ratNT-3	5'-AGAACTACTACGGCAACAGAGACGCTAC-3'	361
	5'-GGCCTGGCTTCTTTACACCTCGTTTCA-3'	

One step RT-PCR was performed as follows. BDNF: 50°C for 30 min, 94°C for 2 min, for 30 cycles of 94°C for 15 sec, 60°C for 30 sec, 68°C for 30 sec and 72°C for 5 min. NT-3: 50°C for 30 min, 94°C for 2 min, for 36 cycles of 94°C for 15 sec, 60°C for 30 sec, 68°C for 30 sec and 72°C for 5 min. GAPDH: 50°C for 30 min, 94°C for 2 min, for 35 cycles of 94°C for 15 sec, 60°C for 1 min, 68°C for 30 sec and 72°C for 5 min.

The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2% agarose gel. Relative band intensities were digitized using FLA2000 MacBAS (Fuji Film, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed with SPSS 11.5 J for Windows. Comparison of the mean mRNA levels of BDNF and NT-3 between each age was assessed by using one-way analysis of variance (ANOVA). When significant differences were found, to examine the age-related changes in mRNA levels of BDNF and NT-3 compared with at 4 weeks old, statistical analysis was performed with Dunnett's test. After that, to examine the expression patterns in mRNA levels of BDNF and NT-3, statistical analysis was performed with Tukey's honestly significant difference test. Values were expressed as a percentage of each muscle at 4 weeks old. $p < 0.05$ was considered to be statistically significant.

RESULTS

Body Weight

Fig. 1 shows the body weight of 4 -, 5 -, 6 -, 7 - and 8 - week - old rats. The body weights at 4, 5, 6, 7 and 8 weeks were 61.3 ± 2.3, 107.2 ± 1.1, 160.8 ± 2.8, 211.0 ± 1.5, and 263.3 ± 4.7 g, respectively. The body weight increased progressively with age and increased approximately fourfold from 4 to 8 weeks.

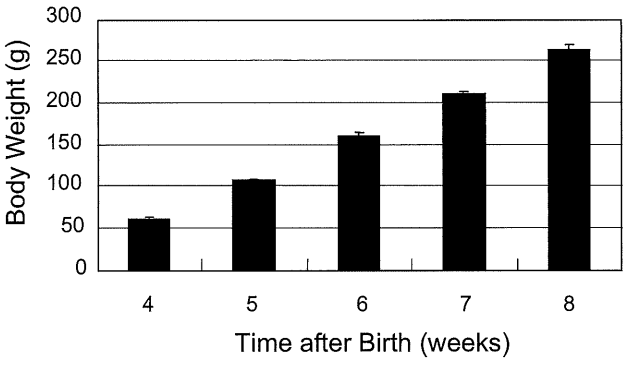

Fig. 1. The body weights of 4 -, 5 -, 6 -, 7 - and 8 - week - old rats (6 rats / group). Data are mean ± S.E.M.

The mRNA level of neurotrophins in skeletal muscles

ANOVA confirmed significant differences in age for BDNF mRNA expression of SOL ($F_{(4, 25)} = 3.370$, $p < 0.05$) and NT-3 mRNA expression of SOL ($F_{(4, 25)} = 4.223$, $p < 0.05$), TA ($F_{(4, 25)} = 3.993$, $p < 0.05$), EDL ($F_{(4, 25)} = 3.453$, $p < 0.05$).

The results of statistical analysis with Dunnett's test showed that there was no significant difference between 4 weeks and other ages in both BDNF mRNA expression in SOL and NT-3 mRNA expression in three muscles.

Fig. 2 shows the mRNA level of BDNF in Soleus, TA and EDL. The levels of BDNF mRNA in soleus muscle at 5, 6, 7 and 8 weeks were 70, 151, 198 and 215% of 4 weeks, respectively. The results of statistical analysis with Tukey's honestly significant difference test showed that the mRNA level of BDNF in Soleus of the 8 - week - old rats was significantly higher than that of the 5 - week - old rats ($p < 0.05$). The levels of BDNF mRNA in TA of 5, 6, 7 and 8 weeks were 54, 82, 126 and 65% of 4 weeks, respectively. The mRNA level of BDNF in EDL of 5, 6, 7 and 8 weeks were 95, 114, 177 and 100% of 4 weeks, respectively.

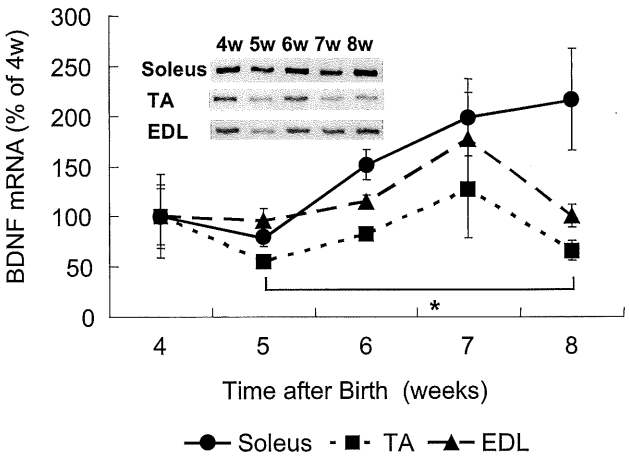

Fig. 2. The expression levels of brain-derived neurotrophic factor (BDNF) mRNA in the soleus muscle (SOL, ◆), the tibialis anterior muscle (TA, ■) and the extensor digitorum longus muscle (EDL, ▲) of 4 -, 5 -, 6 -, 7 - and 8 - week - old rats. Values are expressed as a percentage of each muscle at 4 weeks old. Data are showed as mean ± S.E.M. Each symbol represents a significant difference between stages in the same muscle type ($p < 0.05$; *: SOL).

Fig. 3 shows the mRNA level of NT-3 in Soleus, TA and EDL. The mRNA level of NT-3 in Soleus at 5, 6, 7 and 8 weeks were 46, 48, 128 and 84% of 4 weeks, respectively. The results of statistical analysis with Tukey's honestly significant difference test showed that the mRNA level of NT-3 was significantly higher in Soleus of the 7 - week

- old rats than in that of the 5 - and 6 - week - old rats ($p < 0.05$). The mRNA level of NT-3 in TA of 5, 6, 7 and 8 weeks were 85, 50, 208 and 153% of 4 weeks, respectively. The mRNA level of NT-3 in EDL of 5, 6, 7 and 8 weeks were 63, 53, 135 and 114% of 4 weeks, respectively. For TA and EDL of the 7 - week - old rats, it was significantly higher than the 6 - week - old rats ($p < 0.05$).

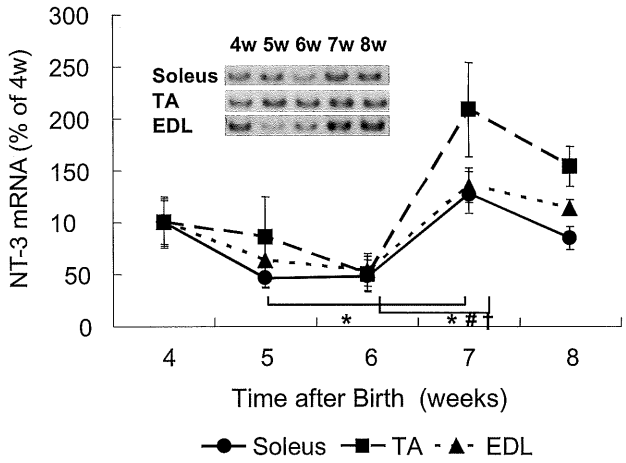

Fig. 3. The expression levels of neurotrophin-3 (NT-3) mRNA in the soleus muscle (SOL, ◆), the tibialis anterior muscle (TA, ■) and the extensor digitorum longus muscle (EDL, ▲) of 4 -, 5 -, 6 -, 7 - and 8 - week - old rats. Values are expressed as a percentage of each muscle at 4 weeks old. Data are shown as mean ± S.E.M. Each symbol represents a significant difference between stages in the same muscle type ($p < 0.05$; *: SOL, #: TA, †: EDL).

DISCUSSION

In this study, we showed the mRNA expression patterns of BDNF and NT-3 in hind limb skeletal muscles of rats from 4 to 8 weeks of age.

Because motoneurons transport BDNF retrogradely[2,12,13,24], the level of neurotrophin protein is unlikely to reflect the level of neurotrophin protein synthesized in skeletal muscles. Therefore, we examined the mRNA expression of neurotrophins in consideration of the influence of retrograde transportation.

The growth of body weight is correlative with the growth of skeletal muscle weight in Wistar rats[26]. In the present study, the body weight increased progressively with age while the expression patterns of neurotrophin mRNA did not reflect the change of body weight. The ratio of neurotrophin mRNA expression to muscle weight may decrease during development. It is known that the neurotrophin mRNA express abundantly in the hind limb skeletal muscle of embryonic Wistar rats to support motoneuron survival and, after birth, rapidly decrease[8]. There may be a different manner of expression before and after birth. Before birth, neurotrophin mRNA may mainly express innately, while after birth the manner of expression shifts to activity dependent. Because of the decrease of innate expression, neurotrophin mRNA expression may not increase in proportion to the body weight during the young developmental period.

From 5 to 8 weeks of age, the expression of BDNF mRNA in Soleus gradually became higher with age, and was significantly higher at 8 weeks than at 5 weeks of age. Nagano and Suzuki[19] reported that BDNF mRNA expression in Soleus was significantly higher at 3 months of age than at 6 and 15 days of age. Our results showed that BDNF mRNA expression in Soleus increases with growth from 5 weeks of age. In TA and EDL, there was no change in mRNA expression of BDNF with growth. On the other hand, the expression of NT-3 mRNA in Soleus, TA, and EDL did not show a specific tendency during this period. These muscles consist of different types of muscle fibers, i.e., Soleus mainly consists of slow fibers containing slow myosin heavy chain (MHC) isoform, while TA and EDL mainly consist of fast fibers containing fast MHC isoform[1,22]. These hind limb muscles are involved in different movements during locomotion, i.e., Soleus is antagonistic to TA and EDL across the ankle joint[21]. Punkt et al[22] reported that muscle fibers showed differentiation after birth, and different fiber types and subtypes were detectable in EDL and Soleus of rats at the age of 21 days. In this study, our results showed that BDNF mRNA expression in Soleus increases with growth from 5 weeks of age. The weaning period of rats begins at 3 weeks old. During subsequent development, higher motor activity is required. Presynaptic activation of tyrosine kinase B (TrkB) receptors by BDNF or NT-4 reduces neuromuscular transmission failure resulting in muscle fatigue[17]. BDNF regulates the expression levels of specific synaptic vehicle proteins[20] and enhances synapse transmission and neurotransmitter release[11]. BDNF may be involved in the maturation or maintenance of the neuromuscular function of Soleus after the muscle fiber type differentiation during the young developmental period.

With respect to NT-3, NT-3 mRNA in Soleus, TA, and EDL did not show a specific tendency during the period studied. Ernfors et al[3] reported that a lack of NT-3 leads to the absence of muscle spindles and Golgi tendon organs in mice. Because NT-3 is thought to be related to muscle tone, it may express constantly in different muscles regardless of age. To investigate this possibility, it is necessary to examine NT-3 mRNA expression patterns during the whole life period in a further study.

Differences in muscle fiber types and activity patterns may give rise to differences in the

expression patterns of BDNF and may lead to the maturation and maintenance of distinctive neuromuscular functions during the young developmental period. On the other hand, it is important for NT-3 to express and be used regularly for the survival and maintenance of proprioceptive neurons and muscle spindles.

Motoneurons retrogradely transport BDNF from skeletal muscles to the spinal cord[12]. Considering the roles of neurotrophins, the change in the level of BDNF synthesized in skeletal muscles may affect the neuromuscular function not only at the site of muscles but also the site of the spinal cord during the young developmental stage. Based on the results of this study, we will do further studies to define the role of neurotrophins in neuromuscular function during the young developmental period.

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