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## Effects of acetylcholine and electrical stimulation on glial cell line-derived neurotrophic factor production in skeletal muscle cells

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### Abstract

Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor required for survival of neurons in the central and peripheral nervous system. Specifically, GDNF has been characterized as a survival factor for spinal motor neurons. GDNF is synthesized and secreted by neuronal target tissues, including skeletal muscle in the peripheral nervous system; however, the mechanisms by which GDNF is synthesized and released by skeletal muscle are not fully understood. Previous results suggested that cholinergic neurons regulate secretion of GDNF by skeletal muscle. In the current study, GDNF production by skeletal muscle myotubes following treatment with acetylcholine was examined. Acetylcholine receptors on myotubes were identified with labeled alpha-bungarotoxin and were blocked using unlabeled alpha-bungarotoxin. The question of whether electrical stimulation has a similar effect to that of acetylcholine was also investigated. Cells were stimulated with voltage pulses; at 1 and 5Hz frequencies for times ranging from 30 minutes to 48 hours. GDNF content in myotubes and GDNF in conditioned culture medium were quantified by enzyme-linked immunosorbant assay. Results suggest that acetylcholine and short-term electrical stimulation reduce GDNF secretion, while treatment with carbachol or long-term electrical stimulation enhances GDNF production by skeletal muscle.

### Keywords

Glial cell line-derived neurotrophic factor; acetylcholine; skeletal muscle; electrical stimulation

### 1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) was first purified by Lin et al. (1993) as a survival factor for dopaminergic neurons. GDNF is widely distributed in neuronal and

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non-neuronal tissues (Springer et al., 1995). GDNF exerts its survival effects on other subpopulations of neurons in the central and peripheral nervous systems (Henderson et al., 1994; Moore et al., 1996; Trupp et al., 1995). Specifically, GDNF is characterized as a survival factor for spinal motor neurons (Henderson et al., 1994). The trophic factor is synthesized and released by skeletal muscle, and acts as a muscle-derived neurotrophic factor for spinal motor neurons (Suzuki et al., 1998a).

During development, GDNF rescues motor neurons from programmed cell death (Oppenheim et al., 1995), acts as a chemoattractant, and assists with motor axonal guidance to motor neuron target tissues (Dudanova et al., 2010; Kramer et al., 2006). GDNF facilitates synaptic transmission (Wang et al., 2001), maintains synaptic activity (Zwich et al., 2001), plays a role in enhancing nerve recovery after injury (Cote et al., 2011; Dupont-Versteegden et al., 2004; Hashimoto et al., 2005; Houenou et al., 1996; Naveilhan et al., 1997; Oppenheim et al., 1995; Zhang et al., 2009) and muscle overexpressing GDNF displays hyperinnervation of endplates (Nguyen et al., 1998). These findings support the hypothesis that motor neurons depend on GDNF as a target-derived neurotrophic factor and GDNF secreted by skeletal muscle may be important for motor neuron survival (Angka et al., 2008; Bohn, 2004).

Although much is known about the effects of GDNF on motor neurons, little is known about factors regulating GDNF synthesis and release by skeletal muscle. Denervation of skeletal muscle causes an increase in GDNF expression (Suzuki et al., 1998b; Lie and Weis, 1998), while muscle cells co-cultured with neural cells *in vitro* secrete less GDNF (Vianney and Spitsbergen, 2011). These findings suggest that the innervation status of skeletal muscles plays a role in regulating the amount of GDNF produced by muscle. In cell culture, the ratio of GDNF inside skeletal muscle is higher than that released into culture medium (Vianney and Spitsbergen, 2011), suggesting that GDNF may be synthesized and stored in a manner similar to neurotrophins (Poo et al., 2001). *In vivo* studies have shown that GDNF in skeletal muscle can be regulated in an activity-dependent manner, such as with physical exercise (McCullough et al., 2011; Wehrwein et al., 2002). In the present study the effect of the cholinergic agonists acetylcholine (ACh) and carbachol (CCh) on GDNF production by skeletal muscle were examined. The question of whether electrical stimulation has a similar effect to that of the cholinergic agonists was also investigated. The results suggest that ACh and short-term electrical stimulation reduces GDNF secretion, while long-term stimulation and CCh enhances GDNF production by skeletal muscle.

## 2. Results

### 2.1 Effect of acetylcholine on GDNF production by skeletal muscle cells

In previous studies we showed that cholinergic neurons play a role in regulating GDNF synthesis and release by skeletal muscle (Vianney and Spitsbergen, 2011). Here, we sought to determine whether cholinergic neurons exert their effect via neurotransmitter release. First, cell staining was performed to ensure that the myotubes express ACh receptors (AChRs). Figure 1A shows that C2C12 skeletal muscle cells stain positively using  $\alpha$ -BTX conjugated to AlexaFluor 488, suggesting the skeletal muscle cells express AChRs. Exposure of myotubes to ACh inhibited GDNF protein secretion. GDNF levels secreted at

2h were reduced to 70% of control. No effect of treatment with ACh was observed following 24h of treatment (Fig. 1B). Treatment with ACh had no effect on intracellular levels of GDNF protein (Fig. 1C).

Next whether or not ACh induced its inhibitory effects via AChRs was examined. In this set of experiments treatment with ACh reduced the secretion of GDNF in culture medium to around 60% of control and pretreatment with  $\alpha$ -BTX blocked the inhibitory effects of ACh on GDNF secretion (Fig. 1D). Treatment with ACh and  $\alpha$ -BTX had no effect on GDNF production at 24h (data not shown).

## 2.2 Effect of carbachol on GDNF production by skeletal muscle cells

Since ACh is quickly degraded by acetylcholinesterase (AChE), we sought to test whether CCh, a chemical that mimics ACh effects but isn't broken down by AChE, would exert similar effects to those of ACh. Interestingly, ACh and CCh had different effects on GDNF secretion by muscle cells following 2h and 24h of exposure. While ACh inhibited GDNF secretion following 2h exposure and had no effect after 24h, CCh increased GDNF secretion at both time points (Fig. 2A). However, when CCh was added to the cells for only 5 minutes and then removed, a significant decrease in GDNF secretion was observed 30 minutes following treatment (Fig. 2A). Similar to what was observed for ACh, CCh had no effect on intracellular GDNF protein content (data not shown). Pretreatment with  $\alpha$ -BTX abolished the action of CCh on GDNF secretion (Figure 2B).

## 2.3 Effect of electrical stimulation on GDNF production by skeletal muscle cells

Direct electrical stimulation can be used to elicit muscle contraction, bypassing the effect of ACh. Electrical stimulation has also been shown to alter protein expression in skeletal muscle (Bayol et al., 2005; Donnelly et al., 2010; Thelen et al., 1997). In this study, cells were exposed to 24V pulses of 30ms duration, applied at frequencies of 1 or 5Hz. With electrical stimulation, myotubes contracted synchronously at a rate similar to the stimulation frequency. Cells viewed after the stimulation period were found to be intact following all stimulation protocols. For these studies, 30 and 90 minutes were regarded as short-term stimulation and 12h to 48h of stimulation were regarded as long-term stimulation. Figure 3 summarizes the changes in GDNF production by skeletal muscle cells following short-term and long-term electrical stimulation. Thirty minutes of electrical stimulation at either 1 or 5Hz caused a significant inhibition in GDNF secretion by muscle cells, with no difference in effect being observed between stimulation frequencies (Fig. 3A). Unlike the effects observed following treatments with ACh or CCh, electrical stimulation also altered intracellular GDNF protein content. Although stimulation at 1Hz and 5Hz significantly decreased GDNF protein levels in cells, the effect following stimulation at 1Hz was more marked than that observed following stimulation at 5Hz (Fig. 3B). As the duration of stimulation increased, the inhibitory effect on GDNF production was diminished and changed to a stimulatory effect following 48h of stimulation (Fig. 3A and 3B).

## 2.4 Role of voltage-gated sodium channels

In order to test whether voltage-gated sodium channels were important for the effect of electrical stimulation on GDNF production, channels were blocked using TTX. Thirty

minutes of stimulation in the presence of TTX was chosen because we sought to examine whether TTX could reverse the inhibitory effect of electrical stimulation on GDNF production. Although GDNF production was still inhibited following electrical stimulation, the results showed that the inhibitory effect was significantly reduced following TTX treatment, both for GDNF secretion (Figure 4A) and GDNF content within myotubes (Figure 4B).

### 3. Discussion

The goal of the current study was to determine whether motor neurons may regulate their own supply of neurotrophic factors produced by skeletal muscle via effects of the neurotransmitter ACh and whether the regulatory effects of ACh are dependent on electrical activity in the muscle tissues. The major findings suggest that: 1) Both ACh and short-term electrical stimulation inhibited GDNF production by skeletal muscle. 2) Carbachol, a chemical that mimics ACh action, inhibited GDNF production at early times following exposure, while increasing GDNF production at later times following exposure. 3) The results suggest that both ACh and CCh act via AChRs, as blocking the receptors prevented the action of both chemicals. 4) Blocking voltage-gated sodium channels with TTX reduced the effect of electrical stimulation on GDNF production. 5) Finally, the magnitude and direction (inhibition vs excitation) of the effect of electrical stimulation was dependent on the duration of stimulation, where short-term stimulation inhibited GDNF production and prolonged stimulation enhanced GDNF production.

The inhibition of GDNF production caused by short-term depolarization may explain results of previous studies which show that GDNF production is inhibited when skeletal muscle is co-cultured with cholinergic neurons (Vianney and Spitsbergen, 2011). The inhibitory effect of ACh or short-term electrical stimulation may also help explain why GDNF mRNA levels increase in skeletal muscle following denervation (Lie and Weis, 1998), and why ACh inhibits extra cholinergic nerve branching during development (An et al. 2010). Taken together it can be suggested that signaling pathways activated following skeletal muscle innervation, treatment with exogenous ACh, or short-term direct electrical stimulation, inhibit GDNF production by skeletal muscle.

The inhibitory effect of short-term electrical stimulation appears to involve activation of voltage-gated sodium channels, as blocking these channels with TTX partially reversed the inhibitory effect. It is interesting to note that TTX was less effective at blocking the effects of electrical stimulation on intracellular GDNF, possibly suggesting that intracellular GDNF content may be regulated differently than secreted GDNF.

Unlike effects observed following treatment with ACh, exposure to CCh increased GDNF secretion by the muscle. C2C12 skeletal myotubes used in the current study express AChE (Choi et al., 2003; Lee et al., 2005; Siow et al., 2002; Tung et al., 2004), thus, these differences may be due in part to differences in the way these molecules are degraded (Taylor and Brown, 1999). Unlike ACh, CCh is not broken down by AChE (Jankovic et al., 1998). Because CCh is resistant to AChE, long-term exposure to CCh increases muscle contraction (Protas et al., 1998) and increase the time-course of other cellular responses

(Jankovic et al., 1998, Longmore et al., 1986). Therefore, we suggest that the opposite response of ACh compared to CCh, on GDNF expression by myotubes, may be due to the differences in metabolism of the two drugs, as a very short-term exposure to CCh exerts similar effects to that of ACh. If CCh continuously activates the muscle this may signal the muscle to increase GDNF secretion.

A similar phenomenon was observed with short-term vs long-term electrical stimulation, where the inhibitory effect of electrical stimulation was diminished following increased duration of stimulation, converting to a stimulatory effect following 24–48hr of stimulation. These observations suggest that following prolonged treatment the effect of electrical activity on GDNF production changes from inhibition to stimulation. A stimulatory effect following prolonged treatment, either with exposure to CCh or long-term electrical stimulation, may help explain the increase in GDNF content found in skeletal muscle following exercise training (Wehrwein et al., 2002, McCullough et al., 2011).

The results show that treatment with ACh, brief exposure to CCh, or short-term electrical stimulation all inhibit GDNF secretion by muscle cells, while longer term exposure to CCh or increased duration of electrical stimulation increases GDNF secretion by muscle cells. The similarities in the pattern of response, early inhibition followed by late stimulation, may suggest that both treatment modalities are altering GDNF secretion via similar mechanisms. However, the observation that CCh can enhance GDNF secretion following as little as 2h of exposure, while electrical stimulation does not increase GDNF secretion until 48h of stimulation, may suggest that different signaling mechanisms are involved. The observation that electrical stimulation alters both intracellular GDNF content and secretion, while treatment with cholinergic agonists only affects GDNF secretion, also supports the notion that different signaling mechanisms may be involved. Additional studies are needed to elucidate the signaling pathways by which electrical and chemical treatments alter GDNF secretion by skeletal muscle.

Previous work has demonstrated that fast and slow type skeletal muscles display different changes in GDNF protein content in response to low intensity exercise and to low frequency field stimulation (McCullough et al., 2011). Walk training or low frequency field stimulation (0.1Hz) of skeletal muscle decreases GDNF protein content in extensor digitorum longus, a muscle comprised primarily of fast-type muscle fibers, while these stimuli increase GDNF protein in soleus muscle, a muscle comprised primarily of slow-type muscle fibers (McCullough et al., 2011). A switch from fast to slow muscle fiber-type has been observed following electrical stimulation of C2C12 myotubes (Nedachi et al., 2008); and these changes alter physiological demands of the cells in vivo and in vitro (Williams and Neuffer, 2011, Zebedin et al., 2004). Thus changes in muscle fiber-type could also help to explain the change in response following long-term stimulation.

These findings, and results of studies by Xie et al. (1997), show that different families of neurotrophic factors may be regulated differently by muscle depolarization. Xie et al. showed that neurotrophin 3 expression in skeletal muscle increased with electrical stimulation or treatment with ACh (Xie et al., 1997), while our result show that GDNF production is inhibited by ACh or short-term electrical stimulation. Our observations that a

sizeable pool of intracellular GDNF is maintained in skeletal muscle and that intracellular GDNF content is regulated differently than secreted GDNF may suggest that muscle retains an intracellular store of GDNF that may be released under certain circumstances. This has been shown to be the case with the neurotrophins (Poo, 2001), where these neurotrophic factors appear to be synthesized, stored and released upon demand.

In summary, these results suggest that GDNF production in skeletal muscle cells is regulated in an activity-dependent manner and can be modulated by chemical or electrical stimulation. Furthermore, the results demonstrate that the regulatory effect may change from an inhibitory effect on GDNF production at low levels of stimulation to an excitatory effect with long-term stimulation, suggesting that levels of GDNF protein being produced and/or secreted depend on physiological demands on the skeletal muscle cells.

## 4. Experimental Procedure

### 4.1 Cell culture procedure

Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The C2C12 mouse skeletal muscle cell line was purchased from American Type Culture Collection (ATCC: Manassas, VA, USA). The C2C12 cell line was extracted from 2-month old mouse thigh muscle and the cells have been used by researchers because of their *in vivo* skeletal muscle phenotype, including expression of contractile proteins (Ling et al., 2005; Yafel and Saxel, 1977). Culturing procedures were performed according to the ATCC protocols and as described by Vianney and Spitsbergen (2011), with some minor modifications. Briefly, C2C12 myoblasts were initially seeded on 100-mm plates and maintained in Dulbecco's Modified Eagle's Medium (DMEM: ATCC) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Carlsbad, CA, USA). Cells were incubated at 37°C in a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub>. For experiments, myoblast cells were seeded in 6-well plates (USA Scientific, Inc. Ocala, FL, USA). Differentiation of myoblasts to myotubes was induced by replacing the growth medium with DMEM supplemented with 10% horse serum and 1% antibiotic-antimycotic. The medium was renewed every one to two days. All experiments were run on myotubes that had been maintained in culture for 6 to 8 days.

### 4.2 Sample collection and cell harvesting

To determine GDNF protein concentration in culture medium, a 1ml sample of medium was collected from each culture dish at 2, 4, and 24 hours following treatment and kept at -20°C. To harvest cells, culture medium was removed, cells were washed with calcium/magnesium-free buffer and 1ml of sample buffer (a mixture of phosphate buffered saline, 0.005% Tween-20, 0.5% bovine serum albumin, 0.1mM benzethonium chloride, 2mM benzemidine, 0.4M NaCl, 2mM EDTA and 164µl/100ml aprotinin) was added to each culture dish. The cells were scraped from the dish using a cell lifter. To examine intracellular GDNF, cells were spun in a cold centrifuge at 13.5 × g and supernatant was removed and stored at -20°C. GDNF protein content in each experiment was measured by an enzyme-linked immunosorbent assay (ELISA) as described below.

### 4.3 GDNF protein detection by ELISA

Determination of GDNF protein content in culture medium and cell supernatant was measured by ELISA. Briefly, GDNF primary antibody (1 µg/ml: R&D Systems, Minneapolis, MN, USA) was added to 96-well ELISA plates and then incubated overnight at room temperature. Plates were blocked with Phosphate Buffer Saline (PBS) containing 1% Bovine Serum Albumin (BSA: Thermo Fisher Scientific, Waltham, MA, USA) and sucrose 5%. Plates were rinsed three times with wash buffer (a mixture of PBS and 0.05% Tween-20), then GDNF standard (R&D Systems) or samples (conditioned culture medium or harvested cells) were added to each well, and plates were incubated for two hours at room temperature. Plates were washed and 100ng/ml anti-GDNF secondary antibody conjugated to biotin (R&D Systems) was added and incubated for two hours at room temperature. Following incubation plates were washed and beta-galactosidase conjugated to streptavidin (Life Technologies) was added and incubated for 20 minutes at room temperature. Plates were washed and 1mg/ml chlorophenol red-β-D galactopyranoside (CPRG: Roche Diagnostics GmbH, Indianapolis, IN, USA) was added and incubated at room temperature. Plate readings were taken every two hours until the standard curve was developed. For each assay a standard curve was calculated from known GDNF concentrations.

### 4.4 Detection of acetylcholine receptors using alpha-bungarotoxin

Procedures were adopted as previously described in Vianney and Spitsbergen, (2011) with minor modification. Briefly, cells were grown on cover slips pre-treated with 0.1% gelatin (Thelen et al, 1997). Cells were maintained at 37°C in a standard incubator and allowed to differentiate into myotubes. To examine whether myotubes express AChRs, live cells were treated with alpha bungarotoxin (α-BTX) using a procedure adopted from Yang and Nelson (2004), with minor modifications. Briefly, myotubes were treated with fresh medium containing 200nM α-BTX conjugated to AlexaFluor® 488 (Life Technologies) and maintained at 37°C in a standard incubator for 1h. Cells were washed and fixed with 4% paraformaldehyde for 30 min. Cells were washed with PBS and the coverslip with cells was mounted on a glass slide with 50% glycerol/50%PBS and sealed. Images were captured using a Zeiss laser scanning confocal microscope.

### 4.5 Treatment with Acetylcholine or Carbachol

Cells were grown as previously described. On the day of each experiment, fresh medium containing acetylcholine or carbachol (ACh or CCh, respectively; 0.1µM, 1µM, 100µM) was added to myotubes. Samples of conditioned culture medium and harvested cells were taken after 2, 4, and 24 hours. Control plates were processed in the same manner except that no ACh or CCh was added. In a separate experiment, CCh was added to myotubes for 5 minutes then removed and fresh culture medium was added. Samples of conditioned culture medium and harvested cells were collected after 30 minutes. Both cells and culture medium samples were stored at -20°C. GDNF protein content was measured by ELISA.

### 4.6 Block of acetylcholine receptors with alpha-bungarotoxin

C2C12 myoblasts were grown on 12-well plates and allowed to differentiate into myotubes. Myotubes were treated with fresh medium containing 200nM unlabeled α-BTX (Biotium,



Hayward, CA, USA) for 25 min. Following incubation with  $\alpha$ -BTX the cells were washed twice with fresh culture medium and fresh medium containing 100nM ACh or 100nM CCh was added. Controls consisted of plates without treatments and plates that were treated with 100nM ACh or 100nM CCh without  $\alpha$ -BTX. Samples were collected at intervals of 2, 4, and 24 hours. GDNF protein content was measured by ELISA.

#### 4.7 Electrical stimulation of the skeletal muscle cells

The approach of Marotta et. al. (2004) inspired the electrical stimulation apparatus used in this work, including its use of switched semicircular electrodes as part of a culture dish lid, a series carpling capacitor (0.47 $\mu$ F rather than the 220 $\mu$ F) and current sampling resistors (approximately 100 $\omega$ ). Following Donnelly et al. (2010) stainless steel electrodes were used instead of platinum electrodes used by Marotta et. al. (2004) and Thelen et al. (1997). A Grass Technologies S88 stimulator provided voltage pulses to a custom interface box. Electrodes were secured to the culture dish lid with bolts, which also provided convenient wire connection points using wires with spade lugs. Within the interface box a unity gain voltage buffer provided current to up to six pair of electrodes connected to six switches, which enabled each pair of electrodes to be individually selected. Stimulator pulse characteristics were experimentally selected to cause noticeable contraction of the muscle cells. Cells were stimulated at 1Hz or 5Hz for 30min, 90min, 12h, 24h, and 48h. Typical pulse amplitudes were in the range of 20–25V, with approximate 30 ms widths at the voltage buffer output. Using this apparatus three wells were stimulated simultaneously and three wells served as controls. All cultures were maintained at 37°C in water-saturated incubator of 95% air and 5% CO<sub>2</sub> during stimulation periods. GDNF protein content was determined by ELISA.

#### 4.8 Block of voltage-gated sodium channels

Cells were grown in 6-well plates and allowed to fuse into myotubes as in previous experiments. For each experiment, fresh medium containing 100 $\mu$ M tetrodotoxin (TTX) was added to myotubes and cultures were maintained at 37°C in a water saturated incubator of 95% air and 5% CO<sub>2</sub> for 30 minutes before electrical stimulation. Cells were electrically stimulated as described above. Samples of conditioned culture medium and harvested cells were collected at 0, 2, and 24hours following electrical stimulation.

#### 4.9 Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's test, or Student's t-test. P values  $\leq$  0.05 were considered statistically significant. All data values are reported as the mean  $\pm$  standard error of the mean (SEM).

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## Abbreviations

<b>ACh</b>	acetylcholine
<b>AChRs</b>	acetylcholine receptor
<b><math>\alpha</math>BTX</b>	alpha-bungarotoxin
<b>ATCC</b>	American Type culture Collection
<b>BSA</b>	bovine serum albumin
<b>CCh</b>	carbachol
<b>CPRG</b>	chlorophenol red- $\beta$ -D galactopyranoside
<b>DMEM</b>	Dulbecco's Modified Eagle's Medium
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>GDNF</b>	glial cell line-derived neurotrophic factor
<b>PBS</b>	phosphate buffer saline
<b>TTX</b>	tetrodotoxin

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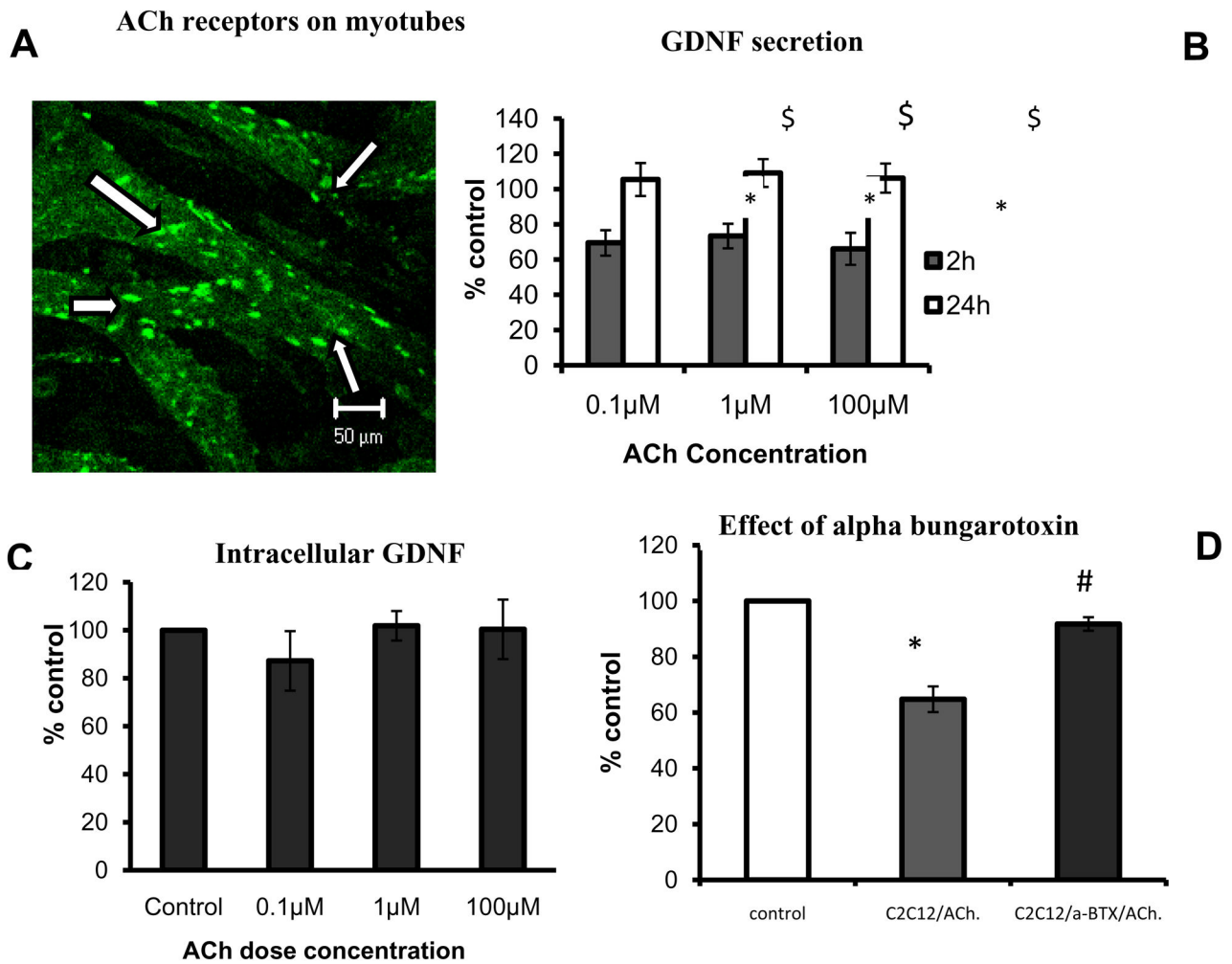
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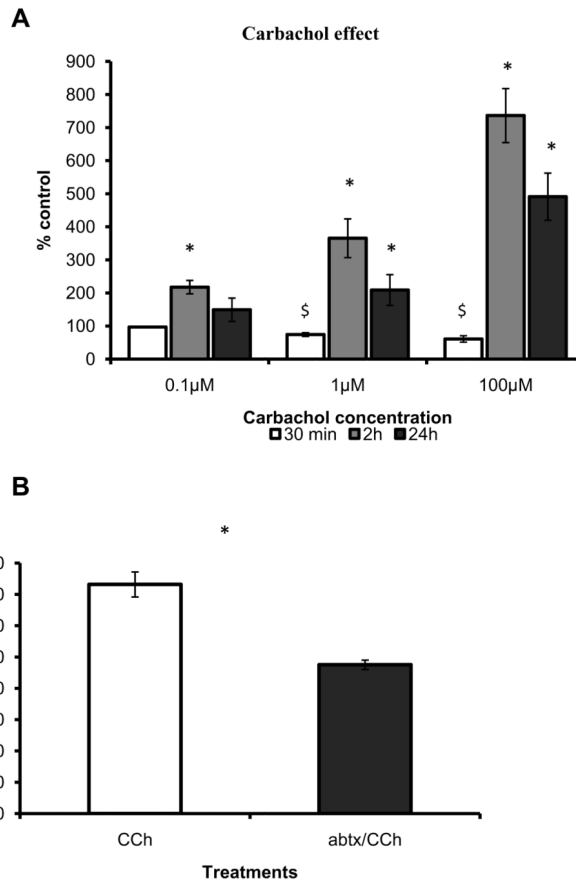
### Highlights

- Acetylcholine inhibits GDNF secretion by myotubes.
- Short-term exposure to carbamylcholine inhibits GDNF secretion by myotubes.
- Short-term electrical stimulation inhibits GDNF production by myotubes.
- Long-term exposure to carbamylcholine enhances GDNF secretion by myotubes.
- Long-term electrical stimulation enhances GDNF production by myotubes.



**Figure 1. Effect of ACh on GDNF production by skeletal muscle cells**

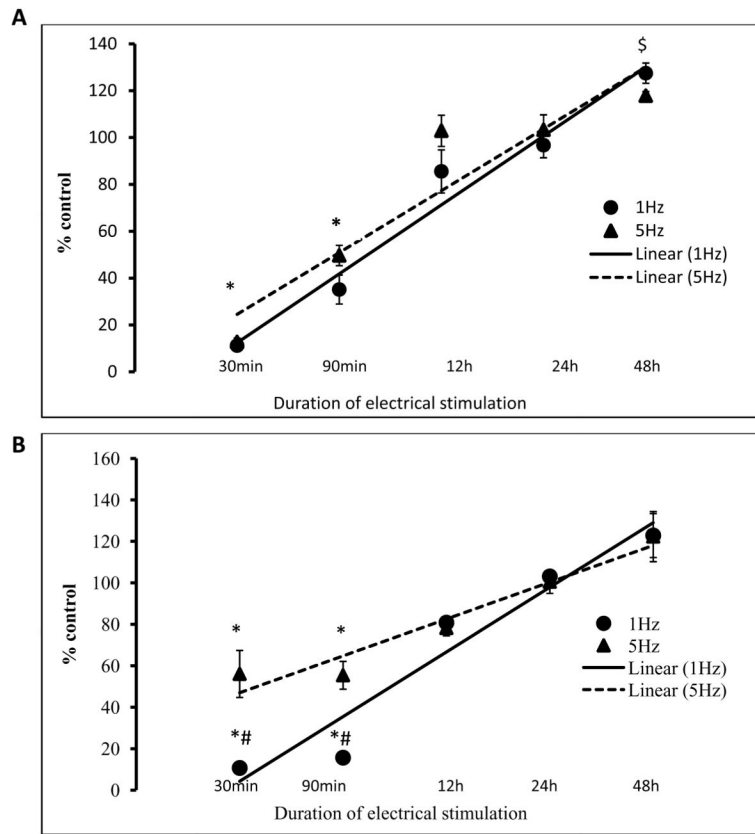
Myoblast cells were grown and allowed to differentiate into myotubes. **A. AChRs on myotubes (white arrows)**. Culture medium containing  $\alpha$ -BTX (200nM) conjugated to AlexaFluor 488 (green) was added to myotubes and incubated for 1h in a standard incubator. Following 1h of treatment cells were fixed with 4% paraformaldehyde and viewed on a confocal microscope. **B-C**, 7-day-old myotubes were treated with culture medium containing ACh at concentrations of 0.1  $\mu$ M, 1 $\mu$ M, and 100 $\mu$ M. Conditioned culture medium and cells were collected at 2h and 24h. **B.** ACh inhibits GDNF secretion following 2h but not 24h of exposure. **C.** ACh had no effect on intracellular GDNF content. **D.** Blocking AChRs with  $\alpha$ -BTX prevented the effects of ACh on GDNF secretion at 2h. An asterisk (\*) indicates a significant decrease from control, dollar sign (\$) indicates a significant difference in GDNF levels between samples collected at 2h and that collected after 24h, pound sign (#) indicates a significant difference in GDNF levels between cells treated with or without  $\alpha$ -BTX. Values are presented as means  $\pm$  S.E.M, P 0.05.



**Figure 2. Effect of carbachol on GDNF production by skeletal muscle cells**

Seven-day-old myotubes were treated with culture medium containing CCh at concentrations of 0.1 μM, 1 μM, and 100 μM. Cells were either treated with CCh for only 5 min, followed by removal of treated medium and replacement with fresh culture medium, or the exposure to CCh was prolonged up to 24 h. Conditioned culture medium and cells were collected at 30 min for a short-term exposure, and 2 h and 24 h for a long-term exposure.

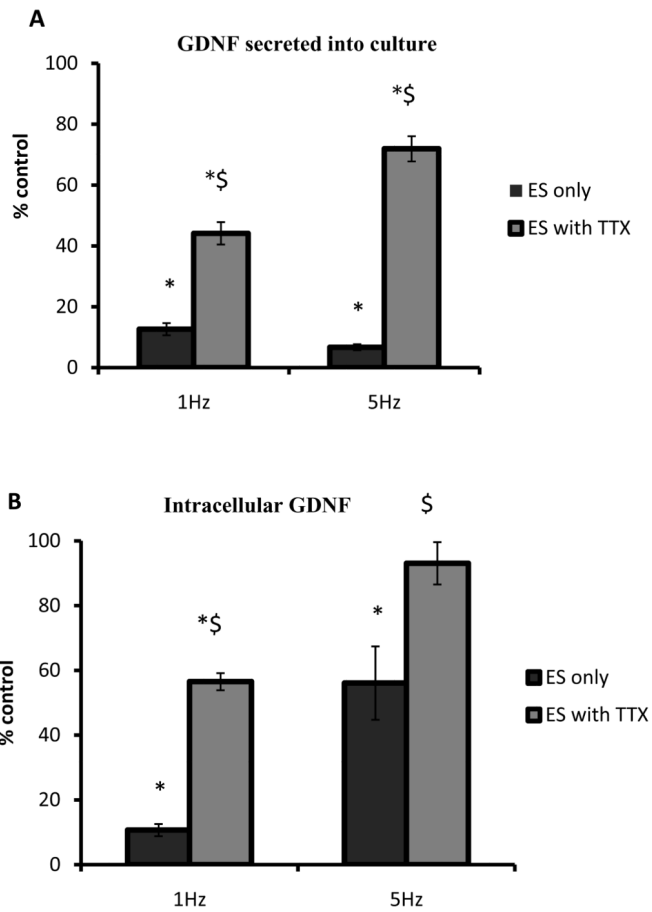
**A.** GDNF secretion decreases in the sample collected 30 minutes following a 5 min exposure to CCh, but increases 2 h and 24 h following exposure to CCh. **B.** Blocking AChRs with α-BTX prevented the action of CCh. An asterisk (\*) indicates a significant increase from control. A dollar sign (\$) indicates a significant decrease from control. Values are presented as mean ± S.E.M.



**Figure 3. Effects of electrical stimulation on GDNF production**

Short-term electrical stimulation decreases GDNF secretion (Panel A) and GDNF content in muscle cells (Panel B). The inhibitory effect of electrical stimulation is reduced as the duration of stimulation is increased. Electrical stimulation for 24 and 48 hours shifts the inhibitory effect of electrical stimulation to a stimulatory effect leading to an increase in GDNF production. An asterisk (\*) indicates a significant decrease from control. A dollar sign (\$) indicates a significant increase from control. A pound sign (#) indicates a significant difference on GDNF levels between 1Hz and 5Hz groups. Values are presented as mean  $\pm$  S.E.M.





**Figure 4. Effect of blocking voltage-gated sodium channels in C2C12 myotubes**  
 Cells were electrically stimulated in the presence or absence of tetrodotoxin (TTX) for 30 minutes. The inhibitory effect on GDNF production caused by electrical stimulation was reduced in cells exposed to TTX. **A.** GDNF secreted into culture medium and **B.** Intracellular GDNF. An asterisk (\*) indicates a significant decrease from control and a dollar sign (\$) indicates a significant difference on GDNF levels between TTX-treated and non-treated groups. Values are presented as mean  $\pm$  S.E.M. (P < 0.05).