Proceedings of The National Conference
On Undergraduate Research (NCUR) 2013
University of Wisconsin La Crosse, WI
April 11 – 13, 2013

Age-related Changes in GDNF Content of Skeletal Muscle

Stephanie E. Jerger
Department of Biological Sciences
Western Michigan University
Kalamazoo, MI 49008 USA

Faculty Advisor: Dr. John M. Spitsbergen

Abstract

Glial cell line-derived neurotrophic factor (GDNF) is a very potent neurotrophic factor that promotes the maintenance and survival of peripheral motor neurons and is expressed in skeletal muscle. Progressive loss of motor neurons occurs with aging, and over time this degeneration elicits devastating consequences on the physical performance and quality of life of the elderly. One possible explanation for motor neuron degeneration is a reduction in neurotrophic factor support. The primary objective of this study is to examine the age-related changes in GDNF content of skeletal muscle of sedentary Sprague-Dawley rats. Acquiring a better understanding of how GDNF expression in skeletal muscle changes with age will provide insight into the role that GDNF plays during the process of age-induced motor neuron loss. To accomplish this, we are using an inverted confocal scanning microscope to view GDNF protein in the extensor hallucis longus (EHL) skeletal muscle from rats aged 3 weeks to 24 months. The muscles were fixed with paraformaldehyde, and the size and shape of neuromuscular junctions were measured using alpha-bungarotoxin to detect acetylcholine receptors, antibodies against synaptic vesicle protein 2 (SV2) to detect nerve terminal and antibodies against GDNF to determine where and how much GDNF is in the muscle. The results of our study thus far have shown that neuromuscular junctions (NMJ) are immature and undergo initial development in rats aged 3 weeks, and the staining of trophic factor was strongest at this age. The end plates from rats aged 5 and 8 weeks were more developed, and the presence of trophic factor was clearly defined at the end plates. In the EHL muscle from rats aged 24 months, there appeared to be multiple end plates at each fiber, some of which were small and immature, which is indicative of possible degeneration followed by regeneration. Staining for GDNF in rats aged 24 months appeared less defined and dispersed around the NMJ rather than localized at the end plates, which further supports the possibility of end plate degeneration and regeneration. These results suggest that the presence of trophic factor is strongest while the NMJ is forming in young rats, and that the dispersal and decrease of GDNF in old rats appears to correlate with degeneration and regeneration. This work was supported by NIH grant 1 R15 AG022908-01A2, NSF grant DBI 0552517 and Western Michigan University.

Keywords: GDNF, Aging, Neuromuscular Junctions

1. Introduction

Skeletal muscle fibers are innervated by peripheral motor neurons. The specific site of contact between motor neurons and muscle fibers occurs at motor endplates, which are clusters of ACh receptors embedded in the sarcolemma[1]. Prior to the complete development of neuromuscular junctions, individual skeletal muscle fibers are often innervated by more than one motor neuron, referred to as polyneuronal innervation[1,2, 3, 4]. As development proceeds, additional synapses are eliminated by axonal competition to ensure that each fiber remains innervated by only one motor neuron[2, 3, 4, 5]. With advancing age, the number of motor neurons progressively declines as a result of age-induced neuronal degeneration, leading to a reduction in both the number and diameter of skeletal muscle fibers[6, 7, 8]. The accumulated loss of motor neurons contributes to a measurable loss of skeletal muscle mass and

strength[8, 9, 10, 11, 12] which in turn, evokes devastating consequences on the physical performance and quality of life of elderly individuals.

Neurotrophic factors are a family of extracellular signaling proteins vital to the maintenance, survival and plasticity of neurons in both the central and peripheral nervous systems[13]. It has been proposed that neurotrophic factors are derived from target tissues, which produce molecular signals capable of enhancing neuron survivability and development and are recognized by the innervating neurons[14]. In particular, glial cell line-derived neurotrophic factor (GDNF) plays a profound role as one of the most potent neurotrophic factors for promoting the development, maintenance and survival of peripheral motor neurons[15, 16, 17]. GDNF expression has been observed in Schwann cells[18, 19], nerve terminal and motor endplates[20, 21] and previous studies have also demonstrated the critical importance of GDNF expression at an early age for proper formation and development of the NMJ[22]. It has been observed that GDNF concentration determines the number of neurons forming synapses with skeletal muscle during development[16, 17]. Thus, exceptionally high levels of GDNF expression may be responsible for the early presence of polyneuronal innervation in skeletal muscle, which would also suggest that GDNF plays an important role in modulating and supporting synapses between peripheral motor neurons and motor endplates in skeletal muscle fibers throughout NMJ development. As a result of the imperative association between GDNF expression and survival of the NMJ, one possible explanation for age-induced motor neuron degeneration and the subsequent degradation of skeletal muscle may be a loss in neurotrophic factor support.

Previous studies in our lab, which have compared GDNF protein content in slow-twitch and fast-twitch muscle fibers, demonstrated that the predominantly slow-twitch soleus (SOL) muscle from young, postnatal animals express higher GDNF protein content than both the predominantly fast-twitch extensor digitorum longus (EDL) and extensor hallucis longus (EHL) muscles. Evidence from other studies suggests that skeletal muscles are able to alter fiber-type composition[6, 23, 24] over time. In other words, skeletal muscles representing predominantly fast-twitch muscle fibers in young animals may convert to predominantly slow-twitch fibers with advancing age. Fiber type transition occurs after a fast-twitch fiber loses innervation by a degenerated fast-type motor neuron[25]. The fiber then becomes reinnervated by a slow-type motor neuron[7, 26]. It has been suggested that GDNF support may promote reinnervation following injury-induced degeneration in adults[27]; thus, an increase in GDNF expression may also be anticipated in the presence of fiber type conversion.

Because of the strong correlation between GDNF expression and motor neuron maintenance and survival, examining the age-related changes in the location and content of GDNF in skeletal muscle, as well as the structural changes of neuromuscular junctions, may provide further insight into the role of GDNF during the process of age-induced neuronal degeneration and muscle degradation.

2. Methods

2.1 subjects

Twenty eight male SASCO (Sprague-Dawley) rats (Charles River, Kalamazoo, MI) were given access to food and water *ad libitum*, and were maintained on a 12 hour light/dark cycle. All studies were performed on sedentary control animals that were housed in individual standard living chambers and remained sedentary throughout the study. All of the animal experiments were performed in compliance to the "Guide for the Care and Use of Laboratory Animals" (National Research Council) and all protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University.

2.2 Immunochemistry

EHL skeletal muscles were chosen because they represent predominantly fast-twitch muscles. The tissues were removed and fixed in 4% paraformaldehyde for 15 minutes followed by three subsequent washes in phosphate buffered saline (PBS). The tissues were then frozen in 2-methylbutane cooled on dry ice. Primary antibodies were applied to the tissues and the tissues were then incubated overnight at 4ºC. EHL tissues were bound to rabbit anti-GDNF (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a secondary antibody conjugated to AlexaFluor 594 (Life Technologies Corp., Carlsbad, CA) for determining the location and expression of GDNF; α-bungarotoxin conjugated to AlexaFluor 488 (Life Technologies) was used to detect nicotinic acetylcholine receptors for endplate visualization and mouse anti-SV2 (University of Iowa Hybridoma Bank) was used against synaptic vesicle protein II, followed by a secondary antibody conjugated to AlexaFluor 647 (Life Technologies) for viewing the nerve

terminal. All stained tissues were mounted onto slides using a 1:1 solution of PBS:glycerol and sealed with a cover slip.

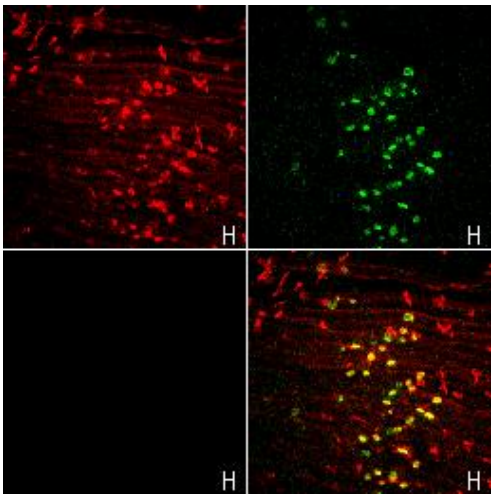
2.3 Histology

Tissues were viewed using a Zeiss LSM 510 confocal laser scanning microscope. Samples were optically sectioned and images were constructed using LSM510 software to obtain a projection incorporating all optical sections. For visualization of Alexa Fluor 488, the filter was set to band-pass 505-550 nm. For visualization of Alexa Fluor 594 filters were set to band pass 585-615 nm. For visualization of Alexa Fluor 647 filters were set to low pass 650 nm. Samples were optically sectioned in 1µm slices and then digitally reconstructed via Zeiss LSM 510® software to obtain a Z-stack projection encompassing all layers of the end plate. The amplifier gain settings for red and blue signals were adjusted separately for each tissue. Images of whole-mounted EHL muscle were obtained from a minimum of three animals per age group, and image brightness and contrast was adjusted using the LSM software. GDNF, SV2 and ACh receptors were viewed in independent channels, and the merged channel showing an overlay of staining was examined to assess for co-localization.

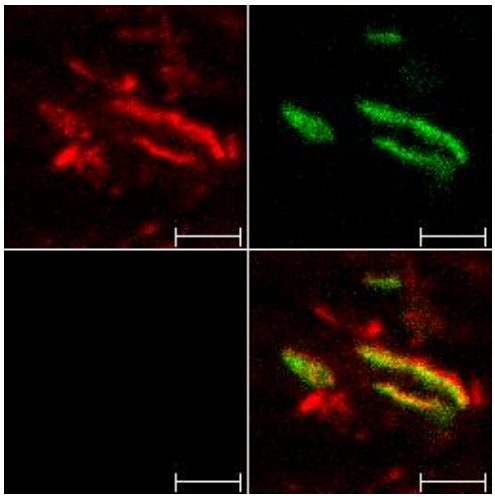
3. Results

Images captured from the confocal microscope convey the age-related changes in GDNF expression and neuromuscular junctions in animals aged 3 weeks to 24 months.

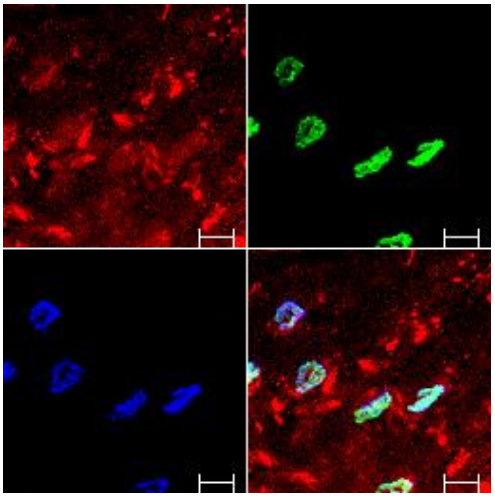
(a) 3-week-old (20x)



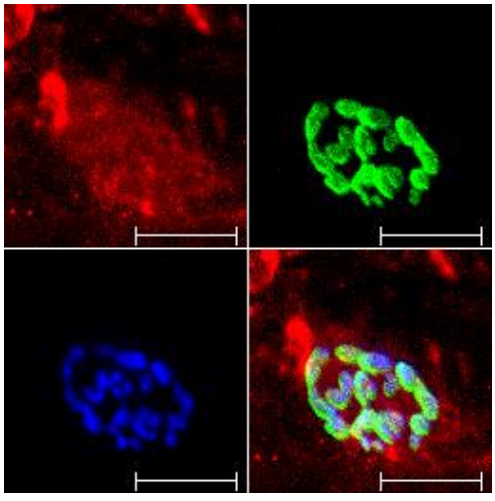
(b) 3-week-old (20x-6x)



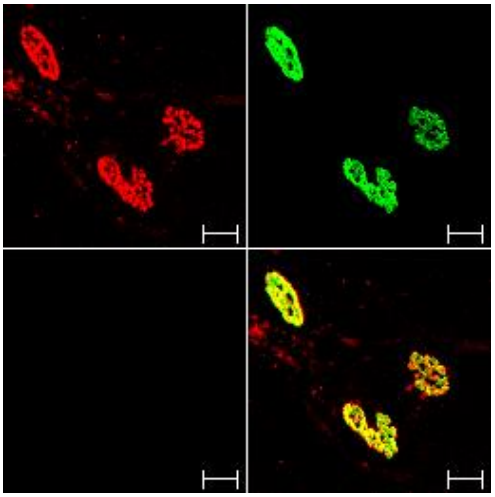
(c) 5-week-old (63x)



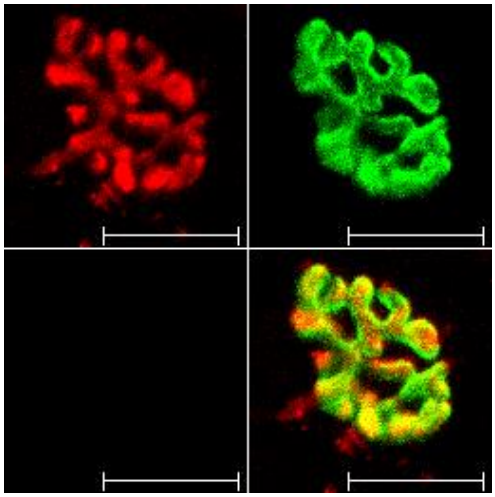
(d) 5-week-old (63x-3x)



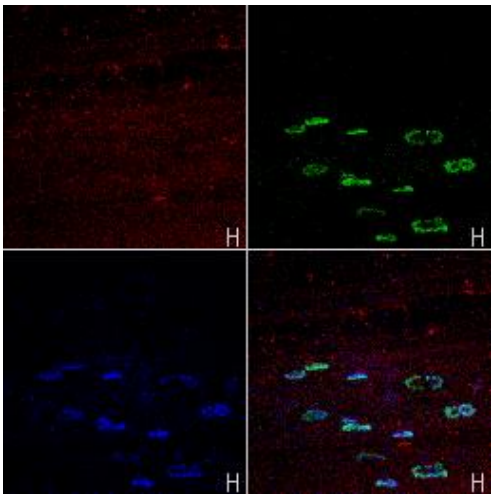
(e) 8-week-old (63x)



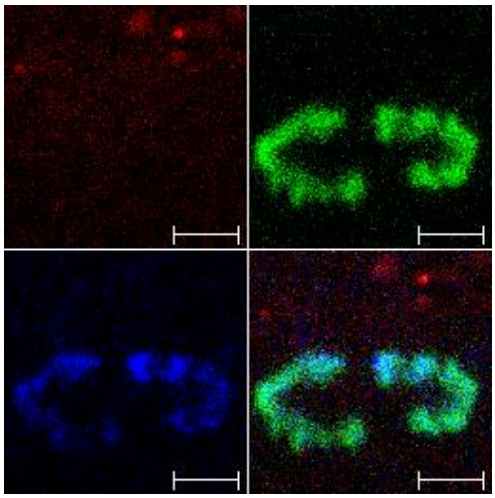
(f) 8-week-old (63x-4x)



(g) 6-month-old (20x)



(h) 6-month-old (20x-6x**)**



(i) 24-month-old (20x)

(j) 24-month-old (63x)

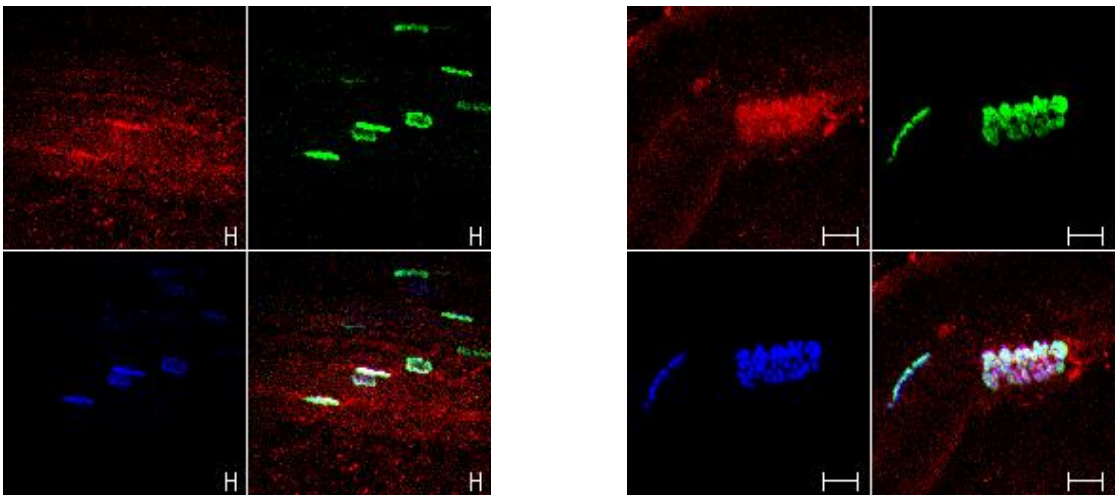


Figure 1. Immunohistochemistry from the EHL of animals aged 3 weeks to 24 months

Figure 1. GDNF staining changes with age in the EHL muscle. Confocal images of whole-mounted EHL tissues from (a) and (b) 3-week-old, (c) and (d) 5-week-old, (e) and (f) 8-week-old, (g) and (h) 6-month-old, and (i) and (j) 24-month-old animals were stained with rabbit anti-GDNF (red), α-bungarotoxin (nicotinic acetylcholine receptors, green) and mouse anti-SV2 (blue). In 3-week-old animals, the endplates are immature and GDNF is present at nearly every endplate region and throughout the tissue. The endplates are more developed in 5-week-old animals and GDNF is distributed at and around the NMJ. GDNF is primarily localized to the endplates in 8-week-old tissues and endplates appear fully developed. In 6-month-olds, GDNF staining significantly decreases and is not visible at the NMJ and is only slightly detectable in surrounding tissue, and NMJs appear to be deteriorating, indicated by endplate dispersion. More endplates appear to be intact in tissues from rats aged 24 months, and more GDNF is located at and around the NMJs. The Scale bar represents 20μm.

GDNF is visible at and around each endplate and throughout the tissue in young animals as the NMJs are actively developing. GDNF staining is localized primarily to the endplate region in animals with recently developed NMJs. In older animals, a significant decline in GDNF is visible and many of the endplates appear to be deteriorating. This is followed by the appearance of some intact endplates and a moderate increase in GDNF staining at and around the endplate regions in the oldest age group.

4. Conclusion

Our results show that the presence of GDNF is distinct in the EHL from young animals aged 3 – 8 weeks, followed by a significant decline in 6-month-old animals and a substantial increase in rats aged 24 months. We were unable to visualize SV-2 in 3 and 8-week-old animals, which may be due to a lack of synaptic vesicle protein II in these age groups, or our staining procedure may have failed in these two age groups.

At 3 weeks of age, animals were in the initial stages of neuromuscular junction development and tissues were densely populated with immature endplates located in very close proximities to one another. In addition, we could see clear localization of GDNF at every visible endplate and there appeared to be a greater number of concentrated GDNF regions than number of visible endplates. One possible explanation for the greater ratio of GDNF to endplates as well as the large population of endplates located in close proximities may be related to polyneuronal innervation. The presence of hyperinnervation of muscle fibers has been tied to GDNF over expression in muscle[16, 17]. Elimination of polyneuronal innervation in rats typically progresses during the first few weeks of age[4] as development of the NMJ proceeds to completion. Therefore, it is possible that each fiber contains more than one

endplate[17] and is innervated by multiple axons at 3 weeks of age when endplates are still very immature. Thus, the appearance of hyperinnervation may result from the high level of GDNF expression. It is likely that the predominant location of GDNF in 3-week-old animals was either in the tissue directly underlying the endplates or in the nerve terminal directly above the endplates as suggested by the observation of two distinct layers of staining in the merged channel.

The endplates in 5-week-old tissues were slightly more developed and GDNF appeared distinct near the NMJ and in the surrounding tissue. Concentrated regions of GDNF staining outside of the NMJs were also detected, although this was not as common as in 3-week-old animals. The increased concentration of GDNF at the end plate region may be due to altered expression of GDNF only in areas where it is specifically needed. The distribution of distinct GDNF staining at the NMJ exceeded the size of the NMJ and extended out into surrounding tissue. One possibility for the excess size of GDNF staining may be that GDNF presence precedes further development and growth of the NMJ. It has been proposed that GDNF support is critical for motor neuron growth and development[22] which may explain the excess distribution of GDNF staining prior to further development and size expansion of the NMJ. Our observations from animals aged 8 weeks appear to support this possible explanation.

Endplates in animals aged 8 weeks appeared fully developed, suggested by the increase in size and structural complexity. In these animals, the distribution of GDNF was co-localized closely to the acetylcholine receptors as suggested by the nearly identical staining pattern for GDNF and the end plate. GDNF presence appeared confined primarily to vicinities of the NMJ, which suggests that GDNF expression may occur only where it is specifically needed following development. This may suggest that GDNF support is most essential at the fully developed NMJ in order to maintain proper synaptic connections and intact structures.

In 6-month-olds, many of the endplates showed signs of dispersion, suggestive of NMJ deterioration. GDNF staining was minimal and only sparsely visible in one or two areas of tissue but not within the NMJ vicinity. The significant reduction and lack of neurotrophic factor support at the NMJ may be related to the deterioration of the NMJ and the dispersion of endplates observed in 6-month-olds. One possible explanation for the significant reduction in neurotrophic factor support may be related to the animals remaining sedentary. Minimal activation of EHL muscle fibers due to lack of physical activity may contribute to a decrease in GDNF expression. The significant reduction in GDNF support at the NMJs may account for the appearance of deteriorating endplates in this age group.

In 24-month-olds, GDNF staining increased substantially from that seen in 6-month-olds. Distinct GDNF staining was clearly visible at the endplate regions. Further, many NMJs appeared intact with complex endplates. The increase in GDNF support as well as mature, intact NMJs suggests the possibility of muscle type conversion. Based on the appearance of deteriorating NMJs in 6-month-old tissues, muscle type conversion would likely take place around this time[6], resulting from the denervation of fast-type muscle and reinnervation[23, 25] by slow-type neurons. It may be that the increase in GDNF expression observed in these animals helps to promote reinnervation of the muscle fibers, allowing the subsequent phenotypic conversion to transpire. Based on the appearance of fully developed endplates at 24 months of age, the process of fiber conversion likely reaches completion near this age.

The present study examined the age-related changes in GDNF content in the EHL skeletal muscle from sedentary rats. Our findings suggest that GDNF content in skeletal muscle is high in young animals as muscle fibers are actively being innervated. GDNF content in skeletal muscle appears to decrease as synaptic contact with motor neurons matures and continues to decline with aging, possibly contributing to a loss of synaptic contacts. Finally, in 24-month-old animals more GDNF is observed at and around synaptic contacts, possibly as remaining slow-type motor neurons are reinnervating fast-type muscle fibers that have undergone denervation due to degeneration of fast-type motor neurons. An accumulated loss of peripheral motor neurons ultimately leads to the measureable deterioration of skeletal muscle mass and strength. A better understanding of the changes in neurotrophic factor expression occurring with age will aid in understanding the roles for altered neurotrophic factor expression in nervous system function that naturally occurs with age. These studies may also help to identify possible therapeutic targets to enhance GDNF expression in aging individuals in order to slow the progression of age-induced motor neuron degeneration, thereby enhancing the quality of life for elderly individuals.

5. Acknowledgements

The author wishes to express her sincere appreciation to her faculty mentor, Dr. John Spitsbergen, for his continued guidance, assistance and encouragement throughout the duration of the project. The author also wishes to express her appreciation to the Lee Honors College of Western Michigan University for sponsoring the trip to NCUR, the

Office of the Vice President for Research Undergraduate Research Excellence Award, the College of Arts and Sciences Undergraduate Research and Creative Activities Award, and the Biological Sciences Imaging facility at Western Michigan University.

6. References

1. Hunt, C.C. and Kuffler, S.W. 1954. Motor innervation of skeletal muscle: multiple innervation of individual muscle fibres and motor unit function. *Journal of Physiology*, 126, 293-303.

2. Brown, M.C., Jansen, J.K. and Van Essen, D. 1976. Polyneuronal innervation of skeletal muscle in newborn rats and elimination during maturation. *Journal of Physiology*, 261, 387-422.

3. Favero, M., Massella, O., Cangiano, A. and Buffelli, M. 2009. On the mechanism of action of muscle fibre activity in synapse competition and elimination at the mammalian neuromuscular junction. *European Journal of Neuroscience*, 29, 2327–2334.

4. O'brien, R.A.D., Ostberg, A.J.C. and Vrbova, G. 1978. Observations on the elimination of polyneuronal innervation in developing mammalian skeletal muscle. *Journal of Physiology*, 282, 571-582.

5. Bennett, M. R., McGrath, P. A., Davey, D. F. and Hutchinson, I. 1983. Death of motorneurons during the postnatal loss of polyneuronal innervation of rat muscles. *Journal of Comparative Neurology*, 218, 351–363.

6. Alnaqeeb, M.A. and Goldspink, G. 1987. Changes in fibre type, number and diameter in developing and ageing skeletal muscle. *Journal of Anatomy*, 153, 31-45.

7. Larsson, L. 1995. Motor units: remodeling in aged animals. *Journal of Gerontology A Biol Sci Med Sci*, 50, 11-16.

8. Tauchi, H., Yoshioka, T. and Kobayashi, H. 1971. Age change of skeletal muscles of rats. *Gerontologia*, 17(4), 219-227.

9. Doherty, T.J., Vanderhoot, A.A., Taylor, A.W. and Brown, W.F. 1993. Effects of motor unit losses on strength in older men and women. *Journal of Applied Physiology*, 74(2), 868-874.

10. Janssen, I., Heymsfield, S. B. and Ross, R. 2002. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. *Journal of the American Geriatrics Society*, 50, 889–896.

11. Lexell, J., Henriksson-Larsén, K., Winblad, B. and Sjöström, M. 1983. Distribution of different fiber types in human skeletal muscles: Effects of aging studied in whole muscle cross sections. *Muscle Nerve*, 6, 588–595.

12. Rosenberg, IH. 1997. Sarcopenia: origins and clinical relevance. *Journal of Nutrition*, 127(5 Suppl): 990S-991S.

13. Kablar, B. and Belliveau, A.C. 2005. Presence of neurotrophic factors in skeletal muscle correlates with survival of spinal cord motor neurons. *Developmental Dynamics*, 234, 659-669.

14. Purves, D., Snider, W.D. and Voyvodickk, J.T. 1988. Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. Nature, 336(6195), 123-128.

15. Henderson, C.E., Philliphs, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M., et al. 1994. GDNF: a potent survival factor for motorneurons present in peripheral nerve and muscle. *Science*, 266(5187), 1062-1064.

16. Nguyen, Q.T., Parsadanian, A.S., Snider, W.D. and Lichtman, J.W. 1998. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science*, 279(5357), 1725-1729.

17. Zwick, M., Teng, L., Mu, X., Springer, J.E. and Davis, B.M. 2001. Overexpression of GDNF induces and maintains hyperinnervation of muscle fibers and multiple end-plate formation. *Experimental Neurology*, 171(2): 342-250.

18. Hase, A., Saito, F., Yamada, H., Aria, K, Shimizu, T. and Matsumura, K. 2005. Characterization of glial cell line-derived neurotrophic factor family receptor alpha-1 in peripheral nerve Schwann cells. *Journal of Neurochemistry*, 95, 537-543.

19. Iwase, T., Jung, C.G., Bae, H., Zhang, M., Soliven, B. 2005. Glial cell line-derived neurotrophic factor-induced signaling in Schwann cells. Journal of Neurochemistry, 94, 1488-1499.

20. Suzuki, H., Hase, A., Miyata, Y., Arahata, K. and Akazawa, C. 1998. Prominent expression of glial cell line-derived neurotrophic factor in human skeletal muscle. *J Comp Neurol*, 402, 303-312.

21. Wehrwein, E. A., Roskelley, E. M. and Spitsbergen, J. M. 2002. GDNF is regulated in an activity-dependent manner in rat skeletal muscle. *Muscle Nerve*, 26, 206–211.

22. Ribchester, R. R., Thomson, D., Haddow, L. J. and Ushkaryov, Y. A. 1998. Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF). *Journal of Physiology*, 512, 635–641.

23. Lexell, J. 1995. Human aging, muscle mass, and fiber composition. *Journal of Gerontology A Biol Med Sci*, 50, 91-95.

24. Pette, D. and Staron, R.S. 2001. Transitions of muscle fiber phenotypic profiles. *Histochem Cell Bio*, 115, 359-372.

25. Li, Y., Lee, Y.I. and Thompson, W.J. 2011. Changes in aging mouse neuromuscular junctions are explained by degeneration and regeneration of muscle fiber segments at the synapse. *Journal of Neuroscience*, 31(42): 14910-14919.

26. Kawabuchi, M., Zhou, C.J., Wang, S., Nakamura, K., Liu, W.T. and Hirata K. 2001. The spatiotemporal relationship among Schwann cells, axons and postsynaptic acetylcholine receptor regions during muscle reinnervation in aged rats. *The Anatomical Record*, 264, 183-202.

27. Blesch, A. and Tuszynski, M.H. 2001. GDNF gene delivery to injured adult CNS motor neurons promotes axonal growth, expression of trophic neuropeptide CGRP, and cellular protection. *Journal of Comparative Neurology*, 436, 399-410.