Neural Regulation of Muscle Acetylcholine Receptor ϵ- and α-Subunit Gene Promoters in Transgenic Mice

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Abstract. The effects of denervation were investigated in mice with transgenes containing promoter elements from the muscle acetylcholine receptor ϵ- and α-subunit genes. The promoter sequences were coupled to a nuclear localization signal–β-galactosidase fusion gene (*nlacZ*) as a reporter. While many postsynaptic specializations form in the embryo, expression of the ϵ subunit is induced during the first two postnatal weeks. When muscles were denervated at birth, before the onset of ϵ expression, ϵnlacZ still appeared at the former synaptic sites on schedule. This result suggests that the nerve leaves a localized "trace" in the muscle that can continue to regulate transcription. An additional finding was that ϵ*nlacZ* expression was much stronger in denervated than in intact muscles. This suggests that the ϵ promoter is similar to the other subunits in containing elements that are activated on cessation of neural activity. However, even after denervation, ϵ*nlacZ* expression was always confined to the synaptic region whereas α*nlacZ* expression increased in nuclei along the entire length of the fiber. This suggests that while the ϵ gene is similar in its activity dependence to other subunit genes, it is unique in that local nerve-derived signals are essential for its expression. Consequently, inactivity enhances ϵ expression only in synaptic nuclei where such signals are present, but enhances expression throughout the muscle fiber. Truncations and an internal deletion of the ϵ promoter indicate that *cis*-elements essential for the response to synaptic signals are contained within 280 bp of the transcription start site. In contrast to these results in young animals, denervation in older animals leads to an unexpected reduction in nlacZ activity. However, mRNA measurements indicated that transgene expression was increased in these animals. This discordance between *nlacZ* mRNA and enzyme activity, demonstrates a previously unknown limitation of *nlacZ* as a reporter gene in transgenic animals.

MOTOR neurons not only evoke contractions in muscles, but also control the expression of a broad range of muscle proteins including many crucial for mechanical activity and synaptic transmission (for example see Gorza et al., 1988; Gundersen et al., 1988; Lømo and Gundersen, 1988). This type of neural regulation of muscle phenotype is often referred to as neurotrophic control. Since Heidenhain's observation in 1883 that muscles become supersensitive to nicotine after denervation, it has become clear that the nicotinic acetylcholine receptor (AChR)[1] is particularly sensitive to neurotrophic control. Our current view is that AChRs are present throughout the fetal myotube, and that these receptors have a low conductance, long channel open time, and the subunit composition $\alpha_2\beta\gamma\delta$. In contrast, adult animals have AChRs in high concentrations only at the synapse, and these channels have high conductance, short open time, and the subunit composition $\alpha_2\beta\epsilon\delta$. After denervation, AChR expression is increased, receptors reappear along the entire length of the muscle fiber, and the vast majority of these receptors are of the fetal type. Formation and maintenance of a high density of the mature type of AChRs exclusively at synaptic sites are thought to be mediated in part by the release of chemical substances from the nerve. Independently, nerve-evoked electrical activity suppresses γ-subunit expression everywhere in the muscle, and α-, β-, and δ-subunit expression in nonsynaptic areas. Both local synaptic control and activity-dependent control seem to be mediated, at least in part, through transcriptional regulation of the AChR subunit genes (for reviews see Schuetze and Role, 1987; Changeux, 1991; Hall and Sanes, 1993).

Indirect evidence for selective regulation of AChR genes near synaptic sites has been provided by the observation that high levels of AChR mRNA accumulate near the synapse (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989; Brenner et al., 1990). Because muscle fibers are multinucleated, these results raise the possibility

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; EDL, extensor digitorum longus; CAT, chloramphenicol acetyl transferase.

that the few synapse-associated nuclei differ in their transcriptional activity from the majority of nuclei, which occupy extrasynaptic areas. In fact, experiments with transgenic mice have indicated that high levels of ε- and δ-subunit transcription are confined to synaptic nuclei in normally innervated muscles (Sanes et al., 1991; Simon et al., 1992). Similar experiments with the α-subunit promoter have been less conclusive (Klarsfeld et al., 1991; Sanes et al., 1991), but it seems likely that this subunit is selectively transcribed at the synapse as well.

In contrast to the general agreement that AChR genes are selectively transcribed at synapses, the role of the nerve in establishing this transcription has been less clear. The ε subunit is particularly suitable for addressing the role of the nerve, since it is induced after birth, unlike many other postsynaptic specializations, which form during embryogenesis (Brenner et al., 1990; Martinou et al., 1991; Sanes et al., 1991). Schuetze and Vicini (1984), using noise analysis, concluded that neonatal denervation prevented or delayed the switch from embryonic to adult AChR channels in the rat. Similarly, Martinou and Merlie (1991) analyzed RNA in mouse muscle extracts, and found that the increase in ε mRNA that normally occurs during the first two postnatal weeks could be prevented by neonatal denervation. These results suggested that the presence of the nerve is necessary for ε induction. In contrast, Brenner et al. (1990) showed accumulation of ε mRNA at synaptic nuclei in the rat even if the nerve was transected at birth, suggesting that nerve presence is not necessary for ε induction. To further test the importance whether the nerve must be continuously present for induction of ε, we have now performed neonatal denervations in transgenic mice with 5′ flanking sequences from the ε gene coupled to a nuclear localization signal–β-galactosidase fusion gene (*nlacZ*) as a reporter (*εnlacZ*). Synaptic expression of *εnlacZ* was neither prevented nor inhibited by neonatal denervation in these mice, indicating that ε-promoter elements can be induced in synaptic nuclei even after the nerve has been removed. Thus, previous nerve contact seems to be sufficient to stably modify the synaptic site in a way that permits subsequent local gene induction.

The increase in extrasynaptic AChR levels that occurs after denervation is due largely to increased transcription. Thus, it has been observed that α-, β-, γ-, and δ-subunit mRNA levels increase upon denervation (Merlie et al., 1984; Evans et al., 1987), and increased transcription has been demonstrated directly for the α, δ, and γ subunits by nuclear run-on experiments (Tsay and Schmidt, 1989). This induction seems to be caused by the removal of nerve-evoked electrical activity since it can be mimicked by pharmacological paralysis and prevented by electrical stimulation (Goldman et al., 1988). Experiments with transgenic mice have shown that sensitivity to denervation can be conferred by ~830 and ~1850 bp of 5′ flanking sequences from the α and δ subunits, respectively (Merlie and Kornhauser, 1989; Simon et al., 1992). In contrast, measurements of ε mRNA after denervation have shown small and variable effects (Witzemann et al., 1987, 1989, 1991; Martinou et al., 1991). Moreover, even after denervation, ε-subunit mRNA is detected in substantial amounts only near the synaptic site. The apparent lack of a clear denervation effect on ε expression, has suggested that this restriction to the synaptic region is due to an inability of the ε gene to respond to the activity-dependent signals which turn on extrasynaptic expression of the other subunit genes. We report here that the level of *εnlacZ* expression was dramatically increased by denervation in young transgenic mice, but that expression remained confined to the synaptic region even after being strongly increased. In contrast, when *nlacZ* expression was regulated by an α-promoter element (*αnlacZ*), denervation in young mice led to increased expression in nuclei along the whole length of the muscle. Thus, we hypothesize that the synaptic localization of the ε subunit is not caused by an inability to respond to decreased electrical activity, but rather by an absolute requirement for local synaptic signals.

Finally, we report that paradoxical observation that denervation of adult animals often led to a reduction in nlacZ activity. We show that this was not due to an aberrant decrease in transgene transcription, but rather reflected an effect at a posttranscriptional level. This posttranscriptional modulation might limit the usefulness of *nlacZ* in certain experiments, and should be taken into consideration when interpreting results obtained with this widely used reporter.

Materials and Methods

Fusion Genes

The transgenes used in this study are diagramed in Fig. 1. The structures of *α829nlacZ* and *ε3500nlacZ* have been described previously (Sanes et al., 1991). Briefly, to make *α829nlacZ*, the 5′ flanking sequence of the chicken AChR α-subunit gene (Klarsfeld et al., 1987) consisting of the sequence from −829 to +19 bp relative to the transcriptional start site was placed upstream of a reporter gene, *lacZ*. The *lacZ* gene product, the enzyme β-galactosidase, generates a blue precipitate upon histochemical staining using X-gel as the substrate (see Sanes et al., 1991 for details). The *lacZ* gene had been modified by adding coding sequence for a nuclear transport signal from the SV-40 T-antigen at the amino terminus (Lanford et al., 1988). This nuclear-lacZ (nlacZ) protein is targeted to the nucleus (Mercer et al., 1991). *ε3500nlacZ* was made by inserting the mouse AChR ε-subunit gene sequence extending from nucleotide ~3500 to +82 upstream of *nlacZ*. *ε830nlacZ* was made by cutting *ε3500nlacZ* with PstI which truncates the ε promoter at nucleotide −830 bp relative to the cap site. An *εnlacZ*-construct with an internal deletion was made by cutting ε3500nlacZ with NsiI (unique sites at nucleotides −280 and −1318) and then re-ligating the remaining promoter fragments. This construct, called *εIDnlacZ*, is identical to *ε3500nlacZ* except that it lacks the fragment between nucleotides −280 and −1318. *ε280nlacZ* was made by truncating *ε3500nlacZ* with NsiI at nucleotide −280 (Sunyer, T., and J. P. Merlie, 1993). The promoterless *nlacZ* construct was prepared by using the poly linker Acc I site located between the ε promoter and the nlacZ cassette.

Transgenic Mice

DNA was linearized, and the fusion genes were inserted into the genome of mice by conventional transgenic techniques (Hogan et al., 1986), with modifications described previously (Gundersen et al., 1993). One line (α-16) was established with the *α829nlacZ*-construct. Two line (ε-29, and ε-51) were investigated with *ε3500nlacZ*. Embryos for DNA injection were derived from matings between B6CBA F1 hybrids. Subsequent generations were bred by back crossing to B6CBA F1 hybrids. Thus, the transgene appears on a somewhat variable hybrid genetic background. Mice with *ε280nlacZ*, *ε830nlacZ*, and *εIDnlacZ* transgenes were in most cases analyzed in the F_0 generation (see Results). Transgene positive animals were identified by a polymerase chain reaction assay of DNA from tail tissue digests (Hanley and Merlie, 1991).

Denervation

Neonatal animals were anesthetized by cooling on ice, and older animals by ether inhalation or intraperitoneal injection of Nembutal. An incision was made in the thigh, and the nerve was exposed by blunt dissection through the vastus lateralis muscle. A piece of the nerve was cut out, or

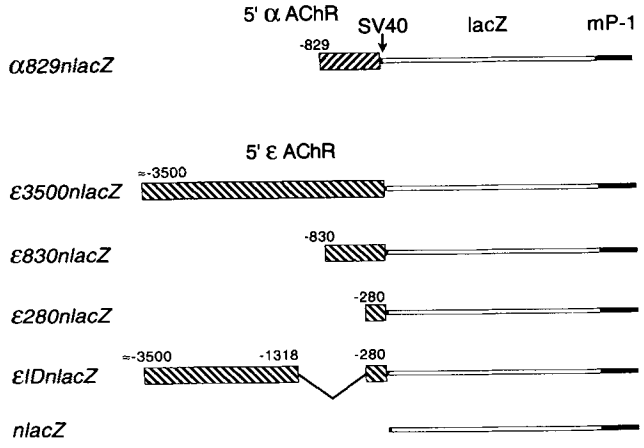

Figure 1. Maps of the transgenes used in the present study. 5' α AChR indicates 5' flanking sequence from the chicken acetylcholine receptor α-subunit gene and 5' ε AChR from the mouse ε-subunit gene. (*lacZ*) The *E. coli lacZ* gene; (SV-40) the SV-40 nuclear localization signal; and (*mP-1*) sequence from the mouse protamine gene. Further details are provided in Materials and Methods.

the nerve was reflected and sutured under the skin. In neonatal animals the wound was sutured; in older animals it was closed with clips. During the terminal experiment animals subjected to long-term denervation were checked for re-innervation by electrical stimulation of nerve stumps and along the nerve path. No animals showed any sign of re-innervation. Denervation was performed unilaterally with the muscles in the opposite leg serving as controls. The age at denervation and sacrifice is given in postnatal days, with day 0 being the day of birth.

Histochemistry and Muscle Preparation

Animals were killed by cervical dislocation, and muscles were dissected free and pinned out in a slightly stretched condition during fixation. Paraformaldehyde/glutaraldehyde fixation and X-gal staining were performed as described previously (Sanes et al., 1991). Muscles from the intact leg and the denervated leg were always processed together. Whole muscles were photographed through a dissecting microscope. Single muscle fibers were teased, mounted in glycerol, and photographed through a compound microscope. Individual teased fibers were stained for acetylcholinesterase according to Karnovsky and Roots (1964) after the muscles had been stained for β-galactosidase.

RNAse Protection Assay

RNA was extracted from the lower hind limb muscles as described by Chomczynski and Sacchi (1987). An RNAse protection assay (Melton et al., 1984) was used to quantify mRNA from the *αnlacZ* transgene, using a ~150-bp antisense *lacZ* probe transcribed from the plasmid pT7lacZ (A. Patapoutian, unpublished observations).

Results

Synaptic Induction of εnlacZ Expression Occurs in the Absence of Nerve

Unlike the other AChR subunits, expression of the endogenous ε subunit is induced after birth (Brenner et al., 1990; Martinou and Merlie, 1991). Consistent with this finding, no *εnlacZ* expression was detected in the transgenic mouse lines ε-29 and ε-51 during the first five postnatal days (Sanes et al., 1991). Since the nerve defines the area where ε is induced it was of interest to determine if *εnlacZ* expression could still be induced following removal of the nerve terminal. To this end, the sciatic nerve in *εnlacZ* mice was transected in the thigh unilaterally at day 0-4, with the intact leg serving as control. Five muscles from each leg were subsequently dissected out: soleus, plantaris, gastrocnemius, tibialis anterior, and extensor digitorum longus (EDL). When these muscles were stained for nlacZ, both the intact and the denervated leg showed staining of nuclei in the synaptic region of the muscles (Fig. 2, *a–d*). The identity of the synaptic sites was confirmed by co-staining with acetylcholinesterase as a synaptic marker (data not shown). We conclude that the post natal induction of *εnlacZ* still occurs at the original nerve contact sites even when the nerve is removed at birth.

Denervation Increases Both αnlacZ and *εnlacZ Expression in Young Mice*

When comparing intact and denervated muscles of young animals we observed a pronounced increase in εnlacZ staining. Similar increases were seen in two independently derived lines; ε-51 (Fig. 2, *c* and *d*) and ε-29 (Fig. 2, *e* and *f*), indicating that this effect was not insertion-site specific. In the less intensely stained ε-51 line even when expression was undetectable in the intact leg, the denervated leg showed strong staining. Table I summarizes the effects of denervation on tibialis and gastrocnemius muscles of young εnlacZ mice. These observations suggest that ε, like the other subunits, is regulated by activity. Notably, however, εnlacZ staining was never observed in nonsynaptic portions of muscle fibers, indicating that even though expression of this subunit is subject to activity regulation, its expression is restricted to synaptic nuclei.

The only nlacZ-positive structures in ε-nlacZ mice, other than synapse-associated nuclei were the intrafusal muscle fibers of muscle spindles (Sanes et al., 1991). Stained spindles generally disappeared after neonatal denervation (Fig. 2, *c* and *d*), but not following denervation in the adult. This is consistent with previous morphological studies which have shown that neonatal, but not adult denervation leads to "disintegration" of spindles (Zelená, 1964; Kucera and Walro, 1988).

Denervation of young *αnlacZ* animals also led to an increase in staining (Fig. 2, *g–j*; Table I). The increase was particularly dramatic in the soleus which showed little or no staining in the intact leg (Fig. 2, *i* and *j*). In contrast to the εnlacZ activity, however, the increase in αnlacZ activity was uniform along the entire length of the muscle fibers. These findings extend previous findings with the same promoter element coupled to CAT (chloramphenicol acetyl transferase) as a reporter (Merlie and Kornhauser, 1989). CAT was also induced by denervation, but since it is a readily diffusible molecule, its spatial distribution could not be determined. The present findings show that the 829-bp chicken α promoter conferred increased transcription in all parts of the muscle fiber after denervation, while the ε promoter responded only in the synaptic area.

The Effect of Denervation on nlacZ Staining Differs with Animal Age

It has been shown previously that denervation of adult animals leads to a strong increase in the level of mRNA of the endogenous α subunit gene (Merlie et al., 1984), and of the reporter CAT regulated with the same α-promoter element we are using here (Merlie and Kornhauser, 1989). We

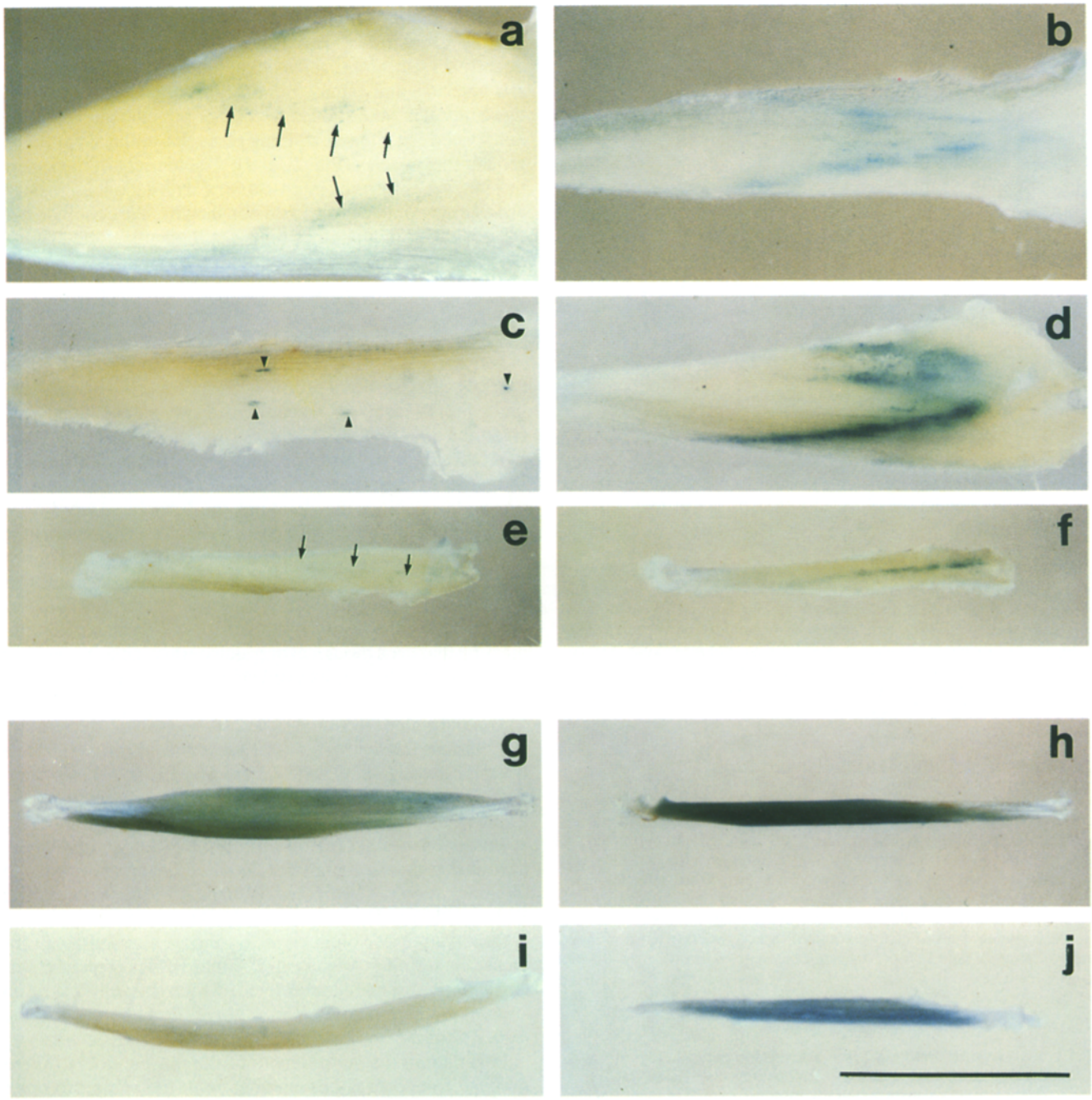

Figure 2. Denervation increases nlacZ activity in young transgenic mice. Intact control muscles are shown on the left (*a*, *c*, *e*, *g*, and *i*), and denervated contralateral muscles from the same animals on the right (*b*, *d*, *f*, *h*, and *j*). The muscles are from 15-d-old mice of lines ϵ-29 (*a*, *b*, *e*, and *f*), ϵ-51 (*c* and *d*) and α-16 (*g–j*). Tibialis muscles were denervated on postnatal day 0 (*b*) and day 4 (*d*), a plantaris muscle was denervated on day 7 (*f*), and a soleus (*h*), and an EDL (*j*) muscle were denervated day 4. Arrows point to the synaptic zone where synaptic staining is visible. Arrowheads point to stained muscle spindles (see Results and Sanes et al., 1991 for further details). Bar: (*a–d*) 2.5 mm; (*e–j*) 5 mm.

were therefore surprised to find that denervated muscles from older αnlacZ animals frequently showed a reduction in the intensity of staining (Fig. 3). This led us to perform a series of denervations at various ages. We found that denervation performed during the first month usually led to an increase in staining, while denervation in older animals usually led to a decrease (Fig. 4). The data show that the change in denervation response occurs around week five for the posterior crural muscles (soleus, gastrocnemius, and plantaris), and around week three for the anterior crural muscles (tibialis and EDL).

The ϵnlacZ staining showed an age dependence that was qualitatively similar to that of the αnlacZ: in young animals denervation usually led to an increase in staining (Table I), while in the adult a decrease was often seen (Fig. 3). The response was however more variable than for the αnlacZ stain-

Table I. The Effect on nlacZ Staining of Denervation in Young Animals

Promoter	Line	(n)	Percent of muscles		
			Increase	No change	Decrease
α	16	(48)	77	21	2
ε	29	(55)	58	13	29
	51	(40)	95	0	5

All denervations were performed in animals less than a month old. *n* indicates the number of muscle pairs for which staining was compared. Results from gastrocnemius and tibialis muscles are included. Pairs where neither the denervated nor the intact muscle showed any staining were excluded.

ing, and a significant increase in εnlacZ staining was occasionally observed even after denervations in the adult.

Reduced αnlacZ Staining in Adults Reflects a Posttranscriptional Phenomenon

To investigate whether the paradoxical reduction in nlacZ staining after denervation in adult animals resulted from reduced transcription of the transgene, the level of lacZ mRNA was measured in 2.5-month-old α-16 mice that had been denervated for 5 d. An RNAse protection assay showed that lacZ mRNA was increased after denervation, indicating that the transgene responded positively to denervation (Fig. 5). This contrasts with the decreased histochemical nlacZ staining documented above and indicates that denervation reduces β-galactosidase enzyme activity by some posttranscriptional mechanism. Similar observations for εnlacZ would have been desirable. However, given the low level of ε transcripts in total muscle mRNA, and the problems of interpreting results based on bulk RNA for a gene with selective synaptic expression (see Discussion), we decided against attempting such experiments. Nonetheless, in that the transgenes in the αnlacZ and εnlacZ mice code for identical proteins, and since the mRNA sequences differ only over a small 5′ portion, it seems likely that any posttranscriptional mechanism would influence both of them in a similar fashion.

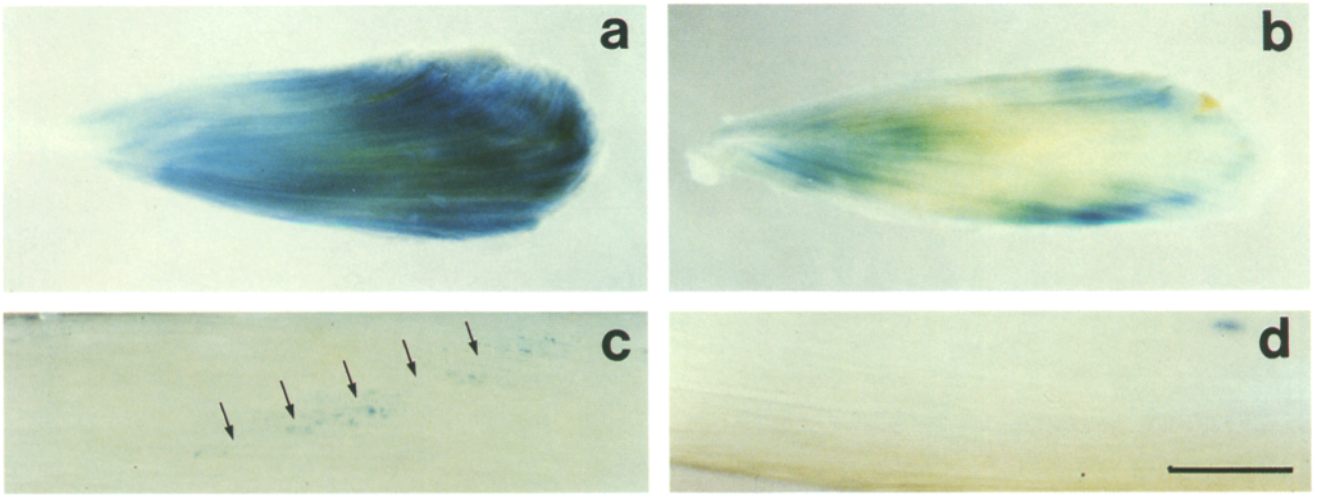

Figure 3. Denervation decreases nlacZ activity in adult transgenic mice. Intact control muscles are shown on the left (*a* and *c*), and denervated contralateral muscles from the same animal on the right (*b* and *d*). The α-16 mouse (*a* and *b*) was denervated on postnatal day 34 and the ε-29 mouse (*c* and *d*) on day 126. 15 d after denervation the tibialis (*a* and *b*) and EDL (*c* and *d*) muscles were dissected out and stained. Arrows in *c* point to the synaptic zone where nlacZ staining is visible. Bar: (*a* and *b*) 2 mm; (*c* and *d*) 0.5 mm.

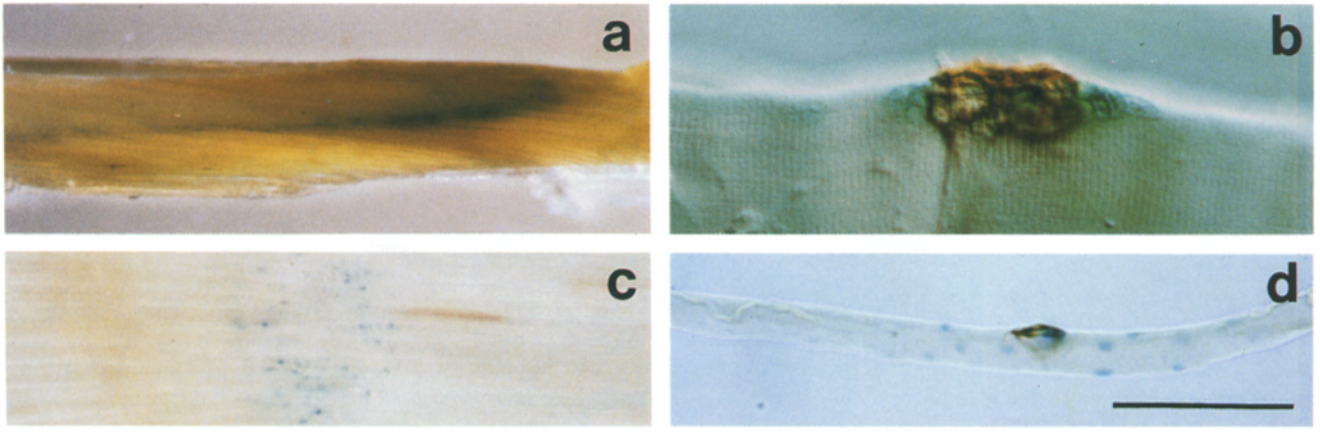

Figure 6. nlacZ expression in mice with the *εIDnlacZ* transgene (*a*), *ε280lacZ* (*b*), and the *ε830nlacZ* (*c* and *d*). The preparations are: whole mount EDL (*a*) and diaphragm (*c*), and teased single fibers double stained for acetylcholinesterase (*b* and *d*) according to Karnovsky and Roots (1964). Bar: (*a*) 1.5 mm; (*c*) 0.7 mm; (*d*) 100 μm.

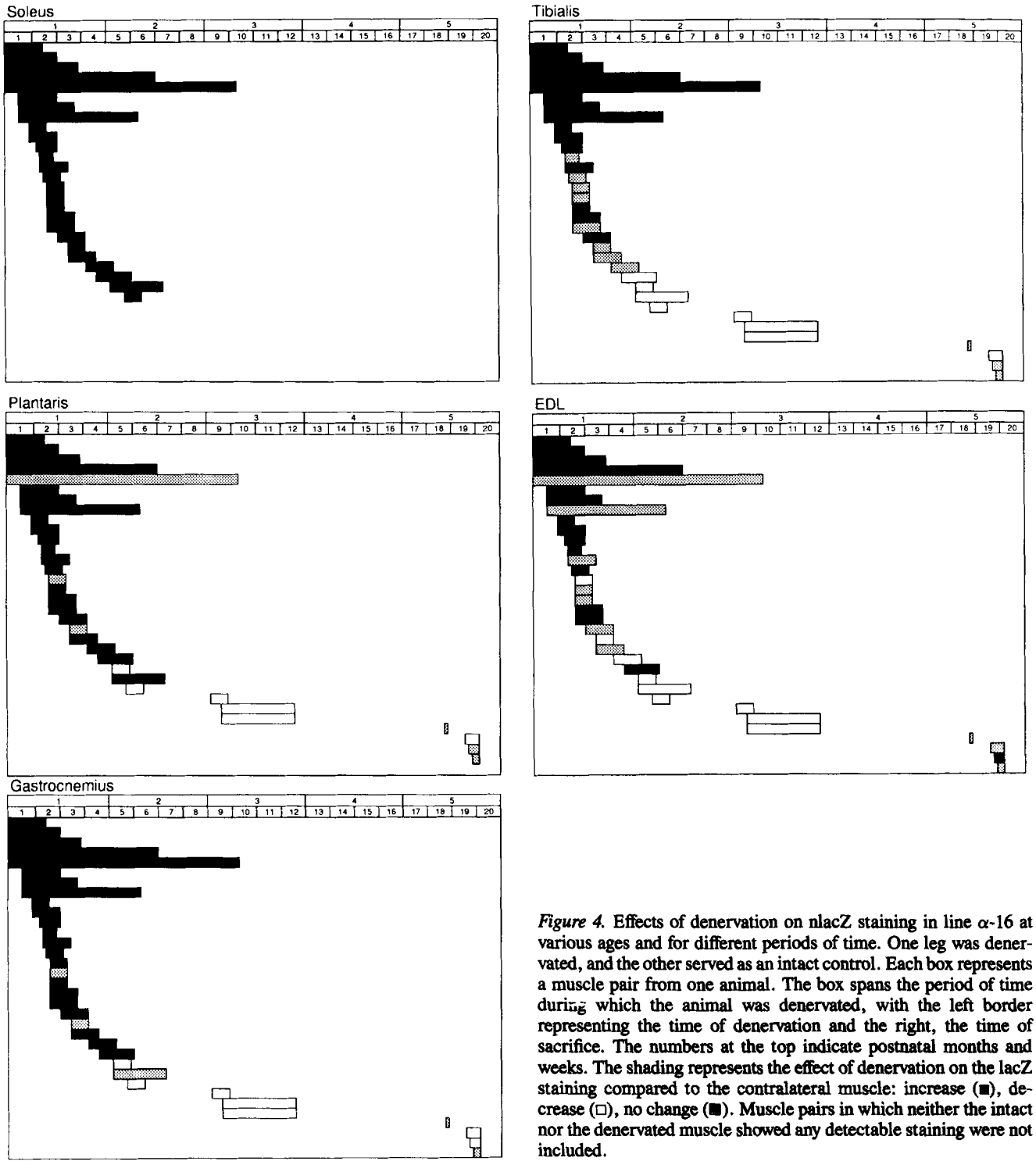

Figure 4. Effects of denervation on nlacZ staining in line α-16 at various ages and for different periods of time. One leg was denervated, and the other served as an intact control. Each box represents a muscle pair from one animal. The box spans the period of time during which the animal was denervated, with the left border representing the time of denervation and the right, the time of sacrifice. The numbers at the top indicate postnatal months and weeks. The shading represents the effect of denervation on the lacZ staining compared to the contralateral muscle: increase (■), decrease (□), no change (■). Muscle pairs in which neither the intact nor the denervated muscle showed any detectable staining were not included.

Cis-Elements Responsible for Synaptic Localization of ϵ Expression

Our data suggest that the ϵ-subunit promoter differs from the other subunit promoters in that it requires local synaptic signals to be expressed even after denervation. To initiate analysis of *cis*-acting elements responsible for this specificity, we investigated a series of ϵ-promoter constructs. First we tested the *ϵ830nlacZ* transgene which lacks ~2,670 bp of distal 5′ sequence contained in the original *ϵ3500nlacZ* transgene (Fig. 1). Of 18 F_0 founders, 10 showed no staining, six showed staining in spindles only, and two showed staining in both spindles and synapses. The synaptic staining was no less localized than for the 3,500-bp ϵ transgene, and no expression was detected in extrasynaptic regions of extrafusal (nonspindle) muscle fibers (Fig. 6). This was confirmed by teasing out single fibers and double staining for cholinester-

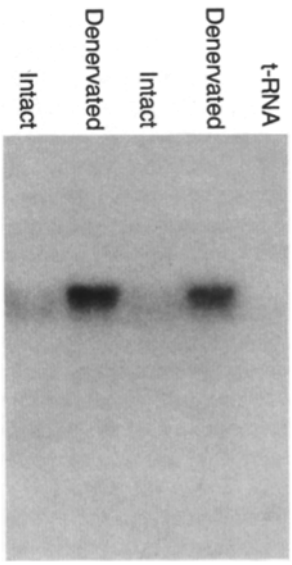

Figure 5. RNAse protection assay of 50 μg total muscle RNA extracted from the lower hind limbs of two 73-d-old α-29 mice denervated unilaterally for 5 d with the intact leg serving as a control. The RNA was probed with a radioactive antisense RNA sequence from the *lacZ* gene (see Materials and Methods). The right lane represents probe hybridized with yeast t-RNA, and the lack of a signal indicates that the bands are specific to the mouse muscle RNA.

ase as a synaptic marker (Fig. 6). Thus, sequences upstream of position −830 are not necessary for synaptically localized expression.

Next, we tested *ε280nlacZ*, a construct that lacks all but 280 bp 5′ of the start of ε gene transcription. This construct, like *ε830nlacZ*, gave a very low yield of expressing founder mice (Table II). Of the 18 independently derived *ε280lacZ* mice, eight showed easily detectable spindle staining, while one showed synaptic staining. Nonetheless, the staining pattern of *ε280lacZ* was highly specific and only slightly less intense than the *ε3500lacZ* mice (Fig. 6), indicating that 280 bp of the ε promoter is sufficient to confer synapse specific expression.

It is perhaps noteworthy that the frequency of mice expressing lacZ was very low with both the *ε280nlacZ* and the *ε830nlacZ* constructs. In contrast, the frequency of expressing founders with the *ε3500nlacZ* was quite high (Sanes et al., 1991). This difference suggested to us that upstream elements, although not required for synaptic or spindle expression, may affect the probability that a particular site of transgene integration will lead to expression. A final construct, *εIDlacZ* which contains an internal deletion of sequences between −830 and −1318 lends support to such a notion. With *εIDnlacZ* mice staining was once again synapse and spindle specific (Fig. 6). Now, however, the frequency of expressing founder mice was equal to that of the *ε3500lacZ* construct (Table II). A promoterless *nlacZ* construct showed no expression in any tissues in 15 independently derived transgenic mice. Thus, our data support a role for upstream sequences in conferring integration site independence on the synapse specific elements within the 280-bp ε promoter (see Palmiter et al., 1993 for a recent discussion of such effects). It is also striking from the data in Table II that spindle staining is less insertion site sensitive than synaptic staining, but this could be a trivial consequence of the spindle staining usually being stronger and easier to detect.

In summary, our data indicate that *cis*-acting elements within 280 bp of the transcriptional start site of the ε-subunit gene are sufficient to confer synapse specific staining. This conclusion differs from that of Duclert et al. (1993). In their studies, 5′ flanking ε-promoter sequences as large as 2.2 kb, in addition to synaptic staining, conferred extrasynaptic staining in 20% of the fibers. This lesser specificity, of DNA constructs analyzed by direct intramuscular injections, could be explained by the high plasmid copy number as the authors point out (Duclert et al., 1993), or from the difference in the physical nature or location of the DNA (linearly integrated into the genome in the transgenic mice versus non-integrated supercoiled plasmid in the case of intramuscular DNA injection).

Discussion

The Nerve Deposits a Stable Signal That Activates ε Transcription

Our experiments show that the *εnlacZ* transgene was induced during early postnatal development even when the nerve was transected at birth. Thus the developmentally determined induction does not require the continuous presence of the nerve. Previous experiments have shown that when new synapses are formed ectopically in adult animals, postsynaptic specializations become stable after only a brief nerve contact (Lømo and Slater, 1980*a*,*b*). Moreover, studies of muscles regenerating after muscle damage have shown that when new fibers form within old basal lamina sheaths, postsynaptic specializations are induced at the original synaptic sites (Burden et al., 1979; McMahan and Slater, 1984). Recently, these studies have been extended to show that signals bound in the basal lamina can induce local accumulation of AChR mRNA (Goldman et al., 1991; Brenner et al., 1992) and localized δ-subunit transcription (Jo and Burden, 1992). Based on such experiments it has been suggested that the

Table II. The Effect of Promoter Length on the Yield of nlacZ Expression in Synapses and Spindles

	Number of animals				
Transgene	ε3500nlacZ	ε830nlacZ	ε280nlacZ	εIDnlacZ	nlacZ
With staining in:					
Synapses and spindles	4	2	1	3	0
Synapses only	0	0	0	1	0
Spindles only	0	6	7	0	0
No staining	1	10	12	1	15
Founders	5	18	18	5	15
Percent with synapses	80	12	6	80	0

nerve leaves a "trace" deposited in the basal lamina of the muscle fiber that triggers postsynaptic specializations. Our findings suggest that the nerve deposits this stable trace during embryogenesis and that it provides signals necessary to induce the ε promoter during postnatal development. This conclusion is consistent with findings obtained with in situ hybridization with ε mRNA in synaptic nuclei of neonatally denervated muscles (Brenner et al., 1990). In contrast, when ε mRNA was measured in extracts from whole muscles, the level of ε mRNA appeared to be dependent on the nerve supply in neonates (Martinou and Merlie, 1991). These measurements of bulk mRNA might not, however, reflect changes in transcription that only occur in a few synaptic nuclei (see below).

The molecular nature of the trace is unknown, although the chicken brain protein, ARIA (Falls et al., 1993), and the extracellular matrix component, agrin (Rupp et al., 1991; Tsim et al., 1992), are candidates for such a role. ARIA has been shown to increase ε expression in mouse muscle primary cultures (Martinou et al., 1991), but it remains to be demonstrated when this substance appears at synaptic sites. Agrin is clearly present in the synaptic cleft before birth, and induces AChR clustering, but has not been shown to be involved in transcriptional regulation. The transgenic animals described in this paper should be useful in further investigation of the molecular nature of the trace. We have recently made stable muscle cell lines from these animals (Moscoso, L., J. P. Merlie, and J. R. Sanes, unpublished) which may allow in vitro testing of molecules that are candidate components of the trace.

The ε Promoter Has Activity Dependent Elements

At least in young animals the *εnlacZ* transgene responded to denervation by increasing expression; thus, the ε promoter seems to be similar to the other subunit promoters in being responsive to activity. This was surprising since quantitative measurements performed on bulk RNA in muscle extracts from denervated muscles have shown only small and variable effects; Martinou et al. (1991) observed a small decrease after denervation in both neonatal and adult mice, while Witzemann and co-workers (1987, 1989, 1991) observed a small increase in adult rats. However, analysis of bulk RNA might not well reflect expression in those nuclei that are associated with the synapse, which constitute only ~1% of all the muscle cell nuclei (Merlie and Sanes, 1985). Even low levels of ε expression in the ~99% nonsynaptic nuclei (see Pinset et al., 1991), could mask accumulation of ε mRNA by synaptic nuclei. The use of nlacZ as a reporter overcomes these difficulties, and the data indicate that the 3,500-bp ε sequence contains *cis*-acting elements that respond to denervation by increasing transcription.

The intracellular mechanisms that mediate activity regulation of AChR have been investigated intensively. It has been shown that expression of the myogenic factors (myoD, myogenin, MRF4 and myf5) is increased by denervation, and that this increase is counteracted by electrical stimulation (Duclert et al., 1991; Eftimie et al., 1991; Witzemann and Sakmann, 1991; Buonanno et al., 1992). In vitro studies of the chicken α promoter have shown that myogenic factors can bind to sites with the consensus sequence CANNTG, and cotransfection studies have shown that such binding can transactivate the promoter (Piette et al., 1990; Prody and Merlie, 1992). The proximal sequence of the ε promoter contains a similar binding site (Numberger et al., 1991; Sanes et al., 1991; Sunyer and Merlie, 1993). It is therefore tempting to speculate that myogenic factors are also responsible for the denervation response of this subunit. Consistent with this idea, is the finding that overexpression of myogenin in transgenic mice leads to higher levels of ε mRNA (Gundersen, K., and J. P. Merlie. 1993. *Soc. Neurosci. Abstr.* 19:223).

The present study indicates that the α829-bp promoter fragment contains *cis*-acting elements that, in contrast to ε-promoter elements, can turn on transcription in nuclei along the entire length of the muscle fiber after denervation. Simon et al. (1992) have reported similar results for ~1,850 bp of 5′ flanking sequence of the δ-subunit gene using growth hormone as a localized reporter.

In fact, all of the four AChR subunits genes (α, β, δ, and γ) that are expressed in fetal muscles and in extrajunctional areas of adult muscles after denervation are believed to contain *cis*-elements that are responsive to elimination of nerve-evoked electrical activity. It has been speculated that the unique restriction of the ε gene to synaptic sites was caused by a lack of such activity-dependent elements. The present data indicate, however, that the ε promoter is also upregulated by denervation. Thus, the confinement of ε-gene expression to the synapse seems rather to be due to an essential requirement for a local nerve-derived signal (the "trace"). For the other subunit genes the trace is not essential, since transcription is induced in nonsynaptic nuclei by inactivity. Thus, for the α, β, γ, and δ genes either signals induced by the trace, or signals induced by inactivity are sufficient for expression. For expression of the ε gene, on the other hand, signals induced by the "trace" are essential for expression, and inactivity has an effect only in those nuclei where the trace pathway is also active. This interaction might explain the restricted distribution of mature ε-containing AChR compared to the relative promiscuity of the fetal AChR, which contains the other subunits.

Problems with nlacZ as a Reporter

Our finding that β-galactosidase activity was reduced while *nlacZ* mRNA was increased in *αnlacZ* mice that were denervated later than 3–5 wk of age, demonstrates that nlacZ activity does not always faithfully report transcription. This is an important observation in view of the widespread use of this reporter. Since increased proteolytic activity after denervation is very prominent (for examples see Furuno et al., 1990; Medina et al., 1991), we suggest that the negative effect of denervation could be due to increased degradation of the bacterial β-galactosidase protein. Whether β-galactosidase staining increases after denervation or not, will thus depend on a balance between increased proteolysis and increased synthesis regulated at the transcriptional level. It would not be surprising if this balance changes during development. Consistent with this interpretation is the finding that the downregulation of reporter activity did not occur when a different reporter (CAT) was regulated with identical regulatory sequences (Merlie and Kornhauser, 1989). Moreover, we have observed that in mice with myogenin promoter transgenes, CAT reporter activity increased after denerva-

tion, whereas *lacZ* reporter decreased (Cheng et al., 1992; Gundersen, K., J. Mudd, and J. P. Merlie, unpublished observations).

Posttranscriptional mechanisms like proteolysis might explain some of the surprising findings obtained with AChR-promoter-*nlacZ* constructs, in previous studies. For example, Klarsfeld et al. (1991) using a construct very similar to our α829*nlacZ*, reported that the staining was extinguished after postnatal day 3 while the level of endogenous α-subunit mRNA is virtually unchanged throughout this period (Eftimie et al., 1991). In the same transgenic animals, Salmon and Changeux (1992) recently reported spatio-temporal patterns of nlacZ activity in which the synaptic zone eventually became devoid of staining, and we observed similar patterns after denervation in adult animals (for example see Fig. 3 *b*). In light of our new evidence for posttranscriptional regulation, we suggest that this synapse specific decrease in nlacZ activity might reflect the selective increase in the activity of lysosomal enzymes and protein degradation that has been reported perisynaptically after denervation (Libelius and Tågerud, 1984). In conclusion, although we do not question the general usefulness of *nlacZ* as a reporter in transgenic experiments, variations in posttranscriptional processes like proteolysis, should be taken into consideration when interpreting spatio-temporal expression patterns of *nlacZ*.

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