Progression of hyperexcitability in skeletal muscle – is Huntington’s disease a muscle disorder?

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Abstract

Huntington’s disease (HD) is a progressive and ultimately fatal disorder in which a decline in motor and cognitive function occurs. HD is generally considered a neurological disorder. However, recent findings show diseased skeletal muscle fibers to exhibit prolonged action potential duration, reduced trigger thresholds, and the ability to self-trigger. These defects occur due to a decrease in the resting chloride and potassium conductance in skeletal muscle. As a result, in joint partnership, the Voss and Talmadge Labs are focusing research efforts on gathering data in relation to HD and mammalian skeletal muscle. To examine the role of skeletal muscle in the disease, we utilize the R6/2 transgenic mouse model, which expresses a mutated version of HTT. The focus of this project is to determine the time course over which the chloride channels (ClC-1) defects develop. In order to determine this span of time the Voss Lab utilized electrophysiological techniques to measure individual muscle cell ClC-1 conductance. While the Talmadge Lab employed molecular biological techniques to measure, analyze, and quantify the total inappropriate splice variants in muscle mRNA of exons 5-8 the gene Clcn-1 which codes for the ClC-1 channel. At this time the results of the electrophysiological measurements show a unreliable degree of variation. There is a statistically significant difference in the total inappropriate splice variant in mRNA of exons 5-8 ($p = 1.72 \times 10^{-6}$) between HD and wild-type (WT) in late stage of the disease. However, there is no statistical difference in the total inappropriate splice variant in mRNA of exons 5-8 ($P = 0.188$) between HD and WT of ages 35-55 days.
Introduction

Huntington’s disease (HD) is a degenerative, autosomal-dominant, progressive, and ultimately fatal disorder. The distinct phenotype includes chorea (irregular jerky movements), dystonia (abnormal tonicity often resulting in irregular positioning of head and limbs), rigidity, cognitive decline, and disoriented behavior. Adult-onset HD is the most common form of the disorder, with symptoms typically first appearing between the third and fifth decade of life. The less-common form of HD, juvenile-onset, arises prior to twenty and as early as two years of age. Early signs of the disease include irritability, depression, poor cognitive skill, uncontrolled movements, and inability to make decisions. The disease is found to be most common in those with European ancestry, effecting 3 to 7 in every 100,000 people. HD is the result of >40 expanded trinucleotide repeats CAG polyglutamine (polyQ) of the HTT gene that codes for the human huntingtin protein. Though the complete function of this protein is currently unknown, it is thought to play a pivotal role in both growth and development, as well as in excitable cells throughout the body.

Traditionally HD has been approached as a neurological disorder, with research concentrating on the function and degeneration of the brain. Previous examinations have shown atrophy, metabolic and mitochondrial defects, and loss of strength. Recent findings by Waters, et al. from the Voss laboratory at Cal Poly Pomona have shown there is also a significant reduction in chloride channel (ClC-1) conductance and inward rectifying potassium channel (Kir) conductance in the skeletal muscle the R6/2 transgenic mice model. These findings directly correlate with the phenotypic characteristics of membrane hyperexcitability, involuntary contractions, rigidity, and persistent and prolonged contractions; because the resting membrane potential in the skeletal muscle is maintained by ClC-1 and Kir. Consistent with the decreased conductance, the data also showed aberrant splicing of Clcn1 (gene for ClC-1) mRNA and a reduction in total mature Clcn1 mRNA. A similar mechanism has been reported to cause a decrease in ClC-1 currents in myotonic dystrophy type 1, which is also cause by an expanded trinucleotide repeat. The authors also found a decrease in total mature Kcnj2 (gene for Kir2.1 channels) mRNA.

Theoretical prospective

HD has been traditionally viewed solely as a neurodegenerative disorder and skeletal muscle defects were thought to be only as result of the degeneration of the nerve. The recent findings of ClC-1 and Kir reduction have opened a new opportunity for research of the diseased mammalian skeletal muscle. In order to examine the relationship between the disease progression and the skeletal muscle defects the lab will utilize both electrophYSiological and molecular biological techniques. At this time there are a total of 9 polyglutamine related disorders. The data from this project will contribute to a greater holistic understanding of the role that polyQ expansion plays in skeletal muscle degeneration.
Specific Aims

The aims of this project was to determine the time course over which the chloride channels (ClC-1) defects develop. Utilizing the R6/2 mouse model, the lab took electrochemical measurements from individual muscle fibers disassociated from the flexor digitorum brevis (FDB) and interosseous (IO) muscles in both HD and WT. Preceding these measurements, the total inappropriate splice variant and the individual mis-splice variants in the gene Clcn-1 exons 5-8 were measured and quantified.

Materials and Methods

A description of each buffer is provided at the end of the Materials and Methods.

Animal Care and Use

All animal procedures that took place during this experiment were performed in accordance with the Animal Care and Use Committee of the California State Polytechnic University, Pomona. R6/2 mice purchased from Jackson Laboratory and were used for all experimental procedures. Cynthia McKee is approved to handle animals for the electrophysiological experiments. The protocol for the electrophysiological experiments was submitted by Dr. Voss, with Ms. McKee added as a designated approved user on August 21, 2013 under protocol number 13.017.

Electrical Recordings, Internal and External Buffers

FDB and IO muscle fibers were surgically removed, pinned to Sylgard-bottomed Petri dishes, and enzymatically dissociated at room temperature under mild agitation for ~1 h using collagenase extracellular buffer solution. Fiber dissociation was completed after 1 h resting period at room temperature in extracellular buffer without collagenase.

Fibers were visualized in an Olympus BX51WI microscope, and images were acquired with a CCD camera (ST-7XMEI-C1, Santa Barbara Instruments). Fiber surface area and volume were estimated assuming a cylindrical shape with ImageJ (National Institute of Health), and SigmaPlot 11 (Systat Software). Electrical properties were measured under standard current and voltage clamp conditions using two aluminosilicate intracellular microelectrodes (part 30-0110, Harvard Apparatus), an Axoclamp 900A amplifier, a Digidata 1440a digitizer, and pClamp 10 data acquisition analysis software (Molecular Devises). The voltage-sensing electrode was connected with an Axoclamp HSx1 headstage. The current-passing electrode was connected with an Axoclamp HSx10 headstage that was modified to have a 2-MΩ output resister (HSx5). Both current-passing and voltage-sensing electrodes were filled with internal buffer solution. Data was acquired at 100 kHz. Current and voltage records were low-pass filtered at 2 kHz. After impalement, 20 min was given for equilibration of the electrode solution with the sarcoplasm before data acquisition. EGTA will be used in the internal solutions to prevent contractions.
**Biochemical Recordings**

Total muscle RNA was isolated and 1 μg of RNA was reverse transcribed. To quantify total *Clcn1* mRNA alternatively spliced gene products from exons 5-8, traditional PCR was performed. PCR products were separated and stained with ethidium bromide.

**Internal and Extracellular Buffers**

Internal solution for G ClC-1 (in mM) will be as follows: 17 aspartate, 30 HCl, 30 EGTA, 15 CaCl2, 5 MgCl2, 5 ATP di-Na, 5 phosphocreatine di-Na, 5 glutathione, 20 MOPS, and pH 7.2 with CsOH.

Extracellular solution for action potentials (in mM) will be as follows: 135 NaCl, 2.5 KCl, 5 CaCl2, 2 MgCl2, 5 glucose, 1 NaH2PO4, 10 MOPS, and pH 7.4 with NaOH.

Extracellular GClC-1 solution (in mM) will be as follows: 140 HCl, 10 CsOH, 5 CaCl2, 2 MgCl2, 5 glucose, 1 NaH2PO4, 10 MOPS, 0.0002 tetrodotoxin, 0.02 nifedipine, and pH 7.4 with tetraethylammonium hydroxide. 0.4 anthracene-9-carboxylic acid will be added for GClC-1 blocking solution.

**Statistical Analysis**

All electrophysiological measurements were compared by means of two independent samples using a two-tailed t test, normalized by the Shapiro-Wilk test, and will assess variances based on a folded F variance ratio test. For nonnormal and/or heteroscedastic samples we will used the Mann-Whitney rank sum test. Null hypotheses in all statistical tests will be rejected at $\alpha \leq 0.05$. Mean values will be presented as ±SEM.

**mRNA Analysis**

To quantify total *Clcn1* mRNA alternatively spliced gene products from exons 5-8 were stained with ethidium bromide. The gene products were visualized and recorded using an Alpha Innotech camera and ultraviolet light. Statistical analysis was performed on individual sample percent saturation using FluorChem image software.

**Results**

The electrophysiological data was analyzed with a 2 tailed t-test of HD compared to WT in age groups of $N$ to $N+9$. Age range 20-29 days shows a difference in the mean values of the two groups HD ($n= 13$) and WT ($n=16$) is greater than would be expected by chance; there is a statistically significant difference between the groups ($P = 0.007$). Age range 30-39 days shows a difference in the mean values of the two groups HD ($n=33$) and WT ($n=13$) is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the groups ($P = 0.092$). For the age range 40-49 a Mann-Whitney rank sum test was ran due to the variation in comparative sample data.
between the two groups. The results show a difference in the median values between the two groups HD (n=11) and WT (n=28) is greater than would be expected by chance; there is a statistically significant difference \((P = < 0.001)\). Age range 50-59 days difference in the mean values of the two groups HD (n=17) and WT (n=12) is greater than would be expected by chance; there is a statistically significant difference between the groups \((P = < 0.001)\). Age range 60-69 days shows a difference in the mean values of the two groups HD (n=17) and WT (n=9) is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the groups \((P = 0.224)\).

Thus far the preliminary mRNA analysis shows that there is a statistically significant difference of total inappropriate splice variant between the HD (n=6) and WT (n=6) in the late-stage of the disease \((P = 1.72 \times 10^{-6})\). However, when comparing age groups of between 35-55 days there was not a statistically significant difference between HD (n=5) and WT (n=5) \((P = 0.188)\).

**Discussion**

The variation in electrophysiological statistical data suggests further review is needed. This variation could be observed due to the possibility the current mouse model is not appropriate for a longitudinal study. The R6/2 model does exhibit an advanced form of the disease. The rapid progression of the disease is beneficial when studying the late stages of the disease, however may not be suitable for studying the disease progression over time. Due to the inconclusive nature of the data reported the lab is considering performing the same longitudinal study on a mouse model which exhibits a milder form of the disease which progresses over a longer period of time.

The preliminary biochemical data suggests total inappropriate splice variant increases with the progression of the disease. This is what we would be expected as HD is a characterized as a progressive and degenerative disorder. We expect with further analysis, including increased varying age ranges, the *Clcn1* mRNA alternatively spliced gene products from exons 5-8 will have less of a varying degree of difference in the earlier stages of the disease; whereas the degree of difference is expected to increase with the progression of the disease.

The overall results and data collected during this longitudinal study of the progression of hyperexcitability in Huntington’s disease skeletal muscle does contribute the comprehensive understanding the role skeletal muscle plays in the motor symptom defects characterizing the disease. Though the model used may not have been ideal for a longitudinal study, we do recognize the severe progression of the disease runs parallel with the increase in total inappropriate mRNA splice variants of exons 5-8 which has not been previously reported for Huntington’s disease.
References