**Adaptation of Nicotinic Acetylcholine Receptor, Myogenin, and MRF4 Gene Expression to Long-Term Muscle Denervation**

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**Abstract.** Muscle activity alters the expression of functionally distinct nicotinic acetylcholine receptors (nAChR) via regulation of subunit gene expression. Denervation increases the expression of all subunit genes and promotes the expression of embryonic-type (α2βδγ) nAChRs, while electrical stimulation of denervated muscle prevents this induction. We have discovered that the denervation-induced increases in α, β, γ, and δ subunit gene expression do not persist in muscles that have been denervated for periods extending beyond a couple of months. However, expression of RNA encoding the ε-subunit remains elevated suggesting a return to expression of predominantly adult-type (α3δε) nAChR in long-term denervated muscles; a finding confirmed by single channel patch-clamp analysis. Since the nAChR subunit genes are regulated by the MyoD family of muscle regulatory factors, and the genes encoding these factors are also induced following short-term muscle denervation, we determined their level of expression in long-term denervated muscle. Although MyoD and myf-5 RNA levels remained elevated, myogenin and MRF4 RNAs were induced only transiently by muscle denervation. Surprisingly, Id-1, a negative regulator of transcription, was gradually induced in denervated muscle with RNA levels peaking about two months after denervation. It is likely that this maintained level of increased Id expression, in conjunction with the returning levels of myogenin and MRF4 expression, account for the reduced level of embryonic receptors in long-term denervated muscle. These changing patterns of gene expression may have important consequences for the ability of muscle to recover function after denervation.

The muscle nicotinic acetylcholine receptor (nAChR) is a pentameric integral membrane protein that functions as a ligand-gated ion channel. During muscle development the levels, distribution, and properties of this receptor change (for review see Brehm and Henderson, 1988; Hall and Sanes, 1993). Many of these changes are correlated with muscle innervation. Before innervation, or after denervation of adult muscle embryonic-type nAChRs are expressed throughout the muscle fiber. These receptors are composed of four different subunits with a stoichiometry of αβγδ. After innervation of muscle, the γ subunit is replaced by an ε subunit, and these adult-type receptors (αβδε) are preferentially expressed at the neuromuscular junction (NMJ).

The switch from embryonic to adult-type receptors results in a change in their channel properties. Embryonic-type receptors exhibit a low single channel conductance and long mean channel open time, while adult-type receptors have a higher conductance and faster open time kinetics (for review see Schuetze and Role, 1987; Brehm and Henderson, 1988). Although the reason for the switch in receptor subtypes is not completely clear, it may arise from a requirement for long open time, embryonic-type of receptors in developing muscle (Jaramillo et al., 1988), which are detrimental to adult muscle and therefore replaced with adult-type receptors (Engel et al., 1982; Ohno et al., 1995).

Muscle denervation has been used as a model system to study the molecular mechanisms by which muscle activity regulates synaptic protein expression. Denervation of adult muscle causes a dramatic increase in the sensitivity of the fiber to ACh (Ginetzinsky and Shamarina, 1942; Axelsson and Thesleff, 1959) which is directly attributable to increases in the level of the α, β, γ, and δ subunit RNAs within 24–48 h (Goldman et al., 1988). Increases in these nAChR RNAs lead to high levels of expression of embryonic-type nAChR throughout the muscle fiber (Katz and Miledi, 1972; Neher and Sakmann, 1976; Henderson et al., 1987). In addition, the level of ε subunit RNA, which is...
preferentially expressed in adult muscle, is increased locally at the NMJ within 7 d of muscle denervation (Witzemann et al., 1989, 1991; Gundersen et al., 1993). These changes in RNA levels have been shown to result from altered patterns of nAChR gene expression (Merlie and Kornhauser 1989; Klarsfeld et al., 1991; Simon et al., 1992; Gundersen et al., 1993). In addition, activity-dependent regulation is selective, since RNAs encoding other muscle-specific proteins, such as creatine kinase and myosin light chain, are unaffected by muscle denervation or electrical stimulation (Chahine et al., 1992; Dutton et al., 1993; Gilmour et al., 1995; Su et al., 1995).

The molecular mechanisms mediating the effects of muscle activity on nAChR gene expression are beginning to be elucidated. A calcium-dependent protein kinase C (PKC) and a CaMP-dependent signaling cascade have been implicated in mediating the effects of muscle activity in chick and rat, respectively (Klarsfeld et al., 1989; Huang et al., 1992; Chahine et al., 1993). DNA sequences that ultimately respond to muscle depolarization and alter nAChR gene expression have also been identified. In the mouse δ and chick α subunit genes, DNA sequences conforming to the consensus E-box sequence (CANNTG) have been identified, and those located nearest to the transcriptional start sites have been shown to participate in mediating activity-dependent control of nAChR gene expression (Bessereau et al., 1994; Tang et al., 1994; Su et al., 1995). These elements are bound by members of the helix-loop-helix family of myogenic regulatory factors (MRFs), which include MyoD, myogenin, MRF4, and Myf-5. These proteins are thought to bind E-box sequences as heterodimers consisting of one of the above mentioned proteins complexed with a ubiquitously expressed E protein such as E47 or E12 (Weintraub et al., 1991).

In contrast to the helix-loop-helix proteins described above, one member of this family of proteins, referred to as Id, lacks a DNA binding domain (Benezra et al., 1990). Id can complex with other E-box binding proteins, but these complexes have a low affinity for DNA. Via these protein–protein interactions with other myogenic regulatory factors, Id can act to inhibit helix-loop-helix protein-dependent gene activation.

Muscle denervation has a dramatic effect on muscle-specific gene expression and muscle physiology. Short periods of denervation, up to 1 mo, results in an increase in embryonic-type nAChR and MRF gene expression (Evans et al., 1987; Witzemann and Sakmann, 1991; Voytko et al., 1993). This period corresponds to the time when the muscle can best recover its function after reinnervation. However, prolonged periods of denervation, extending beyond a couple of months, significantly reduce the ability of muscle to become functionally reinnervated (Sunderland, 1978). During this time period, the muscle fiber undergoes dramatic changes in its size, cytoplasmic content, number of nuclei and in its ability to recover function upon regeneration and/or reinnervation (B. M. Carlson, unpublished data). This decreased potential for recovery may be correlated with a concomitant change in the muscle’s ability to maintain a particular pattern of muscle-specific gene expression. To determine if there is a change in muscle-specific gene expression after prolonged periods of denervation which may correlate with the diminished ability to recover function, we assayed for nAChR, creatine kinase and MRF gene expression at various times of denervation. We report here that the changes in gene expression observed after short-term muscle denervation are not maintained in long-term denervated muscles. Most surprising was our finding that the high levels of α, β, γ, and δ subunit RNAs which typify both embryonic and adult denervated muscle, begin to return to innervated levels following 1–2 mo of muscle denervation. In contrast, the adult-type ε subunit and creatine kinase RNAs do not decrease as a result of prolonged periods of denervation. These results indicate that there is a return to predominantly adult-type nAChR gene expression in long-term denervated muscle.

Patch-clamp recordings indicated that changes in subunit gene expression are reflected in changes in both the single channel properties of the receptors and their relative levels of expression in junctional and nonjunctional membrane. In contrast to muscle fibers denervated for short periods of time which express high levels of low conductance, long open time receptors throughout the membrane, fibers denervated for greater than two months expressed predominantly the high conductance, short open time form of the receptor. Moreover, the level of ACh-induced activity was high only in recordings from junctional membrane, suggesting that a high density of receptors is limited to the subsynaptic membrane. Physiological data suggesting that high levels of nAChR expression are restricted to the previous synaptic site was also indicated by the pattern of fluorescence in these fibers stained with rhodamine-conjugated α-bungarotoxin, an irreversible ligand for the muscle nAChR (Chang and Lee, 1963).

The decreased expression of α, β, γ, and δ subunit RNAs after prolonged periods of muscle denervation corresponds to a time when myogenin and MRF4 expression are also declining and Id expression is increasing. Our data suggest that the different patterns of nAChR subunit gene expression observed in short- and long-term denervated muscle may be a consequence of the changing pattern of expression of these myogenic factors and may underlie the decreased ability of muscle to recover function as the time of denervation increases.

Materials and Methods

Muscle Denervation

Denervations were performed on 5-mo-old male Wistar rats of the WI/HicksCar strain maintained at the University of Michigan. Rats were anesthetized with ether. The right legs of the rats were first denervated by sectioning the sciatic nerve high in the thigh, ligating the proximal and distal stumps and implanting the proximal stump into a hip muscle. The distal stump was implanted as far from the proximal stump as possible. This period corresponds to the time when myogenin and MRF4 expression are declining and Id expression is increasing. Our data suggest that the different patterns of nAChR subunit gene expression observed in short- and long-term denervated muscle may be a consequence of the changing pattern of expression of these myogenic factors and may underlie the decreased ability of muscle to recover function as the time of denervation increases.

RNA Isolation and RNase Protection Assay

Total RNA was isolated using the method of Chirgwin et al. (1979). RNase protection assays were carried out as previously described (Saccomanno et al., 1992; Walke et al., 1994). After hybridization of RNA with appropriate probes, RNase T2 was used to digest away single-stranded RNA. RNase-resistant hybrids were analyzed on 6% polyacrylamide, 8 M urea gels. After electrophoresis, gels were dried and exposed to x-ray film. Signals were quantitated by scanning densitometry and values normalized to either total RNA in the hybridization reaction or muscle creatine ki-
Physiological Recordings

Individual muscle fibers were obtained from the flexor digitorum brevis (fdb) muscles of denervated rats. The fdb muscle was dissected and dissociated according to Henderson et al. (1987), and recordings were made in the cell-attached configuration of the patch clamp technique (Hamill et al., 1981) as previously described (Brennan and Henderson, 1993). Irrespective of the time after denervation, the original endplate was morphologically distinguishable at the time of recording, as has previously been reported (Henderson et al., 1987).

Single channel currents were acquired and analyzed as described by Brennan et al. (1992). Mean open times were estimated at $V_{\text{pipet}} = +80$ mV for distributions containing greater than 100 events, except for some non-synaptic recordings where the frequency of events was extremely low. Slope conductances were estimated by linear regression from the current vs. voltage relationship for $V_{\text{pipet}}$, between $+60$ and $+140$ mV. Estimates of the AC-induced activity represent the product of the open probability times the number of channels and were calculated by TAC® software (Instrutech Corp., Emmont, NY). Estimates of the relative percentages of openings corresponding to high and low conductance events, as well as levels of AC-induced activity, were made immediately after seal formation as $V_{\text{pipet}} = +80$ mV. Statistical significance was determined using a Student's two-tailed t test. Where given, $n$ values indicate the number of fibers.

In Situ Hybridization and nAChR Staining

In situ hybridization assays were performed as previously described (Goldman and Staple, 1989; Goldman et al., 1991). nAChRs were stained with rhodamine-conjugated a-bungarotoxin as previously described (Henderson et al., 1987).

Results

Embryonic-Type nAChR RNAs Initially Increase and Then Decrease in Long-Denervated Muscle

It is well documented that sectioning the motor neuron results in increased expression of embryonic-type nAChRs (Katz and Miledi, 1972; Neher and Sakmann, 1976; Henderson et al., 1987) and $\alpha$, $\beta$, $\gamma$, and $\delta$ subunit RNAs in skeletal muscle (Evans et al., 1987; Goldman et al., 1988; Gundersen et al., 1993; Witzemann et al., 1989). However these analyses have generally been confined to the first month of muscle denervation. In the present experiments, we have assayed for nAChR RNAs and nAChR function during a period lasting up to 1 yr after denervation.

To prevent reinnervation of the muscle fiber, we used a denervation procedure that included, in addition to sectioning the sciatic nerve, ligating the proximal and distal nerve stump and implanting the proximal nerve stump into a nearby muscle. Previous experiments, employing silver staining of segments of the distal nerve and electrical stimulation of transected sciatic nerve, have shown that this denervation procedure prevents the return of nerve fibers for a period of time extending beyond 22 mo (Carlson and Faulkner, 1988).

RNase protection assays were used to determine the level of expression of nAChR subunit RNAs in muscle that had been denervated for up to 12 mo. Each assay included a probe for a particular nAChR subunit RNA and MCK. We determined that creatine kinase RNA did not change during 12 mo of muscle denervation by assaying the level of this RNA, normalized to total RNA used in

Figure 1. Long-term muscle denervation has little effect on MCK RNA levels. RNase protection assays were used to assay for creatine kinase RNA levels in muscle denervated for various lengths of time extending up to 1 yr. At the top of the graph is a representative RNase protection result. The graph represents quantitation of the MCK RNA levels as a function of the time the muscle remains denervated. MCK RNA (densitometry value) is normalized to total RNA ($\mu$g) applied to the gel. Values are the average of six experiments. RNA was isolated from both gastrocnemius and tibialis anterior muscles.
Figure 2. Adaptation of nAChR RNA levels to long-term muscle denervation. RNase protection assays were used to assay for embryonic-type nAChR RNA levels in gastrocnemius muscle denervated for various lengths of time extending up to 1 yr. At the top of each graph is a representative RNase protection result. The graph represents quantitation of the nAChR RNA level as a function of the time the muscle remains denervated. nAChR RNA is normalized to MCK RNA levels. Asterisks (*) indicate values were below detectable levels.

The transient nature of the increases in RNAs that encode subunits of the embryonic-type nAChR was in marked contrast to expression of the adult-type ε subunit RNA. Levels of ε subunit RNA were found to increase ninefold shortly after denervation and remained elevated throughout the ensuing 12 mo (Fig. 3). Note that in this particular experiment γ subunit RNA began to decrease between 0.3 and 1 mo after denervation, while in the experiment reported in Fig. 2 this RNA began to decrease after 1 mo of denervation. This difference in time course likely reflects the different muscles used in these two experiments. Experiments reported in Fig. 2 used RNA isolated from the gastrocnemius muscle, while experiments reported in Fig. 3 used RNA isolated from the tibialis anterior muscle.

Prolonged Muscle Denervation Leads to a Decreased Expression of Functional Embryonic-Type nAChRs

To determine if changes in nAChR subunit mRNAs were reflected in concomitant changes in the expression of functionally distinct receptors, single channel recordings were made from denervated fdb fibers isolated from the denervated muscle. Two functionally distinct classes of AChR were present in denervated fdb fibers: high conductance (77.8 ± 2.3 pS; n = 19), short open time (1.56 ± 0.17 ms; n = 20) adult-type nAChR (ε-containing) and the low conductance (49.9 ± 1.7 pS; n = 14), long open time (3.13 ± 0.36 ms; n = 15), embryonic-type nAChR (γ-containing) channel (Fig. 4). The relative percentages of ACh-induced opening attributed to these two distinct classes were determined in both junctional and extrajunctional regions of the muscle fiber (Table I). This analysis revealed a decreased expression of embryonic-type channels as the length of time of denervation increased beyond 1 mo in both junctional and extrajunctional membrane. By 4 mo of denervation, the majority of nAChR channel openings were of the adult-type. In addition, the level of ACh-induced channel activity was 300-fold lower in recordings
from extrajunctional versus junctional membrane from long-term denervated fibers, suggesting a reversal of denervation supersensitivity.

**Adult-type specific e-RNA and nACHRs Remain Localized to Discrete Regions of the Muscle Fiber Four Months After Denervation**

The adult-type specific e-RNA is localized to the neuromuscular junction in innervated and short-term denervated muscle. This localized expression appears to be mediated by the synaptic basal lamina (Goldman et al., 1991; Brenner et al., 1992; Jo and Burden, 1992). We were interested in determining if long-term denervation affected the distribution of this RNA. In situ hybridizations revealed a localized expression of this RNA in muscles denervated for up to 1 yr (Fig. 5 A). In addition, we continue to detect a localized, high level of synaptic expression of nACHR protein in muscles denervated for 4 mo as revealed by fluorescent α-bungarotoxin staining (Fig. 5 B). These results are consistent with continued expression of e-RNA at the old neuromuscular junction of long-term denervated muscle and with the higher levels of ACh-induced activity in junctional than in extrajunctional recordings.

**Long-Term Muscle Denervation Results in a Changing Pattern of MRF RNA Expression**

Since long-term muscle denervation resulted in reduced expression of embryonic-type nACHRs, and because the nACHR genes are known to be regulated by MRFs (Gilmour et al., 1991; Prody and Merlie, 1992; Berberich et al., 1993; Bessereau et al., 1994; Durr et al., 1994; Tang et al., 1994), we examined MRF expression in the long-term denervated muscles. All the MRFs analyzed were induced within 10 d after muscle denervation (Fig. 6). At 10 d after denervation myogenin and MRF4 were induced ~100- and 40-fold, respectively, while MyoD and myf-5 were induced ~7- and 17-fold, respectively. MyoD and myf-5 expression remains high for at least 7 mo after denervation. Myogenin and MRF4 do not maintain their high level of expression beyond 1 mo after denervation, and in this respect are similar to the expression of embryonic-type nACHR RNAs.

Finally, we examined the expression of Id RNA in the denervated muscles. We were surprised to find that this RNA has a relatively slow time course of induction following muscle denervation (Fig. 6). Peak levels of Id RNA were detected ~2 mo after denervation and these levels remained significantly above that found in innervated muscle for at least 7 mo after motor nerve section. The time period in which Id levels were maximal corresponded to the time when nACHR subunit RNA levels and levels of functional receptors were decreasing.

**Table I. Percentage of High-Conductance Openings in FDB Fibers Denervated In Vivo**

| Number of days denervated | Synaptic | Nonsynaptic |
|---------------------------|----------|-------------|
| 0                         | 97% (42) | 97% (10)    |
| 14-17                     | 8% (10)  | 2% (6)      |
| 19-21                     | 16% (13) | 7% (6)      |
| 120-150                   | 70% (10) | 65% (8)     |
| >210                      | 99% (3)  | 67% (6)     |

The values represent the percentage of total openings that were due to activation of the high conductance (e-containing) ACh receptor in synaptic and nonsynaptic recordings from flexor digitorum brevis (FDB) fibers denervated in vivo for the number of days indicated. Values in parentheses indicate the number of fibers examined. Recordings were made in the cell-attached configuration. [ACh] = 250 nM.

*Data are from Brehm and Kulberg (1987) and Brennan and Henderson (1993).

**Discussion**

Before the studies reported here, it has been presumed that embryonic-type nACHR RNA expression remained...
Figure 5. Localization of e-RNA and nAChRs to discrete regions of the 4-mo denervated muscle fiber. (A) In situ hybridization was used to identify e-RNA in tibialis anterior muscle fibers denervated for 4 mo. The white grains correspond to the distribution of e-RNA (similar results were obtained at 1 yr after denervation). (B) Distribution of nAChR in 4 mo denervated muscle fiber. High density of synaptic receptors indicated by rhodamine α-bungarotoxin fluorescence.

elevated in denervated muscle. This is clearly not the case. After 2 mo of denervation, the predominant forms of nAChR RNAs expressed are those which encode the adult-type receptor. These changes in RNA expression are reflected in the expression of functional nAChRs on the muscle's surface. With time after denervation greater than 1–2 mo, the relative percentage of ACh-induced openings attributed to the embryonic-type receptor decreases until at times greater than 210 d, the relative percentages of openings by the adult and embryonic forms of the nAChR at the synapse are indistinguishable from those of innervated fiber. In addition, the observation that the level of extrajunctional ACh-induced activity is quite low (suggesting that denervation supersensitivity has declined) is also consistent with a return to an "innervated condition" in long-term denervated fibers. One caveat is that the relative percentage of openings by embryonic type receptors in extrajunctional membrane (33%) does not completely return to levels characteristic of the innervated state (3%; Brehm and Kullberg, 1987; Brennan and Henderson, 1993). In addition, the overall level of ACh-induced activity at the junction in long-term denervated rat fdb fibers is approximately half that observed in innervated fibers (C. Brennan and L. Henderson, unpublished data), although this may reflect a lower overall capability of long-term denervated fibers to synthesize proteins, rather than a specific effect on nAChR expression.

Is it possible that these results derive from reinnervation of denervated muscle fibers? We do not believe this is the case for the following reasons: (a) a denervation procedure was used that had previously been documented to prevent the return of nerve fibers for a period of time extending beyond 22 mo (Carlson and Faulkner, 1988); (b) the muscle fibers retained, on an average, only 18% of their original mass consistent with their denervated state; (c) morphological studies showed a progressive worsening of the histological quality of the muscle fibers as the time of denervation increased (B. M. Carlson, unpublished observation); (d) MyoD and myf-5 RNAs, which are regulated by muscle activity, remained elevated in the long-term denervated muscles; (e) at the time of dissection of the muscles there was no observable sign of reinnervation, nor did the lower leg contract upon electrical stimulation of the proximal stump of the sciatic nerve; and (f) using the same denervation technique, Billington (1995) has shown that the maximum tetanic force generated by 4 mo and longer denervated EDL muscles drops to 0.3% of control and remains at that level. Therefore, we conclude that the results reported here do not reflect reinnervation of the denervated muscle fibers.

The consequences of expressing adult-type nAChRs in long-term denervated muscle are not known. However, it is interesting to note that the recovery of function of denervated muscle by grafting and reinnervation is ~100% of that of control muscle grafts if the muscle is grafted and reinnervated within the first two months of denervation. However, denervation lasting from 2–7 mo results in a progressive decrease in the ability of the muscle to recover function (B. M. Carlson, unpublished observation). It is possible that one component of this robust recovery of function after short-term denervation is the expression of embryonic-type nAChRs on the denervated muscles surface. Perhaps these receptors are involved in maintaining a permissive state for reinnervation. Alternatively, increased embryonic-type receptor expression may be a consequence of other events that are required for maintaining a muscle in a receptive state for reinnervation and maximal functional recovery. In this case the nAChR genes will serve as useful probes for characterizing the mechanisms leading to poor muscle recovery following long-term denervation.

To gain insight into potential mechanisms mediating the decreased expression of embryonic-type receptor genes during long-term denervation, we assayed for MRF RNA.
Regulation of myogenic factor RNA levels in short and long-term denervated muscle. RNase protection assays were used to determine the level of various myogenic factors in denervated tibialis anterior muscle. Representative RNase protection assays are shown in the upper left of the figure. Quantitation of these RNase protection assays is presented in the graphs. Muscle regulatory factor RNA levels are normalized to MCK RNA levels.

expression in these same muscle samples. We suspected that MRF expression participated in this regulation because: (a) these transcription factors are known to activate nAChR gene promoters in in vitro cell culture experiments (Piette et al., 1990; Gilmour et al., 1991; Prody and Merlie, 1992; Berberich et al., 1993; Durr et al., 1994); (b) myogenin knock-out mice fail to induce nAChR α and γ subunit RNAs (Hasty et al., 1993); and (c) mutations in particular E-box cis-acting sequences, that mediate binding of these factors to nAChR promoter DNA, affect nAChR promoter activity in vivo (Bessereau et al., 1994; Tang et al., 1994). RNase protection assays showed a complex pattern of MRF RNA expression over a 7-mo period of muscle denervation (Fig. 6). Both myogenin and MRF4 were induced shortly after muscle denervation. Consistent with previous reports (Witzemann and Sakmann, 1991; Dulcert et al., 1991; Eftimie et al., 1991; Voytik et al., 1993), we found myogenin was induced to a significantly higher level than any of the other MRFs. However, both myogenin and MRF4 induction were transient and return to a lower level by 2 mo after denervation.

MRFs have been proposed to participate in mediating nAChR gene induction following muscle denervation (Merlie et al., 1994; Tang et al., 1994; Su et al., 1995). Our data are consistent with this possibility and indicate that the decrease in nAChR RNAs beginning ~1-2 mo after denervation may result from lower levels of myogenin and/or MRF4 expression (Fig. 6). Interestingly, in denervated chick muscle, MRF4 is not observed to increase following muscle denervation (Neville et al., 1992), yet nAChR genes are induced. There is evidence to suggest that regulation of nAChR expression in chicks does not precisely parallel that observed in mammals and amphibians. For example, the developmental change in nAChR kinetics does not occur in chick (Schuetze, 1980), and there may be species-specific differences in mechanisms governing myogenic factor, as well as nAChR gene expression (Walke et al., 1994). However, if a mechanism is conserved between birds and mammals in initiating and maintaining nAChR gene induction after muscle denervation, one would predict that myogenin is a more likely candidate for mediating this effect. In support of this hypothesis, Hasty and colleagues (1993) have shown that developmental induction of nAChR α and γ subunit RNAs is prevented in myogenin knock-out mice. However, this study also showed that δ subunit RNA is developmentally induced in myogenin-deficient mice. These data suggest that denervation paradigms may not completely recapitulate early developmental changes in MRF expression, as has previously been reported (Witzemann and Sakmann, 1991). Conversely, different nAChR subunit genes may be differentially regulated by individual MRFs.

Figure 6: Regulation of myogenic factor RNA levels in short and long-term denervated muscle. RNase protection assays were used to determine the level of various myogenic factors in denervated tibialis anterior muscle. Representative RNase protection assays are shown in the upper left of the figure. Quantitation of these RNase protection assays is presented in the graphs. Muscle regulatory factor RNA levels are normalized to MCK RNA levels.

Most interesting is our observation that Id expression gradually increases during the first 2 months after denervation (Fig. 6). This time course is consistent with the hypothesis that Id is responsible for blocking MRF activity that leads to a decline in nAChR gene expression. The effect of Id could be manifested by combining with different MRFs and therefore affecting genes differentially regulated by these MRFs. In addition, Id overexpression has recently been correlated with muscle atrophy in adult transgenic mice (Gundersen and Merlie, 1994). Denervation-induced expression of Id may be contributing to muscle atrophy as well as influencing other events related to the ability of muscle to recover function following long-term denervation. Clearly Id expression is becoming maximal in the same time frame that denervated muscle loses its ability to recover function upon reinnervation. One caveat to these interpretations is that we are assuming that MRF RNA expression reflects MRF protein levels. Although this is generally true, it will ultimately be necessary to demonstrate this correlation by assaying for MRF protein.

In summary, long-term muscle denervation results in a changing pattern of nAChR and MRF gene expression that no longer resembles the embryonic state of developing muscle fibers. It is well known that adult, innervated muscle is usually refractory to further innervation (Elsberg, 1917; Jansen et al., 1978). The changes in gene expression we observe, which in many ways come to resemble that of the innervated state, may contribute to the poor...
recovery of function in long-term denervated muscle subject to reinnervation. It will be of interest to determine if interventions such as electrical stimulation of long-term denervated muscle will delay or prevent these detrimental changes in gene expression, allowing denervated muscle to retain its potential for functional recovery.

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