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Cholinergic Neurons Regulate and Utilize GDNF Secreted by C2C12 Skeletal Muscle Cells in Culture

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Cholinergic neurons regulate and utilize GDNF secreted by C2C12 skeletal muscle cells in culture

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) has been regarded as a potent survival factor for a subpopulation of neurons. It has been shown that GDNF expression is upregulated in skeletal muscle of patients with early stage of neuromuscular diseases such as amyotrophic lateral sclerosis (ALS). Previous results from our laboratory showed that neural cells regulate GDNF secretion by skeletal muscle; non-innervated skeletal muscle appear to secrete more GDNF compared to innervated skeletal muscle. Two aims were examined in the current study. First, to examine whether neural cells inhibit GDNF through acetylcholine release. Second was to examine whether differentiated NG108-15 neural cells secrete GDNF. Acetylcholine receptors on nerve-muscle co-cultured cells were blocked with alpha bungarotoxin (α-BTX). Results showed that α-BTX reversed the action of neural cells, suggesting that neural cells regulate GDNF production through acetylcholine release. ELISA results showed no GDNF in differentiated NG108-15 cells grown alone. However, immunocytochemistry results showed that GDNF was localized in NG108-15 cells co-cultured with myotubes. These observations suggest that upon contact, cholinergic neural cells not only regulate GDNF secretion but also may be utilizing GDNF secreted by skeletal muscle. Taken together, our results suggest that cholinergic neural cells depend on and regulate GDNF secreted by its target. *Supported by NIH Grant 1R15AG022908-01A2, MSU-KCMS, and Western Michigan University.*

Introduction

Glial cell line–derived Neurotrophic Factor (GDNF) is a potent survival factor for subpopulations of neurons in both central and peripheral systems (Lin et al., 1993; Henderson et al., 1994). The presence of GDNF in skeletal muscle at the neuromuscular junction (NMJ) suggests a target-derived action on motor neurons, in which GDNF is retrogradely transported by axons to target neuron's cell body through a receptor-mediated process (Nguyen et al., 1998). GDNF was shown to be a potent survival factor for motor neurons (Henderson et al, 1994).GDNF in skeletal muscle has been shown to be a survival factor for motor neurons during development and may be sufficient for support of the motor nerve innervating muscle after development (Angka et al., 2008). Continuous administration of exogenous GDNF maintains hyperinnervation at the NMJ (Keller-Peck et al., 2001; Zwick et al., 2001). Studies have reported changes in GDNF expression in skeletal muscle of individuals with neurodegenerative diseases, with an increase in early stages followed by a decrease with the progression of disease (Lie and Weis, 1998). GDNF is increased in denervated skeletal muscle (Lie and Weis, 1998), suggesting a response of GDNF in triggering reinnervation; supporting the hypothesis that GDNF may be useful as a potent therapeutic candidate for neuromuscular diseases. Although the effects of exogenous GDNF to neuromuscular synapses have been extensively studied, little is known about factors regulating normal synthesis and secretion of endogenous GDNF in skeletal muscle at the NMJ. This study aims to examine the role that motor neurons play in regulating GDNF production by skeletal muscle at the neuromuscular synapse. Neural cells (NG108-15) were added to skeletal muscle cells (C2C12) and GDNF production was examined. Results from this study suggest that cholinergic neural cells regulate GDNF production by muscle cells possibly through acetylcholine receptors.

Aims

a. **Examine GDNF production by skeletal muscle cells in culture.**

b. **Localize GDNF in skeletal muscle and at the nerve-muscle contact.**

c. **Examine the role that neural cells play in regulating GDNF production by skeletal muscle.**

d. **Examine if cholinergic neural cells induce their effect through acetylcholine receptors.**

Results

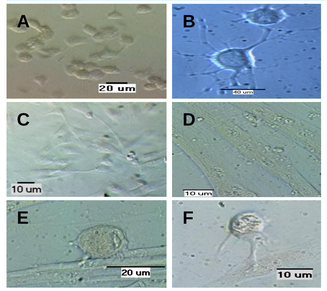
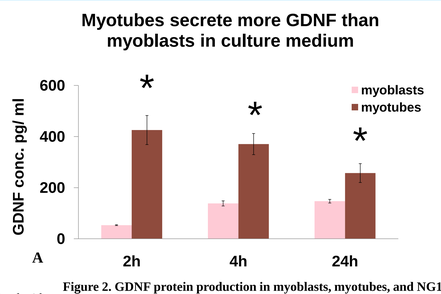


Figure 1. C2C12 and NG108-15 cells in culture. Cells were fixed with 4% paraformaldehyde. **A**. Undifferentiated NG108-15, **B**. Differentiated NG108-15 cells, **C**..Myoblasts, **D**. Myotubes, myoblasts in (c) fused to form myotubes. **E& F**. Nerve-muscle co-cultured cells.



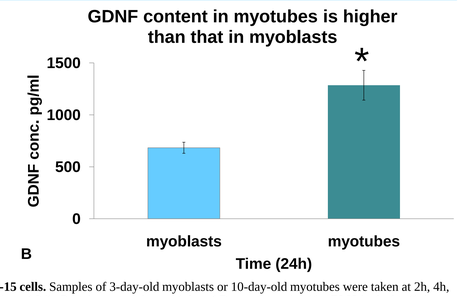


Figure 2. GDNF protein production in myoblasts, myotubes, and NG108-15 cells. Samples of 3-day-old myoblasts or 10-day-old myotubes were taken at 2h, 4h, and 24h after changing medium. Myotubes were scraped from dishes at 2h, 4h, and 24h. Protein content in panel **A** and **B** was determined by ELISA. **A**. At all time points myotubes secreted significantly higher levels of GDNF than myoblasts. **B**. GDNF content within myoblasts and myotubes: Myotubes contain significantly more intracellular GDNF than myoblasts. Values are presented as mean S.E.M. Asterisk indicates significance ($p \leq 0.05$). n=4.

Average GDNF protein content produced by NG108-15 cells versus C2C12 cells in (pg/ml)		
	GDNF secreted into culture medium	*GDNF content in cells*
Non-differentiated	74.3 ± 7	217 ± 13
Differentiated NG108-15	None/ undetectable	None/undetectable
C2C12 Myoblasts	147 ± 7	683 ± 52
C2C12 myotubes	487 ± 89	1047 ± 133

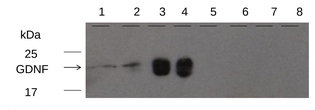


Table 1 ELISA results showed that differentiated NG108-15 do not produce detectable GDNF while non-differentiated express GDNF but in low levels compared to myoblasts and myotubes. **Figure 3** Western blot. Lanes 1& 2 represent GDNF secreted in culture medium by myotubes, lanes 3 & 4 represent GDNF contained within myotubes, lanes 5-6 represent GDNF in NG108-15 culture medium, and lane 7-6 represent GDNF in NG108-15 cells.

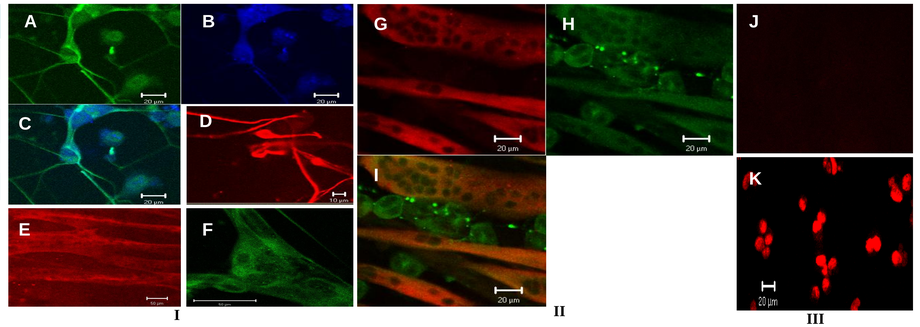
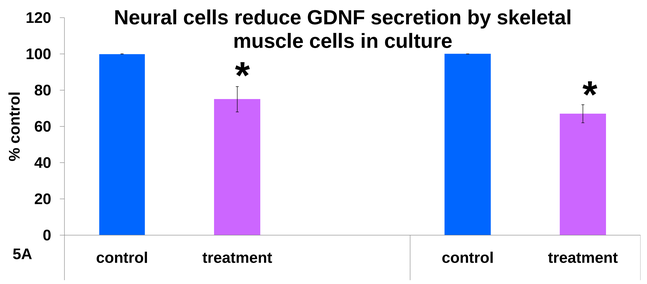


Figure 4. NG108-15 neural cells and C2C12 myotubes express proteins that are normally expressed in neural or skeletal muscle cells. Panel I: A – F. A. NG108-15 cells were bound with Mill Marker FluoroPan Neuronal Marker (green), **B**. anti-ChAT (blue). **C**. Overlay. **D.** NG108-15 express neurofilament. **E**. Myosin in C2C12 myotubes was localized with primary antibody against myosin followed by secondary antibody conjugated to Alexa Fluor 568 (red), **F.** Rabbit anti-GDNF followed by donkey anti-rabbit conjugated to Alexa Fluor 488 (green) used to determine GDNF in myotubes. **Panel II: GDNF in NG108-15 – C2C12 myotubes co-culture. G**. Myosin (red), **H**. GDNF (green), **I**. Overlay, GDNF was observed in both NG108-15 cells and myotubes. **Panel III. J-K . J.** differentiated NG108-15 do not express PCNA while in **K,.**Non-differentiated NG108-15 express PCNA (red), a marker for cell proliferation; it was identified by anti-PCNA antibody.



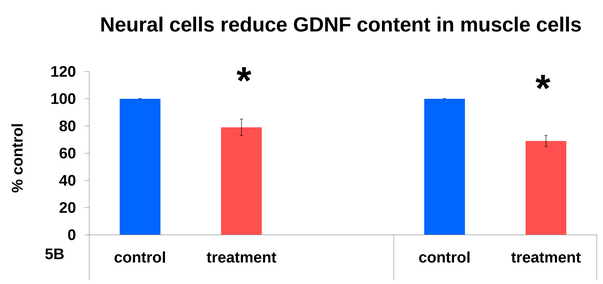
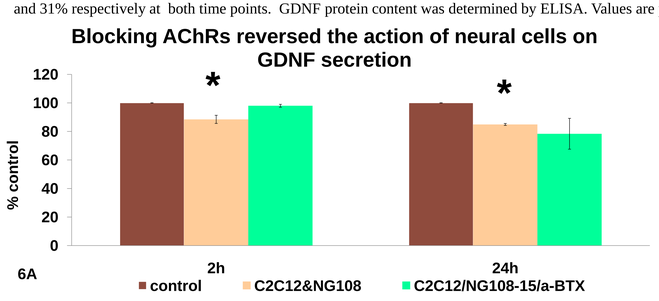


Figure 5. Addition of neural cells inhibited GDNF production. A. GDNF secreted in culture medium was reduced about 25% and 33% at both time points. **B.** GDNF in cells was reduced about 21% and 31% respectively at both time points. GDNF protein content was determined by ELISA. Values are presented as mean ± S.E.M. Asterisk indicates significance ($p \leq 0.05$), n=8.



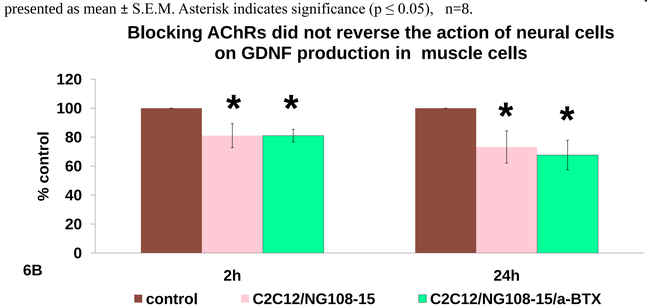


Figure 6.Blocking AChRs with α-BTX on C2C12 muscle cells co-cultured with NG108-15. A. α-BTX reversed the action of neural cells on GDNF secretion by myotubes in culture medium at 2h, but had no effect at 24h. **B.** Blocking AChRs did not have an effect on GDNF production in myotubes. GDNF protein content was determined by ELISA. Data are in percent of control and values are presented as mean S.E.M. Asterisk indicates significance ($p \leq 0.05$) from control at 2hour. n=3

Methods

Cell culture

Mouse skeletal muscle cells (C2C12) and Glioma x Neuroblastoma hybrid cells (NG108-15) were used in this study. About 2.5 x 10^5 C2C12 myoblasts, undifferentiated skeletal muscle cells, were allowed to grow on 35-mm culture plates. Cultured cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Cells were incubated at 37°C in a water-saturated incubator of 95% air and 5% CO_2. Differentiation of myoblasts to myotubes was induced by replacing the growth medium with DMEM supplemented with 10% horse serum . NG108-15 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2% HAT supplement (a mixture of hypoxanthine, aminopterin, and thymidine), and 1% antibiotic-antimycotic. Differentiation of NG108-15 was enhanced by switching from regular medium to a serum free medium.. For Nerve-muscle co-culture, NG108-15 cells were plated onto 10-day old myotubes and the co-cultured cells were incubated for 24-36 hours to allow nerve-muscle contacts to become established. Culturing procedure was the same as described above for blocking acetylcholine receptors, except neural-myotube co-cultured cells were treated with fresh medium containing 200nM unlabeled alpha-bungarotoxin (α-BTX). Cells were incubated for 25min. Following incubation the cells were washed twice with fresh culture medium, and then fresh medium was added . For all studies, culture medium samples were collected at intervals of 2, 4, 24 hours. To examine GDNF in cells, the cells were removed from the dish at the same intervals and were spun in a cold centrifuge at 13.5 xg.. GDNF protein content in all cultures experiments was measured by ELISA and the expression level of GDNF protein were examined by western blotting analysis.

Immunocytochemistry

Detection of choline acetyltransferase (ChAT): For immunocytochemical purposes, the NG108-15 cells were cultured on coverslips and allowed to differentiate by serum starvation. For neuronal protein detection cells were bound with the Mill-Marker FluoroPan Neuronal Marker conjugated to Alexa Fluor 488 (1:125; Millipore). For detection of choline acetyltransferase (ChAT: a marker of cholinergic neurons), cells were incubated with 1:125 ratio of goat anti-ChAT primary antibody (Molecular Probes) followed by labeling with a 1:100 of Alexa Fluor 647 conjugated donkey anti-Goat IgG secondary antibody (Molecular Probes).

Examination of cell proliferation for NG108-15 cells: Detection of proliferation cell nuclear antigen (PCNA) in NG108-15 cells was performed as described in Andrade et al. (1993). At day-3, the neural medium was switched to serum free. After 72 h, serum free medium was replaced by the neural medium for control culture dishes or myotube medium. Cells were fixed at 3h intervals with cold (-20 C) methanol for 5 min followed by acetone for 2 min at -20 C. Plates were blocked with donkey serum followed by incubation with antibody against PCNA (1:250) overnight. Cells were washed followed by 2h incubation with donkey anti mouse secondary antibody (1:125) conjugated to Alexa Fluor 568.

Detection of Myosin and GDNF in Myotubes or nerve-muscle co-cultures: Cells were grown on coverslips. The coverslips were coated with collagen to enhance myotube adhesion. 10-day old myotubes were fixed with 4% paraformaldehyde for 30 min and were incubated overnight at 4°C with mouse anti-myosin or rabbit anti-GDNF primary antibody (1:125). Donkey anti-mouse or donkey anti-rabbit conjugated to Alexa Fluor 488 or Alexa Fluor 568, respectively, was added (1:100). The same immunocytochemical procedure was repeated for nerve-muscle co-cultures. All images were viewed and captured using a Zeiss laser scanning confocal microscope.

Summary

- **C2C12 skeletal muscle cells produce and secrete GDNF in culture medium. However, more GDNF is retained in cells than secreted into culture medium.**
- **NG108-15 neural cells regulate the production of GDNF by C2C12 skeletal muscle by reducing the amount of GDNF secreted in culture medium and contained in cells.**
- **Blockade of acetylcholine receptors blocks effects of neurons on GDNF secretion by skeletal muscle cells but does not block the effect of neurons on GDNF content within muscle cells.**
- **Neural cells grown alone in cell culture do not contain or secrete GDNF; however, neural cells grown in co-culture contain GDNF protein.**

Conclusions

Results from this study suggest that neural cells regulate their own supply of GDNF produced by skeletal muscle, in part, via acetylcholine receptor activation.

Acknowledgements

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