Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 362–366, January 1989
Neurobiology

Decrease in plasminogen activator correlates with synapse elimination during neonatal development of mouse skeletal muscle

(remodeling/basement membrane/degradation/proteases)

DANIEL HANTAÏ*, JASTI S. RAO, CHERYL KAHLER, AND BARRY W. FESTOFF

Neurobiology Research Laboratory, Veterans Administration Medical Center, Kansas City, MO 64128; and Department of Neurology, University of Kansas Medical Center, Kansas City, KS 66103

Communicated by Marshall Nirenberg, September 6, 1988

ABSTRACT **Previous studies have implicated proteases, acting extracellularly, in the mechanism of polyneuronal synapse elimination. Most studies have focused on mammalian, especially rodent, skeletal muscle, where retraction of subordinate nerve terminals occurs during a narrow time window 2–3 weeks after birth. To date no specific protease(s) has been detected that (*i*) coincides in time with maximal synapse elimination and (*ii*) is known to act extracellularly on specific extracellular matrix proteins. In previous studies of denervation in adult mouse muscle, rapid activation of urokinase-type plasminogen activator, a neutral serine protease, was detected. This enzyme, by activation of plasminogen to plasmin, specifically degrades matrix components such as fibronectin, type IV collagen, and laminin in muscle. We now present evidence for an initial increase and subsequent decrease in soluble urokinase-type PA—and, to a lesser extent, tissue PA—in developing muscle, suggesting postnatal developmental regulation of these enzymes during the period of maximal synapse elimination. Although considerably higher in specific activity, membrane-bound PA activity followed the wave of synapse elimination, possibly indicating a longer half-life of membrane-bound enzyme(s).**

Individual skeletal muscle fibers in newborn mammals are innervated at a single endplate by several motor axons. During the first few weeks of postnatal life this pattern of innervation, referred to as polyneuronal innervation, changes dramatically. The number of motor axons at an endplate rapidly decreases until each muscle fiber is innervated by only one axon (1–6). The mechanism involved in the elimination of polyneuronal innervation is still unknown, but some authors have raised the hypothesis that neuromuscular activity might stimulate the release of proteolytic enzymes at the endplate (7–9). These enzymes could then attack the attachment of nerve terminals, causing the withdrawal of all but the most resistant terminal from adhesion to postsynaptic membranes.

As to which enzyme(s) could be involved in this phenomenon, other authors have suggested, but not directly demonstrated, the action of a Ca^{2+}-activated protease (9, 10), but lysosomal (11) and, especially, membrane-bound (12) neutral proteases could be possible candidates. Since tissue development and stabilization in other systems have been associated with increased activity of serine proteases such as plasminogen activators (PAs; refs. 13–16), PAs might also play a role in the neuromuscular system. Previous studies indicated that PA was the predominant neutral protease secreted by cultured clonal murine skeletal muscle cells (17). More recent studies have shown that denervation is followed by a dramatic increase in adult mouse muscle PA activity (18). Denervation results in muscle dedifferentiation in mammals (19), so we asked whether changes in PA levels or activity might also have a role in the differentiation-related events that take place during regression of polyneuronal innervation and stabilization of mature synapses *in vivo*. Our present results demonstrate that high levels of PA are present in mouse skeletal muscle at birth but that PA activity decreases dramatically over the second week of neonatal life, paralleling the elimination of polyneuronal innervation. PA activity remains at low levels in adult muscle as previously described (18) after mature neuromuscular contacts have been established.

These variations in PA activity concern both urokinase-type PA (uPA), which was found to have a molecular mass of 48 kDa (18) similar to uPA of other mouse tissues (20), and tissue PA (tPA), with a molecular mass of 75 kDa (13). In this paper we correlate PA activity with histological data on elimination of polyneuronal innervation during the first month of life in mouse leg muscle and with the developmental regulation of choline acetyltransferase (ChoAcTase).

MATERIALS AND METHODS

Materials. Glu-plasminogen was kindly donated by O. Bertrand (Hôpital Beaujon, Clichy, France). tPA was a generous gift of E. Anglés-Cano (Institut National de la Santé et de la Recherche Médicale, U. 143, Hôpital de Bicêtre, Le Kremlin-Bicêtre, France). The plasmin-specific chromogenic substrate S-2251 (D-Val-Leu-Lys-*p*-nitroanilide), tPA stimulator [fibrin monomer (Fm)], and bovine fibrinogen were purchased from Kabi (Stockholm). Plasminogen-free human fibrinogen was from Helena Diagnostics (Beaumont, TX), ketamine (Ketalar) from Parke, Davis (Chicago), and acepromazine (Vétranquil) from Clin-Midy (Paris). Aprotinin (Trasylol) was a gift of F. Schaumann (AG Bayer, Munich). Purified human thrombin (for zymograms) was a generous gift of D. G. Ozin (Centre National de Transfusion Sanguine, Paris). Human urokinase was a gift of M. Faucher (Institut Choay, Paris).

Preparation of Extracts. Female BALB/c mice (1–40 days old; Charles River Breeding Laboratories) were used for all studies. After decapitation or cervical dislocation depending on age, hindlimb muscles were cleaned of blood vessels and connective tissue, blotted and weighed wet, and then homogenized (1:10, wt/vol) in 100 mM Tris·HCl/2 mM EDTA, pH 7.6, with a Polytron (Brinkman Instruments) tissue homogenizer, all at 4°C. Homogenates were centrifuged at 12,000 × *g* for 30 min. The supernatant was further centrifuged at 100,000 × *g* for 1 hr. The pellet (P_2) was resuspended

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PA, plasminogen activator; uPA, urokinase-type PA; tPA, tissue PA; Fm, fibrin monomer; ChoAcTase, choline acetyltransferase; AcCho, acetylcholine.

*To whom reprint requests should be addressed at: Biologie et Pathologie Neuromusculaires, Institut National de la Santé et de la Recherche Médicale, Unité 153, 17 rue du Fer-à-Moulin, 75005 Paris, France.

(1:5, wt/vol) in 100 mM Tris·HCl/2 mM EDTA/0.4% Triton X-100, pH 7.6. The supernatant (S_2) was used without dilution unless otherwise indicated. After all procedures, fractions were aliquoted, flash-frozen in liquid N_2, and stored at −80°C until used (<1 month).

Amidolytic Assay of PA Activity in Muscle. Activation of plasminogen to plasmin was followed by determination of plasmin activity on the synthetic substrate S-2251 (21, 22). To 50 μl of either muscle extract or purified diluted human uPA or tPA were added 50 μl of S-2251 (150 μg/ml) with or without Fm (130 μg/ml) and 50 μl of Glu-plasminogen (45 μg/ml), all diluted in 0.1 M sodium phosphate/10 mM EDTA/0.01% NaN_3/0.01% Triton X-100, pH 7.3. Incubation was at 37°C in flat-bottomed Microtiter plates (Immulon II, Dynatech Laboratories, Alexandria, VA). The release of *p*-nitroaniline from S-2251 was determined in each well by measuring A_{405} with a micro-ELISA autoreader (Titertek Multiscan, Flow Laboratories). Color development was linear for 6 hr after a 2-hr lag, or if expressed to the fourth power (22). Controls, including buffer alone and extracts without plasminogen, were performed in parallel wells. Generally, two or three replicate wells were included in each assay. Three separate developmental time courses were performed and the results represent the average, with deviations being <10%. Our current results are expressed in absorbance units after 4 hr of incubation at 37°C per mg of protein.

Fibrin Zymography. $NaDodSO_4$/10% PAGE was performed according to Laemmli (23) and was followed by fibrin plate zymography according to Granelli-Piperno and Reich (24). A modification we used was the binding of fibrinogen to GelBond (FMC Bioproducts, Rockland, ME) for casting the fibrin gels. After $NaDodSO_4$/PAGE and removal of $NaDodSO_4$ by washing the polyacrylamide gels in 2.5% Triton X-100 (24), the gels were transferred onto precast fibrin plates, incubated in a moist atmosphere at 37°C for 24 hr, and then stained with amido black. Photography of the fibrin plates was performed with transillumination.

ChoAcTase Assay. We used a radioenzymatic assay developed by Rand and Johnson (25), which involved trapping of the newly formed acetylcholine (AcCho) in the tetraphenylboron layer. A modification we made was that the enzymatic incubation was at 37°C for 2 hr. An adult mouse brain extract was used as standard in all assays. Activity, from duplicate tubes of three separate experiments, is expressed as pmol of AcCho formed per mg of protein per min.

Combined Silver/Cholinesterase Stain for Neuromuscular Junction Innervation. A modification of the method of Hopkins *et al.* (26) was used to determine the degree of multineuronal innervation of mouse sciatic-innervated leg muscles in the postnatal period. After muscles were dissected in Hepes-buffered Ringer solution, they were fixed overnight in 4% paraformaldehyde/0.2 M phosphate buffer, pH 7.4, at 4°C. After rinsing in H_2O, muscles were processed for silver/cholinesterase stain (26); the first dehydration and rehydration step before cholinesterase staining was omitted in order not to prevent visualization of endplate cholinesterase.

RESULTS

Weight Growth and Protein Measurement. Fig. 1*A* shows the expected, almost linear increase in bulk of removed leg muscles with increasing time postnatally. Fig. 1*B* shows the linear increase in noncollagenous proteins postnatally in fraction S_2. Of interest, there was no notable change in fraction P_2.

PA Amidolytic Activity. As measured with a sensitive amidolytic assay using a synthetic peptidyl chromogenic substrate (S-2251) specific for plasmin (21), activity was entirely dependent upon the addition of highly purified

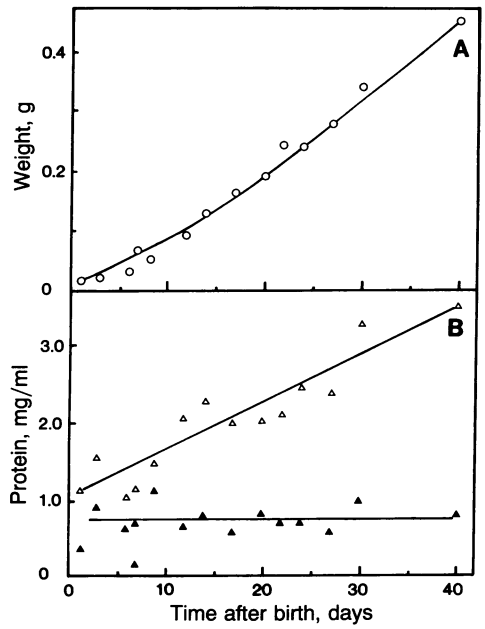

FIG. 1. Change in weight (bulk) and noncollagenous protein of mouse leg muscle (sciatic-innervated) from day 1 to day 40 after birth. (*A*) Variation in weight of leg muscles (per animal). (*B*) Variation in noncollagenous proteins in cytosol (S_2, △) and membrane (P_2, ▲) of leg muscle extracts after birth. Protein was measured by the method of Bradford (27) with duplicates of three separate experiments. Replicates were within 5% of one another.

human plasminogen, as shown earlier for adult muscle (18). Plasminogen-independent activity was barely above the zero value even after 6 hr of incubation (data not shown). Using the ^{125}I-labeled fibrin plate assay (28), previous studies with neutral proteases secreted from G8-1 clonal mouse muscle cells (17) and rat muscle in organ culture (B.W.F. and J.S.R., unpublished work) had indicated considerable PA activity, but plasminogen-independent activity was also detected, especially in muscle homogenates. Moreover, in present as well as in previous experiments (18), the addition of Fm (21, 22) was used to distinguish fibrin-dependent (tPA) and fibrin-independent (uPA) PA activities in muscle extracts.

As previously demonstrated (18), amidolysis of S-2251 was linearly dependent on the concentration of plasminogen from 5 to 40 μg/ml, consistent with other reports (21, 22). Amidolysis was also critically dependent on the pH of the medium, with maximal activity at pH 7.6, and was completely inhibited by aprotinin at 5 kallikrein inhibitor units/ml (data not shown). This study indicated, as did a previous one (18), that amidolytic activity in mouse muscle extracts under these conditions represented only PA.

Evolution of Muscle Plasminogen-Dependent Amidolytic Activity During Neonatal Development. PA activity of S_2 muscle extracts measured in the absence of Fm at day 1 was 6-fold higher than the same activity at day 40 (Fig. 2*A*). PA activity increased slightly from day 1 until day 7 postnatally and then decreased dramatically from 7 to 15 days. After this time, a more gradual decline to the low levels of PA activity already described (18) for adult mouse muscle extracts was found. PA activity measured in the presence of Fm was slightly greater than PA activity in the absence of Fm but paralleled the latter, here again, to reach levels already described for adult muscle (18).

The specific activities of PA in membrane-bound, Triton-extractable activities of P_2 muscle extracts (Fig. 2*B*) were considerably higher than those of aqueous S_2 extracts. PA activity measured in the absence of Fm was high after birth, gradually decreased until day 16, and then decreased dra-

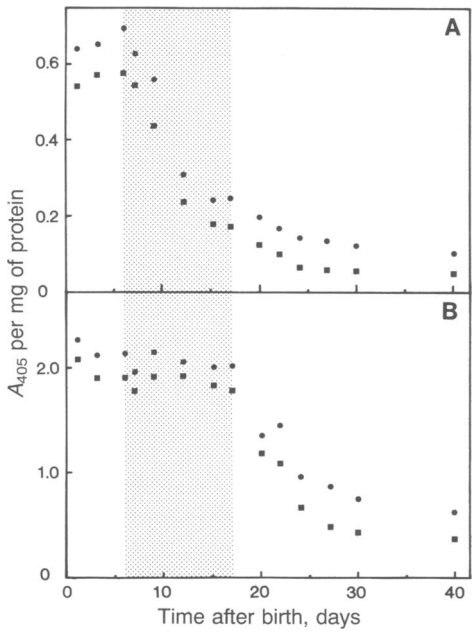

FIG. 2. PA activity in muscle cytosol and membranes during postnatal development. (*A*) PA activity without Fm (■) and with Fm (●) in 100,000 × *g* supernatants of mouse leg muscle extracts. (*B*) PA activity in Triton-extracted pellets from 100,000 × *g* centrifugation of muscle extracts. Activity is expressed as A_{405} per mg of protein and represents duplicate wells of three separate experiments. Replicates agreed within 5–10%. The vertical stippled column represents the range of time for change from polyneuronal to mononeuronal innervation of muscle fibers in rodent leg muscles (3, 5, 7, 29).

matically until day 24, with little change, thereafter, in adult muscle. PA activity measured in the presence of Fm was slightly higher, paralleling the evolution of PA activity in the absence of Fm.

Fibrin Zymography of Muscle Extracts. We used fibrin zymography to more specifically identify the type of PA that was regulated during mouse muscle development postnatally. Fig. 3 shows a typical fibrin zymogram of S_2 and P_2 extracts obtained at various times after birth. This technique enabled us to extend our biochemical results and further offered the possibility of detecting the presence of PA–inhibitor complexes (30). Such complexes have been readily detected with clonal muscle cells (unpublished data), as seen in Fig. 3 (lane 1). The amido black-stained fibrin zymogram shows P_2 and S_2 muscle extracts (at three different times after birth) along with G8-1 cell conditioned medium (lane 1) and purified human uPA (lane 2) showing the high and low molecular mass subunits. Lysis zones at 48 kDa were seen with mouse muscle P_2 (lanes 3–5) and S_2 (lanes 6–8) extracts at all times after birth. They were intense at 6 days and all but disappeared at 27 days postnatally. The major band of fibrinolytic activity at ≈48 kDa migrated just between the position of high (≈55 kDa) and low (≈33 kDa) molecular mass human uPA (lane 2) and comigrated with a major zone in G8-1 conditioned medium (lane 1). A smaller band, evident in the early P_2 muscle extracts (lanes 3 and 4), at 75 kDa comigrated with human tPA (data not shown). These fibrinolytic enzymes are identical to mouse uPA and tPA, respectively, previously identified in denervated muscle (18). Neither the P_2 nor the S_2 muscle extracts showed a high molecular mass lytic zone comparable to activity arising from a complex of tPA and tPA inhibitor at 100–110 kDa (30) or of uPA and uPA inhibitor at 80–95 kDa (ref. 13; unpublished data). G8-1 clonal myoblast conditioned medium contained ≈48-kDa uPA, ≈75-kDa tPA, and additional plasminogen-dependent fibrinolytic zones at ≈110 and ≈160 kDa (lane 1), consistent with tPA–inhibitor and tPA–α_2-macroglobulin complexes, respectively (30, 31).

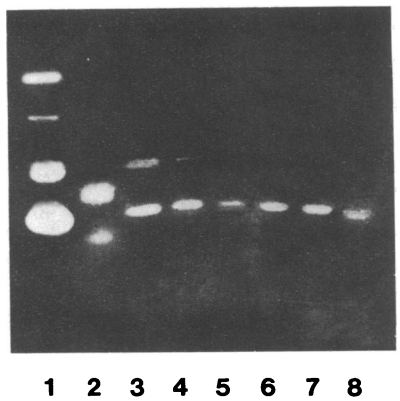

FIG. 3. Zymography of S_2 and P_2 extracts of mouse muscle removed at 6, 12, and 27 days after birth. Lane 1, partially purified and concentrated G8-1 myoblast conditioned medium; lane 2, human uPA (33 and 55 kDa); lanes 3–5, P_2 fractions at 6, 12, and 27 days, respectively; lanes 6–8, S_2 fractions at 6, 12, and 27 days, respectively.

ChoAcTase Activity. Using a radioenzymatic assay, we measured ChoAcTase activity in soluble (S_2) extracts of mouse leg muscle from birth to 40 days (Fig. 4). There was a steady increase in ChoAcTase activity in these muscle extracts from birth to day 17. After day 20, CAT activity decreased, reaching adult values by about day 28. These results are in agreement with results obtained by others (19).

Histological Appearance of Endplate During Postnatal Development. In animals up to 6 days of age many endplates were innervated by two or more axons (Fig. 5*A*), whereas after 12 days almost all endplates were innervated by only one axon (Fig. 5*B*). These results confirm previous electrophysiologic studies of mouse extensor digitorum longus and soleus muscles (7) and electrophysiologic and morphologic studies of neonatal rat soleus muscle (3–5).

DISCUSSION

Our present results indicate that at birth, polyinnervated mouse leg muscle possesses a high level of PA activity. From both S-2251 assays, using addition of Fm to distinguish uPA from tPA (21, 22), and from zymography (24), both types of PA are present in neonatal muscle. After 6 days this activity decreases until 12 days and reaches a low but detectable level, as described in a previous study with adult mouse muscle (18). Although less obvious in the amidolytic assays

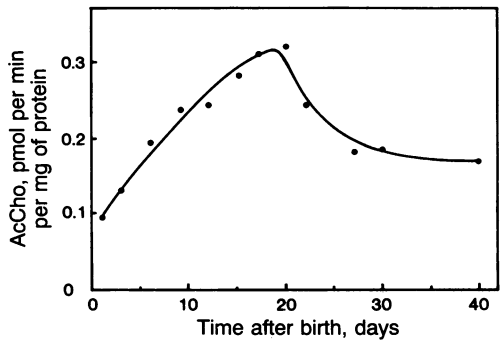

FIG. 4. ChoAcTase activity in S_2 fractions of neonatal mouse muscle from birth to day 40. Data represent averages of duplicates of three separate experiments. Replicates were within 5% of one another.

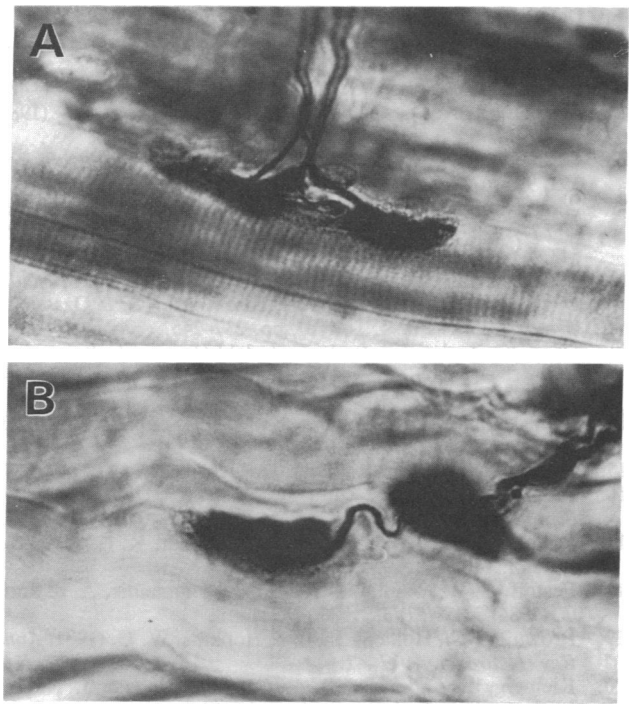

FIG. 5. Histological appearance of leg muscle endplate innervation at day 6 (*A*) and day 12 (*B*). [Combined silver/cholinesterase stain (25), modified as described in *Materials and Methods*; ×140.]

(Fig. 2), tPA is first to decrease, followed by uPA, as seen with zymography (Fig. 3). This is just the reverse of what is found with adult denervated muscle (18).

In addition, we show here that differences exist between soluble (Fig. 2*A*) and membrane-associated (Fig. 2*B*) fractions in respect to regulation of PA activity during the postnatal time course for the first month. Membrane-bound activity is 3-fold higher and stays high until day 20 and then declines. Soluble PA activity initially increases, drops precipitously from day 6 or 7 until day 15, and then gradually reaches adult levels. There is, however, no shift between cellular compartments during development. This result is consistent with data obtained with adult mouse muscle denervated by nerve crush (D.H., J.S.R., and B.W.F., unpublished work). This apparent disparate regulation of soluble and membrane-bound muscle uPA during the neonatal period should be commented upon. It is possible, but by no means certain, that the increased membrane-bound activity represents uPA bound to specific membrane receptors where it is still active (32, 33) and unavailable to inhibition by protease nexin I (32). The soluble uPA, whose activity is one-third that of the membrane-associated uPA and which peaks at 6 days and rapidly declines by day 15, may be affected by a number of factors, including transcription of mRNA, hormonal stimulation, and production and/or release of protease nexin I, just to mention a few.

Neonatal PA activity diminishes by a factor of almost 5 during the time between birth and the adult state. As mentioned above, we also studied the dependence of PA activity on fibrin. In the absence of Fm, there was a consistent decrease in PA activity in both soluble (Fig. 2*A*) and membrane-associated (Fig. 2*B*) compartments. In the presence of Fm, the PA activity was slightly higher and paralleled the decrease of PA activity in the absence of Fm, indicating a small additional PA activity that was dependent on fibrin. When we subtracted PA activity in the absence of Fm from PA activity in the presence of Fm, which should give the tPA activity more accurately, no significant variations in this PA activity dependent on Fm were observed (data not shown).

Because fibrin is required for tPA-induced plasminogen activation (13, 21, 22), these results were compared with those obtained by the fibrin zymography technique (30) by evaluating the intensity of the fibrin lysis area as well as the molecular mass of the activator. With this technique, a prominent lytic band at 48 kDa was seen at birth and at 6 days but decreased steadily thereafter (Fig. 3, lanes 3–8). This decrease over time was found in both soluble (lanes 6–8) and membrane-associated (lanes 3–5) fractions. The PA migrating at 75 kDa was present only in early neonatal (6 days) life and then only in fraction P_2 (lane 3). The molecular masses of these mouse muscle PAs were consistent with previous results in adult mouse muscle (18) and correlated well with uPA and tPA from mouse sarcoma (34), antibodies to which identified this enzyme in a variety of mouse tissues (35). The 75-kDa PA in skeletal muscle fractions correlates well with human and mouse tPA (13, 34, 35). The molecular masses for uPA (48 kDa) and tPA (75 kDa) also correlated with the major plasminogen-dependent fibrinolytic enzymes secreted by G8-1 clonal mouse muscle cells (unpublished work and Fig. 3, lane 1).

The ChoAcTase assays are helpful in following the degree of cholinergic maturation of terminals and endplates during this immediate postnatal period. Our results are in agreement with studies of miniature endplate potential frequency and mean quantal content of AcCho, both of which remain at very low levels in rodent muscle until 2–3 weeks after birth (19). Others have also measured ChoAcTase activity during muscle development and found, as we did, that peak specific activity occurs during the time of maximal synapse elimination. It is possible that AcCho release affects release of PAs from muscle fibers as proposed by Vrbová and colleagues (7, 9, 10). Such a mechanism is similar to results obtained with activated macrophages, where AcCho promotes, while epinephrine and isoproterenol inhibit, release of enzymes, including uPA (13).

Our histological experiments, with double staining of both the endplates and the nerve terminals, showed that in mouse skeletal muscle, endplates undergo a form of differentiation and remodeling during the first days of life, in agreement with the histological findings of others in rat muscle (29). Whereas at birth each muscle fiber is innervated by several neurons, by day 10 after birth almost every muscle fiber is innervated by only one nerve terminal. Dangain and Vrbová (37) described in the mouse extensor digitorum longus muscle a time course of appearance of single innervation that follows closely with our data concerning the time course of reduction in soluble PA activity in skeletal muscle. Subsequently, a low level of PA activity remains detectable throughout life, perhaps to effect turnover of basement membrane macromolecules. In addition, the timing of synapse elimination is not synchronous in any one animal but depends upon which muscle is studied (19, 38, 39). These findings argue against a general, systemic influence and suggest that local changes are more important and critical.

In this regard, denervation (18) and, more specifically, nerve crush (D.H., J.S.R., and B.W.F., unpublished data) result in an up- rather than a down-regulation of local muscle PA activity. Endogenous inhibitors and potentiators of PA are also present in intact and denervated mouse muscle, respectively (40). Therefore, modulation of PA action locally is likely to occur. Protease nexin I, an inhibitor previously identified (41) and showing developmental regulation during myogenesis (42) in G8-1 mouse muscle cells, would be a candidate for regulating PA activity in muscle. Our recent demonstration that protease nexin I is localized at adult mouse neuromuscular junctions (ref. 36; B.W.F., D.H., and J.S.R., unpublished data) takes on greater significance. This

locally regulated system would then be able to influence multiple nerve endings and determine their future removal. The relative concentration of protease nexin I delivered by muscle fibers to synaptic regions would be consistent with target-derived factor(s) diffusing and then anchoring to basal lamina sites (1–6, 19). In the same way, mechanisms for both the establishment of polyneuronal innervation and its subsequent removal are known to persist into adulthood (43). One possible mechanism of action in these attachment/disattachment phenomena would be through the degradation, by locally generated plasmin, of synaptic basement membrane components. We recently showed that muscle fiber basement membrane-associated fibronectin and type IV collagen, and to a lesser extent, laminin, are exquisitely sensitive to the plasmin generated locally by increased uPA in denervated muscle (44). The events observed after muscle denervation and degeneration would then represent a mirror image of those that we have observed in developing skeletal muscle, where the alteration in PA activity parallels neuromuscular differentiation after birth in the present study.

We have focused our discussion on the significance of the change in PA levels and type in the postnatal period during the time of synapse elimination. However, remodeling of the neuromuscular synapse also occurs prior to birth. The acquisition of a separate basement membrane by each primary and secondary myofiber may also involve changes in levels and activities of PAs (6). Likewise, other prenatal remodeling situations such as the formation of postsynaptic folds, ramifications of filo- and lamellopodia of nerve terminals within the junction, and growth of the entire neuromuscular apparatus (6) may also involve the PAs. Since PA activity is quite high at birth it will be important to determine PA levels during prenatal neuromuscular development, as well.

Would these possibilities become more probable if it were known with assurance whether plasminogen is able to enter the synaptic cleft from the circulation? There are presently no data available, but recent studies by Oldfors and Fardeau (45) show that ferritin, a 12-nm-diameter particle of about 450 kDa, is able to pass the basement membrane barrier of the neuromuscular junction. The critical elements of the PA/plasmin system (i.e., plasminogen and uPA) at the neuromuscular junction might then be expected to persist into adulthood, thus allowing for a continuous slow turnover of one or more synaptic basement membrane components. It is possible that alteration in levels of this multicomponent system might take part in the mechanisms underlying the pathogenesis of synaptic abnormalities, such as occurs in amyotrophic lateral sclerosis, as previously suggested (46).

We are grateful to Dr. Brigitte Blondet for her expertise in silver/cholinesterase staining for neuromuscular junction innervation and gratefully acknowledge the help of Mlle Sylvie Le Bihan and Ms. Joyce Capps in preparation of the manuscript. Support for these studies was partially provided to D.H. by the Association des Myopathes de France and the Fondation Philippe; to J.S.R. by the Amyotrophic Lateral Sclerosis (ALS) Association; and to B.W.F. by the Charles Betzelberger ALS Research Fund, the Keith Worthington ALS Research Center, The Myasthenia Gravis Foundation (Greater Kansas City Chapter), the Muscular Dystrophy Association, and the Medical Research Service of the Veterans Administration.

1. Redfern, P. A. (1970) *J. Physiol. (London)* **209,** 701–709.
2. Bagust, J., Lewis, D. M. & Westerman, R. A. (1973) *J. Physiol. (London)* **229,** 241–255.
3. Brown, M. C., Jansen, J. K. S. & Van Essen, D. (1976) *J. Physiol. (London)* **261,** 387–422.
4. Van Essen, D. C. (1982) in *Neuronal Development*, ed. Spitzer, N. C. (Plenum, New York).
5. Hopkins, W. G. & Brown, M. C. (1984) in *Synaptic Plasticity*, ed. Cotman, C. W. (Guilford, New York), pp. 169–199.
6. Dennis, M. J. (1981) *Annu. Rev. Neurosci.* **4,** 43–68.
7. O'Brien, R. A. D., Östberg, A. J. C. & Vrbová, G. (1978) *J. Physiol. (London)* **282,** 571–582.
8. Poberai, M. & Savay, G. (1976) *Acta Histochem.* **57,** 44–48.
9. O'Brien, R. A. D., Östberg, A. J. C. & Vrbová, G. (1980) *Neuroscience* **5,** 1367–1379.
10. Connold, A. L., Evers, J. V. & Vrbová, G. (1986) *Brain Res.* **393,** 99–107.
11. Pennington, R. J. T. (1977) in *Proteinases in Mammalian Cells and Tissues*, ed. Barret, A. J. (Elsevier, Amsterdam), pp. 515–543.
12. Takahasi, K., Ishihara, Y. & Sogawa, K. (1983) in *Muscular Dystrophy: Biomedical Aspects*, eds. Ebashi, S. & Ozawa, E. (Jpn. Sci. Soc. Press, Tokyo), pp. 237–245.
13. Danø, K., Andreasen, P. A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L. S. & Skriver, L. (1985) *Adv. Cancer Res.* **44,** 139–266.
14. Valinsky, J. E. & Le Douarin, N. M. (1985) *EMBO J.* **4,** 1403–1406.
15. Soreq, H. & Miskin, R. (1983) *Dev. Brain Res.* **11,** 149–158.
16. Soreq, H., Miskin, R., Zutra, A. & Littauer, U. Z. (1983) *Dev. Brain Res.* **7,** 257–269.
17. Festoff, B. W., Patterson, M. R., Ronstedt, K. (1982) *J. Cell Physiol.* **110,** 190–195.
18. Festoff, B. W., Hantaï, D., Soria, J., Thomaïdis, A. & Soria, C. (1986) *J. Cell Biol.* **103,** 1415–1421.
19. Brown, M. C., Holland, R. L. & Hopkins, W. G. (1981) *Annu. Rev. Neurosci.* **4,** 17–42.
20. Kielberg, V., Andreasen, P. A., Grøndahl-Hansen, J., Nielsen, L. S., Skriver, L. & Danø, K. (1985) *FEBS Lett.* **182,** 441–445.
21. Rånby, M., Norrman, B. & Wallén, P. (1982) *Thromb. Res.* **27,** 743–748.
22. Weinberg, J. B., Hobbs, M. M. & Pizzo, S. V. (1984) *J. Immunol. Methods* 75, 289–294.
23. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
24. Granelli-Piperno, A. & Reich, E. (1978) *J. Exp. Med.* **148,** 223–234.
25. Rand, J. B. & Johnson, C. D. (1981) *Anal. Biochem.* **116,** 361–374.
26. Hopkins, W. G., Brown, M. C. & Keynes, R. J. (1985) *J. Neurocytol.* **14,** 525–540.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
28. Unkeless, J. C., Gordon, S. & Reich, E. (1974) *J. Exp. Med.* **139,** 834–850.
29. Korneliussen, H. & Jansen, J. K. S. (1976) *J. Neurocytol.* **5,** 591–604.
30. Levin, E. G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6804–6808.
31. Brower, M. S., Harpel, P. C., Hayes, M. B. & Fenton, J. W. (1979) *Blood* **54,** Suppl. 1, 272a (abstr.).
32. Bajpai, A. & Baker, J. B. (1985) *Biochem. Biophys. Res. Commun.* **133,** 994–1000.
33. Vassalli, J. D., Baccino, D. & Belin, D. (1985) *J. Cell Biol.* **100,** 86–92.
34. Danø, K., Nielsen, L. S., Moller, V. & Engelhardt, M. (1980) *Biochim. Biophys. Acta* **630,** 146–151.
35. Larsson, L. I., Skriver, L., Nielsen, L. S., Grøndahl-Hansen, J., Kristensen, P. & Danø, K. (1984) *J. Cell Biol.* **98,** 894–903.
36. Hantaï, D., Rao, J. S. & Festoff, B. W. (1988) *Rev. Neurol. (Paris)* **144,** 680–687.
37. Dangain, J. & Vrbová, G. (1983) *J. Physiol. (London)* **342,** 267–275.
38. Dennis, M. J., Ziskind-Conhaim, L. & Harris, A. J. (1980) *Dev. Biol.* **81,** 266–279.
39. Carry, M. R., Morita, M. & Nornes, H. O. (1983) *Anat. Rec.* **207,** 473–485.
40. Festoff, B. W., Hantaï, D., Soria, C., Soria, J. & Fardeau, M. (1986) in *Molecular Aspects of Neurobiology*, eds. Levi-Montalcini, R., Calissano, P., Kandel, E. R. & Maggi, A. (Springer, Berlin), pp. 162–166.
41. Eaton, D. & Baker, J. B. (1983) *J. Cell Physiol.* **117,** 175–182.
42. Festoff, B. W., Eaton, D., Beach, R. L. & Baker, J. B. (1981) *J. Cell Biol.* **91,** 43a (abstr.).
43. McArdle, J. J. (1975) *Exp. Neurol.* **49,** 629–638.
44. Hantaï, D. & Festoff, B. W. (1987) *Exp. Neurol.* **95,** 44–55.
45. Oldfors, A. & Fardeau, M. (1983) *Neuropathol. Applied Neurobiol.* **9,** 419–432.
46. Festoff, B. W. (1980) *Med. Hypotheses* **6,** 121–132.