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The Histone Deacetylase HDAC4 Connects Neural Activity to Muscle Transcriptional Reprogramming*[S]

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Todd J. Cohen[‡], David S. Waddell[§1], Tomasa Barrientos[‡1], Zhonghua Lu[¶], Guoping Feng[¶], Gregory A. Cox[‖], Sue C. Bodine[§], and Tso-Pang Yao[‡2]

From the [‡]Department of Pharmacology and Cancer Biology and [¶]Department of Neurobiology, Duke University, Durham, North Carolina 27710, the [§]Section of Neurobiology, Physiology & Behavior, University of California, Davis, California 95616, and the [‖]Jackson Laboratory, Bar Harbor, Maine 04609

Neural activity actively regulates muscle gene expression. This regulation is crucial for specifying muscle functionality and synaptic protein expression. How neural activity is relayed into nuclei and connected to the muscle transcriptional machinery, however, is not known. Here we identify the histone deacetylase HDAC4 as the critical linker connecting neural activity to muscle transcription. We found that HDAC4 is normally concentrated at the neuromuscular junction (NMJ), where nerve innervates muscle. Remarkably, reduced neural input by surgical denervation or neuromuscular diseases dissociates HDAC4 from the NMJ and dramatically induces its expression, leading to robust HDAC4 nuclear accumulation. We present evidence that nuclear accumulated HDAC4 is responsible for the coordinated induction of synaptic genes upon denervation. Inactivation of HDAC4 prevents denervation-induced synaptic acetylcholine receptor (nAChR) and MUSK transcription whereas forced expression of HDAC4 mimics denervation and activates ectopic nAChR transcription throughout myofibers. We determined that HDAC4 executes activity-dependent transcription by regulating the Dach2-myogenin transcriptional cascade where inhibition of the repressor Dach2 by HDAC4 permits the induction of the transcription factor myogenin, which in turn activates synaptic gene expression. Our findings establish HDAC4 as a neural activity-regulated deacetylase and a key signaling component that relays neural activity to the muscle transcriptional machinery.

The histone deacetylase HDAC4 and its closely related family member HDAC5 have been implicated in muscle differentiation by virtue of their efficient binding and inhibition of MEF2, a master transcription factor critical for muscle differentiation (1). The potent transcriptional repressor activity of HDAC4 and HDAC5, in turn, is negatively regulated by calcium/calmodulin-dependent kinase (CaMK)[3] (2, 3). CaMK-mediated phosphorylation promotes 14-3-3 association and nuclear export of HDAC4 and HDAC5, resulting in MEF2 activation. The active intracellular trafficking of HDAC4 and related class IIA HDACs was considered a key regulatory mechanism for MEF2 transcriptional activity. As potent repressors of MEF2, HDAC4 and HDAC5 were logically proposed to act as inhibitors of skeletal muscle differentiation, and their nuclear exclusion induced by CaMK was thought to allow differentiation to proceed (2). Surprisingly, our previous analysis, however, showed that HDAC4 became concentrated to rather than excluded from the nuclei upon C2C12 myotube formation (3). This unexpected finding is incompatible with a simple model that nuclear HDAC4 prevents muscle differentiation. Instead, the import of HDAC4 into nuclei of myotubes likely reflects a specific function of HDAC4 in the differentiated myofiber. However, any potential role for HDAC4 in the regulation of muscle function *in vivo* has not been established.

Muscle can undergo remodeling to meet changing functional demands. This remodeling is largely accomplished by specific transcriptional reprogramming induced by neural activity (1). The intimate relationship between neural activity and muscle gene transcription is best illustrated by the dynamic regulation of synaptic gene expression. The expression of synaptic proteins, such as nicotinic acetylcholine receptors (nAChRs), are exquisitely sensitive to innervation status (reviewed in Ref. 4). During embryonic development before muscles are innervated, nAChRs are expressed throughout muscle fibers. This high level of nAChRs confers acetylcholine supersensitivity and may be important for the subsequent formation of synaptic structures upon innervation. After innervation, a dramatic repression of nAChR expression occurs throughout muscle except for a small number of nuclei adjacent to the neuromuscular junction (NMJ), the site of nerve-muscle communication. This active repression of "extra-synaptic" transcription contributes to the high concentration of nAChR at the NMJ, a spatial organization that is critical for efficient propagation of electrical signals from motor neurons. Remarkably, this repression can be readily reversed upon denervation, leading to rapid induction of nAChR and other synaptic genes (5). The tight regulation of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Pharmacology and Cancer Biology, Box 3813, Duke University Medical Center, Durham, North Carolina 27710. Tel.: 919-613-8654; Fax: 919-668-3954; E-mail: yao00001@mc.duke.edu.

[3] The abbreviations used are: CaMK, calcium/calmodulin-dependent kinase; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; nAChR, nicotinic acetylcholine receptors; NMJ, neuromuscular junction; MGN, myogenin; wt, wild type.

nAChR expression illustrates the dominant effect of neural activity on muscle gene transcription and function. The critical issue of how neural signals originating at the NMJ are relayed to the transcriptional machinery in the nucleus remains poorly understood.

The bHLH transcription factor myogenin (MGN) is a critical regulator for nAChR expression. MGN binds and activates E-box elements (CANNTG) present in the promoters of activity-regulated synaptic genes including nAChRε, nAChRδ, and MUSK (6, 7). In normal innervated muscle, myogenin transcription is repressed, ensuring low expression of nAChR in extra-synaptic nuclei. Upon surgical denervation, MGN becomes induced, resulting in the expression of nAChR and other NMJ components (5). Thus, myogenin expression is central to activity-regulated synaptic gene expression. Myogenin expression appears to be regulated by at least two mechanisms. HDAC9 (MITR) is thought to repress myogenin expression through inhibition of MEF2 (20). More recently, a Dachschund related transcriptional co-repressor, Dach2, was reported to inhibit myogenin expression in innervated muscle (8). Interestingly, Dach2 expression itself is suppressed by denervation, suggesting a model whereby reduction of Dach2 leads to transcriptional induction of myogenin and subsequent activation of nAChR in extrasynaptic myofibers (8). How neural activity is connected to the nuclear Dach2/myogenin transcriptional cascade, however, is not known.

Here we present evidence that HDAC4 is a critical component that relays neural input into the nucleus for the regulation of Dach2, myogenin, and nAChR gene expression. In innervated muscle, we found that HDAC4 is expressed at low levels and concentrated at the NMJ, where it co-localizes with CaMKIIδ and 14-3-3 proteins. Importantly, upon reduced neural activity by surgical denervation or defects associated with neuromuscular disease, HDAC4 dissociates from the NMJ, becomes transcriptionally induced and accumulates in nuclei. Thus, HDAC4 levels and subcellular localization are tightly controlled by neural activity. Supporting a critical role for HDAC4 in relaying neural activity into muscle nuclei, we showed that HDAC4 is required for the active repression of Dach2 and the induction of nAChR in response to denervation. Conversely, elevated HDAC4 levels mimic the effect of denervation and induce ectopic nAChR throughout myofibers. We propose that HDAC4 is a critical link between neural activity and muscle gene expression that enables muscle remodeling in response to changing neural input.

MATERIAL AND METHODS

Plasmids and Antibodies—HDAC4wt and HDAC4-3SA DNA fragments containing 5′ EcoRI and 3′ SmaI sites were cloned into the RI/SmaI site on the vector GFP-expressing plasmid, pEGFP-C1, to generate N-terminal GFP-tagged HDAC4wt and HDAC4-3SA mutant used in Fig. 4 electroporation experiments. HDAC4 polyclonal antibody (clone 186) was previously described (3). P-HDAC4-S467 antibody was generated by Pocono Rabbit Farm using the following peptide against human HDAC4-S467: NH_2-CRQHRPLGRTQ<u>S</u>AP-LPQN-COOH phosphorylated on serine residue. Antibody was affinity purified and specificity was confirmed (see supplemental Figs. S1 and S2). 14-3-3 and CAMKIIδ antibodies are from Santa Cruz Biotechnology.

Mouse Procedures—6-week-old C57/BL mice (Jackson Laboratories) were anesthetized with a ketamine/xylazine mixture (25 mg/ml ketamine, 1 mg/ml xylazine in 0.9% NaCl; 100 μl used per mouse. Hair was removed from area surrounding muscle. For denervation, sciatic nerve was exposed and ~5-mM piece of nerve was cut and removed. Incision was sutured, and mice were allowed to recover. For overexpression electroporation analysis, 25 μg of GFP, HDAC4-GFP, or HDAC4-3SA-GFP was used for injection. For siRNA experiments, 500 pmol of siRNA oligo was used per injection. DNA or siRNA were directly injected into TA muscles using cemented MicroSyringe (VWR). ECM 830 electroporator (BTX) was used for all electroporations. Tweezertrodes (Model 520) were coated with transmission gel and were placed outside the skin around the muscle belly and pulsed 5 times at 50 V, 60-ms duration, with 200-ms interval time. Mice were allowed to recover in their cages. Stealth siRNA duplexed oligos are from Invitrogen. Sequences are as follows: mouse HDAC4: 5′-CACCGGAAC-CUGAACCACUGCAUUU-3′, mouse HDAC5: 5′-GGUCCU-CAUCGUGGACUGGGAUAUU-3′. All mice were housed at the Duke University mouse facilities in accordance with the Institutional Animal Care and Use Committee.

Western Analysis—Tibialis muscles were isolated and frozen in liquid nitrogen. Muscles were Dounce-homogenized on ice using 1 ml of glass-on-glass homogenizers and grinded in radioimmune precipitation assay buffer (0.05 M NaCl, 0.02 M Tris, pH 7.6, 1 mM EDTA, supplemented with leupeptin, aprotinin, phenylmethylsulfonyl fluoride, NaF, sodium orthovanadate, and 1 mM dithiothreitol). Lysates were incubated with 1× detergent (1%Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) for 10 min of rocking at 4° C and centrifuged at 14,000 rpm for 10 min to pellet debris. Protein was quantitated using the Bradford assay (BioRad). The HDAC4 polyclonal antibody ab186 was used as described previously (9). Monoclonal α-tubulin antibody (Sigma) was diluted 1:1000. For HDAC4 Westerns, 50 μg of protein lysate was used, and HDAC4 antibody was diluted 1:300.

RNA Analysis—For RNA analysis, TA muscles were Dounce-homogenized in 1 ml of Tri reagent (MRC) and incubated at 4° C overnight. 200 μl of chloroform was added, and after vigorous shaking, samples were centrifuged at 12,000 rpm for 10 min. Top aqueous layer was added to 500 μl of isopropyl alcohol, mixed well, and centrifuged at 12,000 rpm for 10 min. RNA pellet was washed with 70% ethanol, air-dried, and resuspended in DEPC water for RT-PCR analysis. For Northern blotting, 10 μg of total RNA was run on a 1% formaldehyde RNA gel. Gel was transferred onto Hybond N+ membrane and probed using radiolabeled fragments of HDAC4 or Atrogin-1 cDNA generated from Prime-it kit II (Stratagene). Blots were exposed for 2 h at −80°. For RT-PCR analysis, total RNA was DNase-treated using DNA-free kit (Ambion), and 1 μg RNA was used for cDNA synthesis reaction using Iscript Reverse transcriptase kit (BioRad). Samples were diluted 1:50 and 5 μl of cDNA was used per RT-PCR reaction. Real-time quantitative PCR was performed using iQ syber green supermix on the iCycler iQ detection system (BioRad). Sample volume was 20 μl per reaction.

Efficiency and specificity of primers were confirmed by standard PCR and DNA electrophoresis. RT-PCR Program on iCycler was 95° C 15 s, 60° C 30 s, 72° C 30 s. All real-time PCR values were normalized to either actin or 18S rRNA as indicated. All experiments were confirmed with an $n = 3$ animals. *In vivo* overexpression studies are adjusted based on comparison to GFP-alone fibers, which is set to 1 along the *y*-axis. Therefore, all data points reflect a fold-difference compared with GFP-alone fibers.

RT-PCR primer sequences are as follows: G-actin: forward, 5′-ACCCAGGCATTGCTGACAGGATGC, reverse, 5′-CCATCTAGAAGCATTTGCGGTGGACG; 18S: forward, GGACCAGAGCGAAAGCATTT, reverse TGCCAGAGTCTCGTTCGTTAT; Dach2: forward, 5′-ACTGAAAGTGGCTTTGGATAA, reverse, 5′-TTCAGACGCTTTTGCATTGTA; nAChR-a: forward, 5′-CGTCTGGTGGCAAAGCT, reverse, 5′-CCGCTCTCCATGAAGTT; MGN: forward, 5′-CTCAGCTTAGCACCGGAAGCCCGA, reverse, 5′-ATTGCCCCACTCCGGAGCGCAGGAG; MUSK: forward, 5′-CTCGTCCTCCCATTAATGTAAAAA, reverse, 5′-TCCAGCTTCACCAGTTTGGAGTAA; HDAC4: forward, 5′-CAGATGGACTTTCTGGCCG-3′, reverse, 5′-CTTGAGCTGCTGCAGCTTC-3′; HDAC5: forward, 5′-GAAGCACCTCAAGCAGCAGCAGG-3′, reverse, 5′-CACTCTCTTTGCTCTTCTCCTTGTT-3′; HDAC7: forward, 5′-AGCTGGCTGAAGTGATCC-3′, reverse, 5′-TCACCATCAGCCTCTGAG-3′; HDAC9 (MITR): forward, 5′-TCAGAGGTTCCTATGGGCCTG-3′, reverse, 5′-TGGAGACGTTCCACTGAGGG-3′.

Immunohistochemistry—Mouse muscle tissues were excised and placed in trays containing OCT (VWR). Either longitudinal or cross-sections were prepared using a Cryostat at 4° C. Sections were kept at −80 until use. For immunostaining, sections were fixed for 10 min in 4% paraformaldehyde at room temperature. After washing in phosphate-buffered saline 3 × 5 min, sections were blocked in 5% goat serum for 2 h and then incubated overnight with primary antibodies. Primary antibody dilutions are as follows: HDAC4 (clone 186) 1:200, P467-HDAC4 (clone 19111) 1:100, 14-3-3 (K-19, Santa Cruz Biotechnology) 1:100, CAMKIIδ (A-17, Santa Cruz Biotechnology). Sections were washed in phosphate-buffered saline 3 × 5 min, blocked 90 min in goat serum, and incubated in secondary antibody for 2 h, either with or without bungarotoxin as indicated. Secondary antibodies used were from Jackson ImmunoResearch (West Grove, PA) and included goat anti-rabbit RedX, goat anti-mouse Cy3, and goat anti-mouse Cy2. Bungarotoxin-labeled FITC or rhodamine was used (Molecular Probes). Sections were immersed in mounting medium (fluoromount G, Southern Biotech) for visualization. All images shown were taken with a Zeiss Axioskop compound microscope with a ×4, ×20, or ×40 objective.

RESULTS

Skeletal Muscle HDAC4 Expression Is Dramatically Induced in Response to Denervation and Neuromuscular Defects Associated with Disease—Gene array analysis identified HDAC4 as one of the most abundantly induced genes in muscle subject to immobilization (see Table S1 in Ref. 10). As immobilization causes a reduction in neuromuscular activity, we investigated whether HDAC4 is regulated by neural activity in skeletal muscle. To test this, a mouse denervation model of inactivity was employed. Adult mice were right limb-denervated for 7 days, and gastrocnemius muscles were subjected to Western analysis with an HDAC4-specific antibody. As shown in Fig. 1*A*, HDAC4 protein is dramatically increased in denervated muscle compared with the contralateral control muscles. The induction of HDAC4 occurs within 24 h after denervation and appears to be maximal by 7 days post-denervation (Fig. 1*B*). These results demonstrate that HDAC4 protein is rapidly and robustly induced in response to denervation.

To distinguish whether induction of HDAC4 occurs at the protein level or transcription level, RNA from both control and denervated tibialis muscle samples was analyzed by Northern blot and quantitative real-time RT-PCR. As shown in Fig. 1*C*, Northern blotting analysis showed that denervation causes a dramatic induction of the HDAC4 transcript, confirming a transcriptional mode of regulation on HDAC4 (compare HDAC4 to the positive control Atrogin-1). Real-time PCR analysis revealed that HDAC4 mRNA levels are induced by more than 10-fold (see Fig. 1*D*). Interestingly, HDAC5 is also markedly induced by denervation, while HDAC7 and HDAC9 levels are either not affected or modestly reduced. These data suggest that HDAC4 may be a part of a muscle transcriptional program in response to inactivity.

Reduced neural activity caused by denervation is a common hallmark of neuromuscular disease. To assess whether HDAC4 is also involved in skeletal muscle abnormalities associated with neuromuscular disease, we examined HDAC4 levels in two models of neurogenic muscle disease: amyotrophic lateral sclerosis (ALS) mice (SOD1-G93A) and NMD mice (a spinal muscular atrophy (SMARD1) model) (11, 12). As shown in Fig. 1*E*, HDAC4 is dramatically induced in skeletal muscle from both ALS and NMD mice. Interestingly, at ~day 120 when there was no discernible motor defect, HDAC4 was already selectively induced in fast/glycolytic tibialis anterior (TA) muscle but not in the predominantly slow/oxidative *soleus* muscle. This finding is in agreement with previous observations that fast/glycolytic fibers in ALS mice are more sensitive to disease progression than slow/oxidative fibers, further connecting HDAC4 induction to neuromuscular dysfunction. In contrast, DMD (Duchenne Muscular Dystrophy caused by mutation in the dystrophin gene) and LGMD (Limb-Girdle Muscular Dystrophy caused by mutation in the myotilin gene) (22), which are myogenic muscular dystrophies in the absence of neuromuscular defects, showed no significant elevation of HDAC4 levels. Therefore, HDAC4 is specifically induced in response to neural inactivity as opposed to muscle dysfunction.

HDAC4 Undergoes Dramatic Nuclear Accumulation in Response to Denervation—As class IIA HDACs have been shown to dynamically shuttle between the nucleus and the cytoplasm, we next determined whether the subcellular localization of HDAC4 is also regulated by neural activity and affected by denervation. Gastrocnemius muscle sections were prepared from control and denervated muscle, and immunohistochemistry was performed using a specific HDAC4 antibody. As shown in Fig. 2, control muscles display some faint sarcoplasmic staining with weak nuclear staining suggesting a

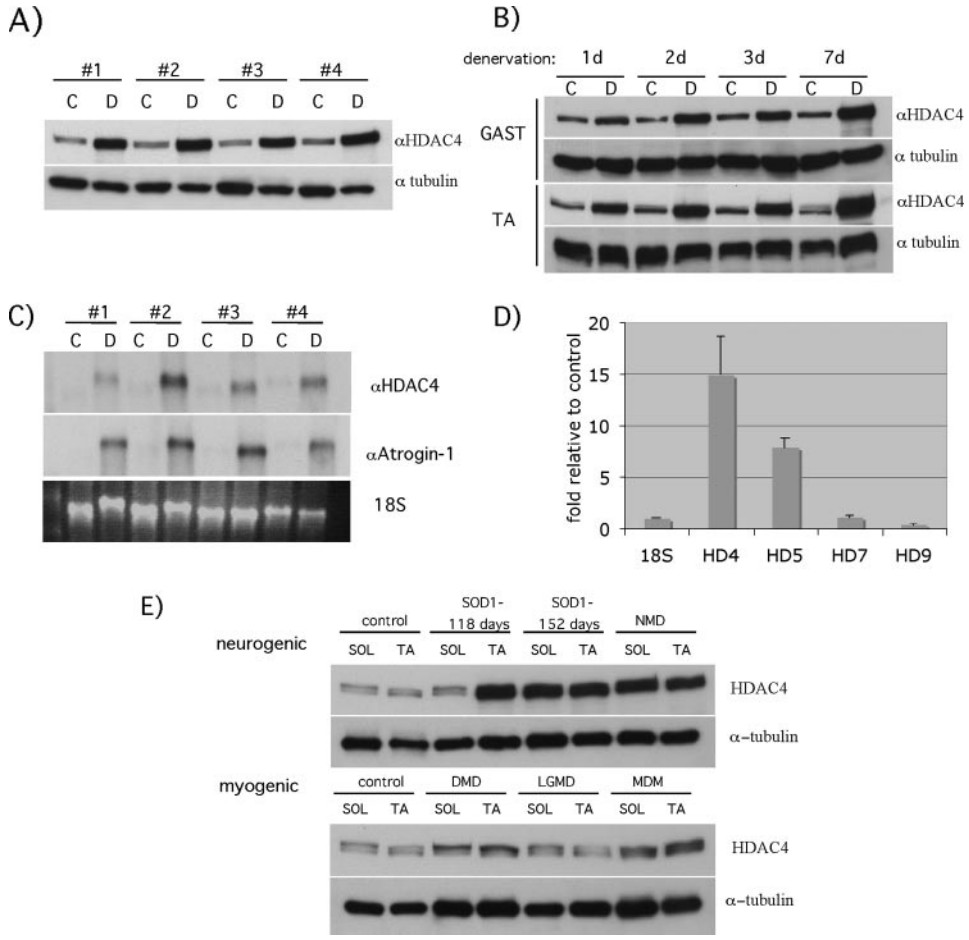

FIGURE 1. **HDAC4 is regulated in an activity-dependent manner.** *A*, mice were right hindlimb-denervated for 7 days. Control C and denervated D mouse gastrocnemius tissue were harvested. Protein was analyzed by Western blotting using polyclonal antibody against HDAC4 and a monoclonal anti-tubulin antibody. *B*, gastrocnemius and tibialis muscles were similarly analyzed from 1-, 2-, 3-, and 7-day denervated mice for expression of HDAC4 and tubulin. *C*, Northern analysis was performed on control and 7-day denervated total RNA samples using radiolabeled probes generated against mouse HDAC4 and Atrogin-1. *D*, RT-PCR analysis was performed on control and 7-day denervated muscle tissue using primers specific for HDACs 4, 5, 7, 9. Values were normalized to the control 18S rRNA. *Error bars* indicate S.E. *E*, mouse models of muscle disease included myogenic models: *DMD*, Duchenne muscular dystrophy; *LGMD*, Limb-Girdle muscular dystrophy; *MDM*, muscular dystrophy with myositis; and the neurogenic models: SOD1-G93A ALS model, and *NMD*, neuromuscular degeneration model. Soleus and tibialis muscles were isolated from diseased mice, and lysate was analyzed by Western blot similar to above.

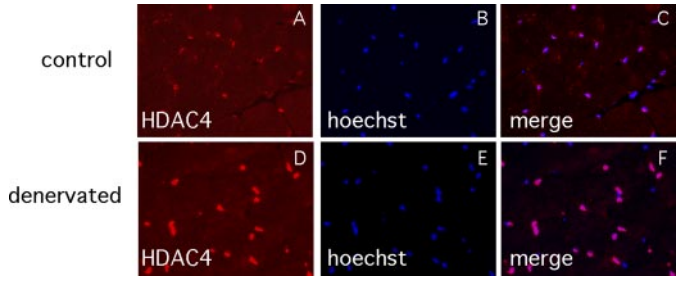

FIGURE 2. **HDAC4 accumulates in nuclei of denervated skeletal muscle.** *Panels A–F*, mouse gastrocnemius muscles from control (*panels A–C*) or 7-day denervated (*panels D–F*) mice were isolated and analyzed by immunohistochemistry. Samples were analyzed using an HDAC4 antibody and were co-stained with Hoechst dye to identify nuclei. The merge indicates accumulation of HDAC4 in nuclei in denervated muscle.

basal level of nuclear HDAC4 under normal conditions (Fig. 2, *panels A–C*). Remarkably, a dramatic accumulation of HDAC4 is observed in myonuclei in muscle subject to 7 days denervation (Fig. 2, *panels D–F*). This finding reveals that HDAC4 becomes accumulated in the nucleus in response to inactivity.

During the characterization of HDAC4 subcellular localization in myofibers, we consistently observed large structures that stained positive for HDAC4. Co-staining with α-bungarotoxin, which binds the nAChR, demonstrated that those structures are neuromuscular junctions, sites where nerve activity is propagated (Fig. 3, *panels A–D*). Interestingly, the NMJ staining is particularly intense using a phosphospecific antibody that recognizes HDAC4 phosphorylated on serine 467, a preferred CAMKII site (Fig. 3, *panels E-H*). The NMJ staining is lost in the presence of immunizing antigens demonstrating antibody specificity (see supplemental Figs. S1 and S2). As CaMKII binds and phosphorylates HDAC4 and is the prime candidate that decodes neural activity, we determined if CaMKII is a component of the NMJ. Indeed as shown in Fig. 3, *panels I–P*, we found that the muscle-specific isoform CaMKIIδ and 14-3-3 are concentrated at the NMJ, revealing that HDAC4 and its regulators are both localized to the NMJ. Because electrical activity is mediated by calcium flux (13–15), we hypothesized that NMJ-localized HDAC4 might be controlled by changes in calcium influx dictated by neural activity. We therefore determined whether HDAC4 localization to the NMJ is regulated by nerve activity. As shown in Fig. 3, *panels Q–T*, a brief 1-day denervation induced a rapid dissociation of HDAC4 from the NMJ, while CaMKIIδ localization to the NMJ remained largely unchanged (data not shown). These results suggest two modes of regulation on HDAC4 in response to nerve activity: first, the activity-dependent localization of HDAC4 to the NMJ as a potential sensor of calcium-elicited nerve activity, and second, the transcriptional induction and nuclear accumulation of HDAC4 in response to inactivity.

HDAC4 Is Required for Proper Activity-dependent Dach2, MGN, and nAChR Gene Expression—The above results revealed that HDAC4, a transcription regulator, undergoes nuclear accumulation upon loss of nerve activity. These properties are consistent with the proposed factor that connects neural activity to muscle transcription. We therefore asked if HDAC4 is required for the transcriptional regulation of the Dach2/myogenin/nAChR cascade in response to denervation.

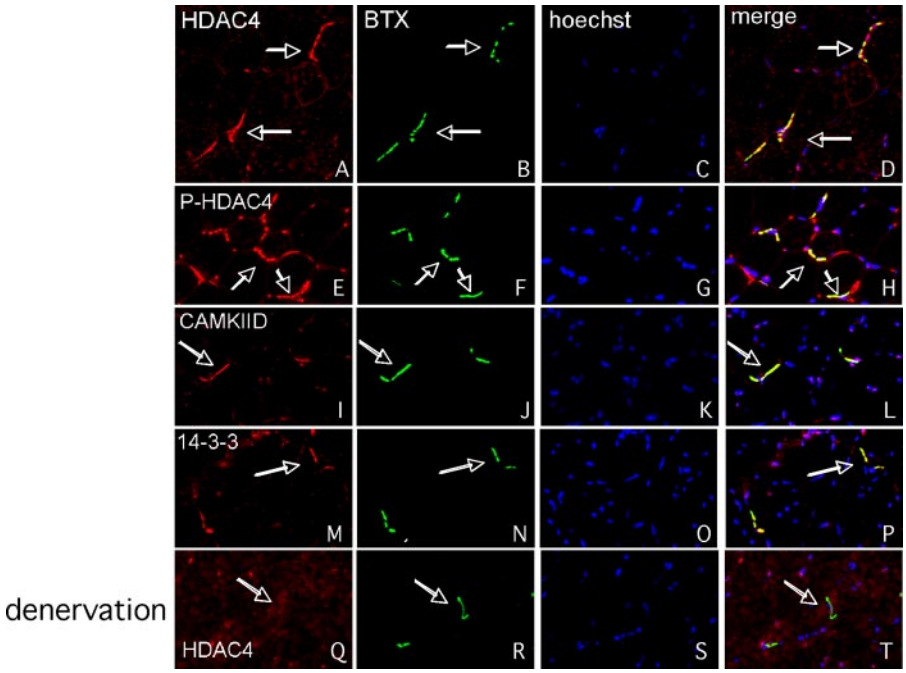

FIGURE 3. **HDAC4, CAMKIIδ, and 14-3-3 proteins localize to the NMJ in mouse skeletal muscle.** *Panels A–T*, gastrocnemius cross-sections from innervated mice were analyzed by immunohistochemistry using antibodies against HDAC4 (*A*, see *panels A–D*), P-HDAC4-S467 (*E*, see *panels E–H*), CAMKIIδ (*I*, see *panels I–L*), and 14-3-3 (*M*, see *panels M–P*). Similar analyses were performed using 1-day denervated gastrocnemius and analyzed with antibody against HDAC4 (*Q*, see *panels Q–T*). Bungarotoxin labeling was used as a marker for the neuromuscular junction and Hoechst staining was used to identify nuclei. The merges indicate that HDAC4 and its regulators localize to the NMJ.

To test this, we first determined the kinetics of HDAC4 induction in relation to Dach2, MGN, and nAChR gene expression, thereby establishing a temporal profile in response to denervation. Mice were denervated, and tibialis muscles were harvested at varying time points for RNA analysis. As shown in Fig. 4*A*, real-time PCR analysis detected abundant HDAC4 levels by 12-h post-denervation, at time at which Dach2 levels begin to decline. Subsequently, MGN and nAChR levels begin to accumulate around 24-h post-denervation. This analysis reveals that the induction of HDAC4 coincides with the repression of Dach2, and that this temporal regulation may represent an early event required for the subsequent induction of myogenin-dependent target genes, including nAChR.

We next determined whether HDAC4 indeed regulates Dach2 expression. To this end, control GFP, wild-type HDAC4 and the constitutively nuclear mutant, HDAC4-3SA, were electroporated into mouse tibialis muscle (see Fig. 4*B* for expression), and RNA samples were prepared and analyzed by RT-PCR. As shown in Fig. 4*C*, both HDAC4-wt and the HDAC4-3SA mutant were capable of repressing Dach2 mRNA levels in this system, although Dach2 is more potently repressed by HDAC4-3SA, consistent with this mutant being constitutively nuclear. Further, both wild-type HDAC4 and HDAC4-3SA expression led to induction of MGN, nAChR, and MUSK mRNA when compared with GFP-electroporated fibers. We note that HDAC4-3SA mutant is not significantly more active than wild-type HDAC4 in this assay. This could be caused by its lower expression (see Fig. 4*B*), possibly because of the toxicity associated with constitutively nuclear class IIA HDACs (16). Regardless, these data reveal that elevated levels of HDAC4 are sufficient to repress Dach2 and induce synaptic gene expression, thereby mimicking the effect of denervation.

To further demonstrate the importance of HDAC4 in the activity-dependent Dach2/MGN/nAChR transcriptional cascade, an *in vivo* knockdown approach was employed using stealth siRNA technology (see Fig. 4*D*). A scrambled control siRNA or an HDAC4 siRNA duplex was electroporated into mouse tibialis muscle, and 7 days after electroporation mice were denervated for 3 days followed by RT-PCR analysis. As previously reported (8) and as shown in Fig. 4, *E* and *F*, Dach2 levels are reduced after denervation with a concomitant induction of the activity-dependent genes MGN, nAChR, and MUSK. Importantly, siRNA-mediated knockdown of HDAC4 significantly alleviates the repression of Dach2 that occurs during denervation. Consistent with this observation, the induction of MGN, nAChR, and MUSK are all markedly blunted in denervated muscles transfected with HDAC4 siRNA (see Fig. 4*F*). Interestingly, double siRNA knockdown of HDAC4 and HDAC5 shows a similar phenotype to that of the HDAC4 single knockdown, further supporting a critical role for HDAC4 in the regulation of this pathway. These data show that HDAC4 is required for activity-dependent induction of synaptic gene expression, and that HDAC4 mediates this effect through down-regulation of the myogenin repressor, Dach2.

HDAC4 Regulates nAChR Distribution in Skeletal Muscle—In denervated muscles, nAChR levels are elevated and distributed along the length of the muscle membrane outside the postsynaptic membrane. To further investigate the importance of HDAC4 in activity-regulated nAChR expression, we analyzed the localization of nAChR in control and HDAC4 siRNA electroporated muscle both before and after denervation. To facilitate the identification of electroporated fibers, we co-electroporated a GFP-expressing plasmid along with either scrambled or HDAC4 siRNA duplexes. As shown in Fig. 5 *panels A–C*, control tibialis muscle does not display any detectable membrane-associated nAChR, consistent with the suppression of nAChR in extrasynaptic muscle. In contrast, 12 day-denervated muscle expressing the control scrambled siRNA showed a dramatic induction of membrane-bound nAChR consistent with the elevation of extrasynaptic nAChR in response to inactivity (see Fig. 5, *panels D–F*). Remarkably, the membrane staining of nAChR is almost completely eliminated in HDAC4 siRNA-expressing myofibers (Fig. 5, *panels G–I*), strongly indicating that HDAC4 is required for extrasynaptic nAChR expression in response to denervation.

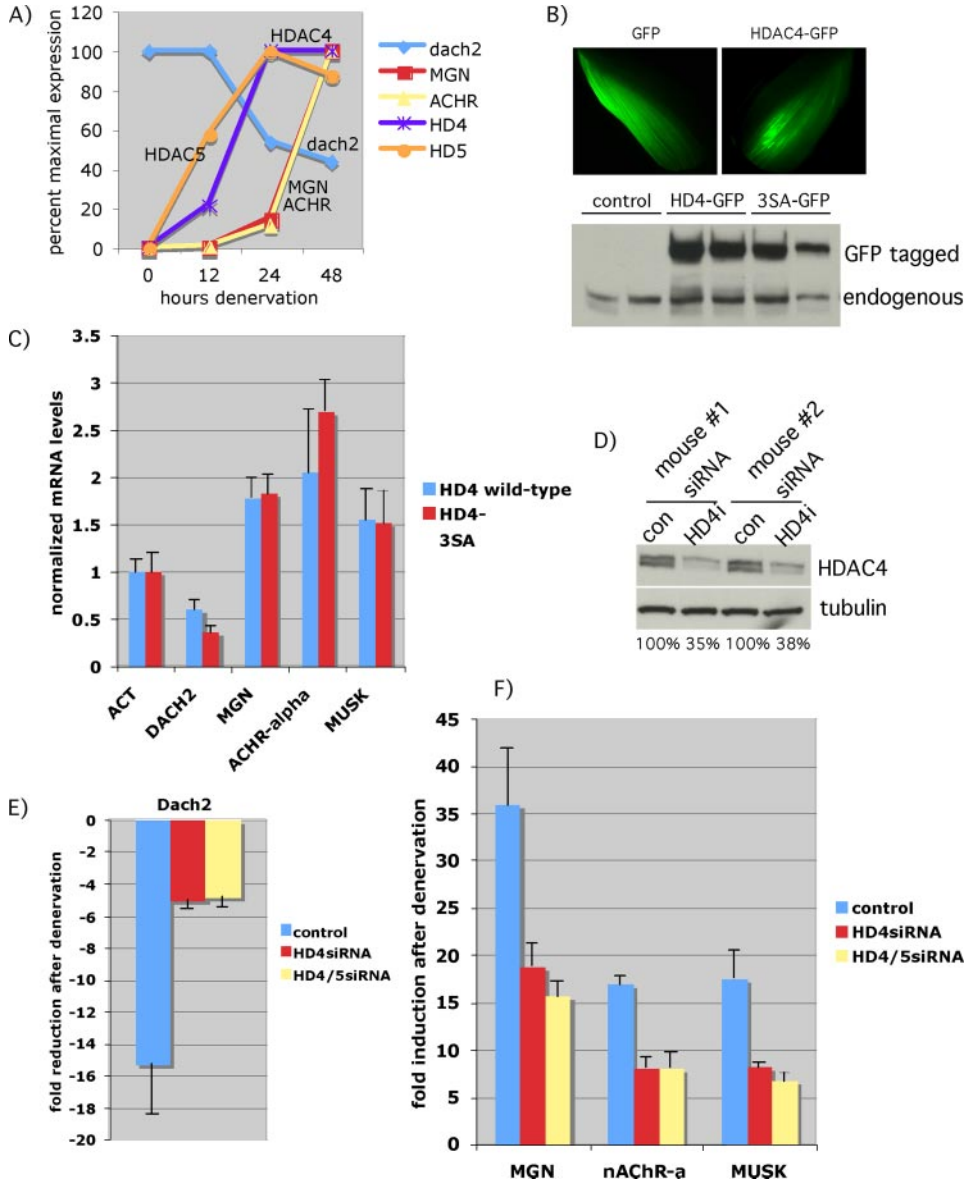

FIGURE 4. **HDAC4 regulates the Dach2/myogenin/nAChR signaling pathway.** *A*, tibialis muscles were harvested at 0, 12, 24, and 48 h after denervation, and RNA was isolated for RT-PCR to determine the relative levels of Dach2, myogenin, nAChR, HDAC4, and HDAC5 by RT-PCR analysis. Values were normalized to actin and represented as percent maximal expression. *B*, *upper panels*, low magnification images of GFP and HDAC4-GFP-containing fibers. *Lower panels*, HDAC4-GFP and HDAC4-3SA were electroporated into tibialis muscles for 7 days, and protein lysate was analyzed by Western blotting using HDAC4 antibody. Both endogenous HDAC4 (*lower band*) and GFP-tagged HDAC4-wt/HDAC4-3SA (*upper band*) are observed due to mobility shift differences. *C*, GFP alone, HDAC4-GFP, or 3SA-GFP was electroporated into tibialis muscles for 7 days. Muscles were subsequently analyzed by RT-PCR for the levels of Dach2, myogenin nAChR, and MUSK as indicated. Samples were normalized to actin as a control, and error is represented as S.E. All *bars* represent fold difference *versus* GFP-electroporated fibers. *D*, scrambled or HDAC4 stealth siRNAs were electroporated into tibialis muscles for 7 days, and protein lysate was used for Western analysis using an HDAC4 antibody and tubulin as a loading control. *E* and *F*, scrambled, HDAC4, or HDAC5 stealth siRNAs were electroporated into tibialis muscles for 7 days, and mice were subsequently denervated for 3 days. Muscle samples were analyzed by RT-PCR using primers for Dach2, nAChRα, MUSK, and myogenin as indicated. Values were normalized to actin and error is reported as S.E. Similar results were obtained with $n = 3$ animals.

DISCUSSION

Neural activity-regulated gene expression is crucial for specifying muscle fiber functionality, a coordinated remodeling process that confers muscles unique ability to adapt to different demands. Identifying the factors that connect neural activity to nuclear gene expression is one critical issue in muscle biology. In this report, we presented evidence that the histone deacetylase HDAC4 is one principal mediator that links neural activity to muscle transcriptional machinery important for synaptic protein gene expression. The unique localization to the NMJ places HDAC4 in proximity to where neural activity is initiated and propagated in muscle. Indeed, we showed that HDAC4 levels and subcellular localization are regulated by neural activity. The importance of HDAC4 in activity-dependent gene expression is demonstrated by its requirement for the repression of Dach2 and the subsequent induction of MGN, nAChR, and MUSK transcription upon denervation. These findings establish HDAC4 as a novel signaling component critical for the execution of activity-regulated gene expression and provide a molecular framework to understand how neural activity is relayed into the nucleus to initiate muscle transcription reprogramming.

HDAC4 and Activity-dependent Skeletal Muscle Remodeling—Activity-regulated gene transcription bestows skeletal muscle the unique functional plasticity for changing demands. The molecule that relays neural activity into muscle nuclei, therefore, is a crucial component that establishes the communication of nerve activity and muscle functionality. Unexpectedly, our study found that HDAC4, a potent MEF2 repressor thought to inhibit muscle differentiation, plays a critical role in connecting neural activity to muscle gene expression. This finding demonstrates that HDAC4 does not simply act to inhibit skeletal muscle differentiation under physiological condition. Rather, the main function of HDAC4, and possibly the related HDAC5 and HDAC9, is to modulate muscle transcription program so that mature muscle fibers can attain specific functionality dictated by neural activities.

Our study provides evidence that one key function of HDAC4 in skeletal muscle is to control synaptic gene expression in response to neural activity. Our analyses demonstrate that HDAC4 is required for the robust transcriptional induction of the synaptic genes nAChR and MUSK by denervation (Figs. 4*F* and 5, *G–I*). In fact, ectopic expression of HDAC4 is sufficient to mimic denervation and induce expression of

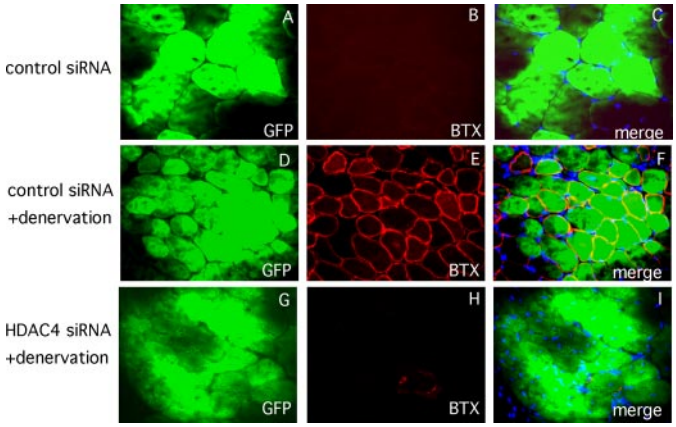

FIGURE 5. **HDAC4 regulates nAChR expression and localization in denervated skeletal muscle.** *Panels A–I*, either scrambled control (*panels A–C*, *D–F*) or HDAC4 siRNA (*panels G–I*) were co-electroporated with pEGFP-C1 vector into mouse tibialis muscle for 4 days and subsequently denervated for 12 days for analysis by fluorescent staining. BTX was used to identify nAChR and GFP+ fibers were used to mark electroporated fibers.

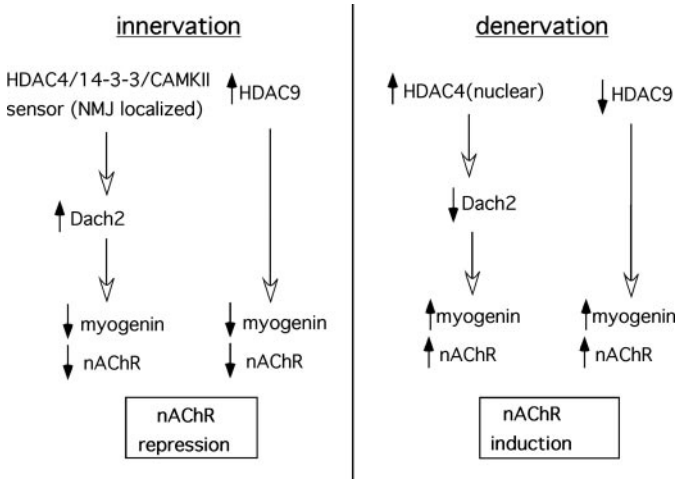

FIGURE 6. **Model for activity-dependent regulation of HDACs.** HDAC4 may sense neuromuscular activity by localizing directly to the neuromuscular junction, allowing elevated levels of Dach2 and subsequent repression of myogenin and nAChR in innervated muscle. In this scenario, HDAC4 acts in parallel with HDAC9, whose levels are elevated in innervated muscle thereby maintaining myogenin in a repressed state. Denervation leads to elevated levels of extrasynaptic HDAC4 and, conversely, reduced levels of HDAC9. In this scenario, induction of HDAC4 represses Dach2 levels leading to increased levels of myogenin. This is reinforced by denervation-dependent reduction of HDAC9 levels, which, in collaboration with HDAC4, achieve potent activity-dependent expression of myogenin, nAChR, and other synaptic genes.

MGN, nAChR, and MUSK (Fig. 4, *C*, *E*, *F*). In this regulatory circuit, we identified Dach2 as one critical transcriptional target of HDAC4. We showed that denervation-dependent repression of Dach2 transcription coincides with the induction of HDAC4, providing a temporal control of Dach2 expression by HDAC4 (Fig. 4*A*). This conclusion is entirely consistent with an earlier report that transcriptional repression of Dach2 required an unidentified HDAC activity (8). Although the specific mechanism by which HDAC4 represses Dach2 expression remains to be established, this regulation is likely a direct one, as chromatin immunoprecipitation assay reveals that HDAC4 associates with the promoter region of Dach2.[4] Most importantly, our results showed that HDAC4 induced by denervation is required for the transcriptional repression of Dach2. The repression of Dach2 by HDAC4 then allows the induction of MGN, which in turn activates nAChR and other genes responsible for proper NMJ formation (17). Consistent with a role for HDAC4 in synaptic gene expression, we have also detected increased expression of HDAC4 in subsynaptic nuclei in innervated muscle (see supplemental Fig. S3). These results establish HDAC4 as a key mediator for neural activity-regulated synaptic gene expression.

Our results suggest that activity-dependent muscle gene transcription involves a regulation of HDAC4 subcellular localization (Figs. 2 and 3). In innervated muscle, we found that HDAC4 and CaMKII are both present at the NMJ, where neural activity initiates in muscle. Interestingly, it was reported that HDAC4, but not the closely related HDAC5, binds active CaMKII (18). CaMKII, being sensitive to calcium frequency (19), is a prime candidate in decoding neural activity based on calcium transients. The presence of HDAC4 and CaMKII at the NMJ thus suggests an intriguing model whereby HDAC4 might "sense and respond" to activity locally at the NMJ by virtue of its functional interaction with CaMKII (see model, Fig. 6). Upon reduced neural activity, HDAC4 dissociates from the NMJ and accumulates in the nucleus where it induces specific transcriptional changes, thereby linking neural activity and muscle gene transcription. In this model, the localization as well as the levels of HDAC4 may control the muscle transcriptional program based on neural input.

Class IIA HDACs and Activity-dependent Transcription, a More Complex Picture—In addition to HDAC4, our analysis revealed that HDAC5 is also markedly induced in response to denervation. Importantly, muscle with HDAC4 single or HDAC4/5 double knockdown showed similar defects in myogenin or nAChR expression (see Fig. 4, *E* and *F*), suggesting that HDAC4 plays a more dominant role in synaptic gene expression in this system. The dramatic induction of HDAC4 observed in denervated muscles, however, is in stark contrast to the repression of MITR, the alternative spliced form of HDAC9 lacking the catalytic domain (20). Mejat *et al.* (20) previously showed that MITR acts as a myogenin repressor, and that denervation leads to MITR repression and subsequent activation of myogenin. Indeed, our RT-PCR analysis confirmed a moderate reduction of HDAC9 expression in denervated muscle (Fig. 1*D*). These findings are quite surprising as HDAC9 (MITR) and HDAC4 both repress MEF2 activity, but are apparently oppositely regulated by neural activity. Indeed, HDAC4 knockdown and MITR knock-out muscle have an opposite effect on MGN expression (Fig. 6 and Ref. 20). More studies will be required to address how the reduction of MITR and the induction of HDAC4 both contribute to denervation-induced nAChR expression. These observations, however, are consistent with the existence of HDAC inhibitor-sensitive and insensitive regulation of nAChR as reported by Tang and Goldman (8). Our results indicate that the HDAC inhibitor sensitive pathway is mediated by HDAC4-dependent Dach2 repression whereas the alternative pathway is controlled by MITR (Fig. 6). Regardless,

[4] T. J. Cohen, unpublished observation.

these findings reveal an important but complex role for class IIA HDACs in activity-dependent muscle gene transcription.

HDAC4 and Neuromuscular Disease—We have found that HDAC4 levels are also dramatically induced in muscles of ALS and NMD neuromuscular disease models. This finding raises an interesting possibility that HDAC4 might be involved in the development of muscle pathology associated with ALS or other neuromuscular disease. If proven true, inhibition of HDAC4 by specific inhibitors could provide potential therapeutic benefit to neuromuscular disease patients. In this context, HDAC inhibitor treatment has been shown to alleviate the disease phenotype in the mouse ALS model (21). Our results suggest that HDAC4 might be a key target for the therapeutic effects of HDAC inhibitors.

Neural activity-dependent muscle remodeling plays a critical role for skeletal muscle to adapt to different functional demands by changing specific transcription programs important for nAChR expression, fiber type-specific gene expression and atrophy. Our study identifies HDAC4 as a key effector that mediates the activity-dependent Dach2-Myogenin-nAChR program. The future characterization of HDAC4 in other aspects of muscle remodeling would determine if HDAC4 is a master regulator of muscle functionality controlled by neural activity.

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The Histone Deacetylase HDAC4 Connects Neural Activity to Muscle Transcriptional Reprogramming

Todd J. Cohen, David S. Waddell, Tomasa Barrientos, Zhonghua Lu, Guoping Feng, Gregory A. Cox, Sue C. Bodine and Tso-Pang Yao

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