

# Regulation of the Size and Distribution of Agrin-Induced Postsynaptic-like Apparatus in Adult Skeletal Muscle by Electrical Muscle Activity

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**We compared acetylcholine receptor (AChR) aggregates induced by neural agrin released from transfected muscle fibers with AChR aggregates induced by transplanted axons in extrajunctional regions of denervated rat soleus muscles. Both neural agrin and transplanted axons induced multiple, irregularly distributed AChR aggregates on muscle fibers. Direct electrical muscle stimulation of transfected muscles for up to 10 weeks removed all agrin-induced AChR aggregates (the losers) except one (the winner) on many fibers. Axon-induced AChR aggregates underwent comparable selection of winners and losers. The results suggest that agrin and acetylcholine-driven muscle activity provided by transplanted axons are sufficient to elicit in a denervated adult muscle fiber processes that regulate the size and distribution of ectopic neuromuscular junctions.**

## INTRODUCTION

According to the agrin hypothesis, agrin released from the axon terminals of motor neurons at developing neuromuscular junctions mediates the motor neuron's induction of protein aggregates in the muscle fiber's postsynaptic apparatus (McMahan, 1990; Reist *et al.*, 1992). The agrin hypothesis has recently received strong support from two types of experiments. First, the postsynaptic apparatus does not form in mutant mice that lack the ability to produce agrin (Gautam *et al.*, 1996) or muscle-specific protein kinase (MuSK), a component of the agrin receptor complex (DeChiara *et al.*, 1996). Second,

when released from muscle fibers transfected with neural agrin cDNA, neural agrin alone induces a postsynaptic-like apparatus on denervated muscle fibers *in vivo* that is structurally and functionally similar to that induced by transplanted axons (Cohen *et al.*, 1997; Jones *et al.*, 1997; Meier *et al.*, 1997; Rimer *et al.*, 1998, 1997).

In addition to agrin, the axon terminals of motoneurons release acetylcholine (ACh), which evokes electrical muscle activity after binding to acetylcholine receptors (AChRs) in the postsynaptic membrane. The role of electrical impulse activity in synapse formation has been extensively studied at the ectopic neuromuscular junctions that form in adult rats between axons of a transplanted fibular nerve and denervated soleus muscle fibers (Brenner *et al.*, 1987, 1983, 1994; Cangiano *et al.*, 1980; Lømo and Slater, 1980a; Lømo and Slater, 1980b; Rotzler and Brenner, 1990; Wærhaug and Lømo, 1994) (Skorpen *et al.*, 1999). Initially, the transplanted axons induce the appearance of AChR aggregates that are small, numerous, multiply innervated, and irregularly distributed along the extrajunctional region of individual muscle fibers. Subsequently, most of the aggregates disappear (the losers), while a few aggregates survive at sites that become permanent mature ectopic neuromuscular junctions of a certain size and distribution along each muscle fiber (the winners) (Lømo *et al.*, 1988). In Skorpen *et al.* (1999) we show that these changes in AChR aggregate appearance and distribution require both electrical muscle activity and a trophic influence from the nerve, which persists in electrically stimulated muscles after the nerve terminals are removed at an early stage of synaptogenesis.

In this study we sought to determine whether the nerve-derived trophic influence, which in conjunction with electrical muscle activity regulates the size and

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distribution of ectopic neuromuscular junctions (Skorpen *et al.*, 1999), could be neural agrin. In addition, we examined whether agrin alone could induce activity-resistant expression of postjunctional proteins in long-term denervated and electrically stimulated muscles. So far, such activity-resistance has been inferred from studies of either innervated muscles or short-term denervated and stimulated muscles (Jones *et al.*, 1997; Meier *et al.*, 1997; Rimer *et al.*, 1997) (see Discussion). We therefore injected an expression vector encoding neural agrin into the extrajunctional region of denervated soleus muscles to obtain release of neural agrin from transfected fibers onto adjacent denervated muscle fibers, as previously described (Cohen *et al.*, 1997; Jones *et al.*, 1997). We then examined the agrin-induced AChR aggregation sites with respect to number, size, and distribution along fibers in stimulated and unstimulated muscles and compared them with the aggregation sites induced by transplanted axons in electrically active or inactive muscles.

## RESULTS

### *Agrin-Induced AChR Aggregates in Unstimulated Muscles*

After intramuscular injection of DNA, encoding rat agrin Y4Z8, about 1% of the muscle fibers expressed immunohistochemically detectable neural agrin in their sarcoplasm, confirming our previous report (Cohen *et al.*, 1997). Figure 1a shows a small bundle of muscle fibers in which one such fiber contains a short agrin-positive segment and other adjacent non-transfected fibers have multiple AChR aggregates on their surface. Figure 1b illustrates the generally uniform appearance of these AChR aggregates as a short series of ovoid patches along individual muscle fibers. The length of agrin positive segments ranged from 30 to 210  $\mu\text{m}$  (median value;  $n = 16$ ). The length of fiber segments having a series of AChR aggregates ranged from 10 to 400  $\mu\text{m}$  (median value 60  $\mu\text{m}$ ,  $n = 139$ ).

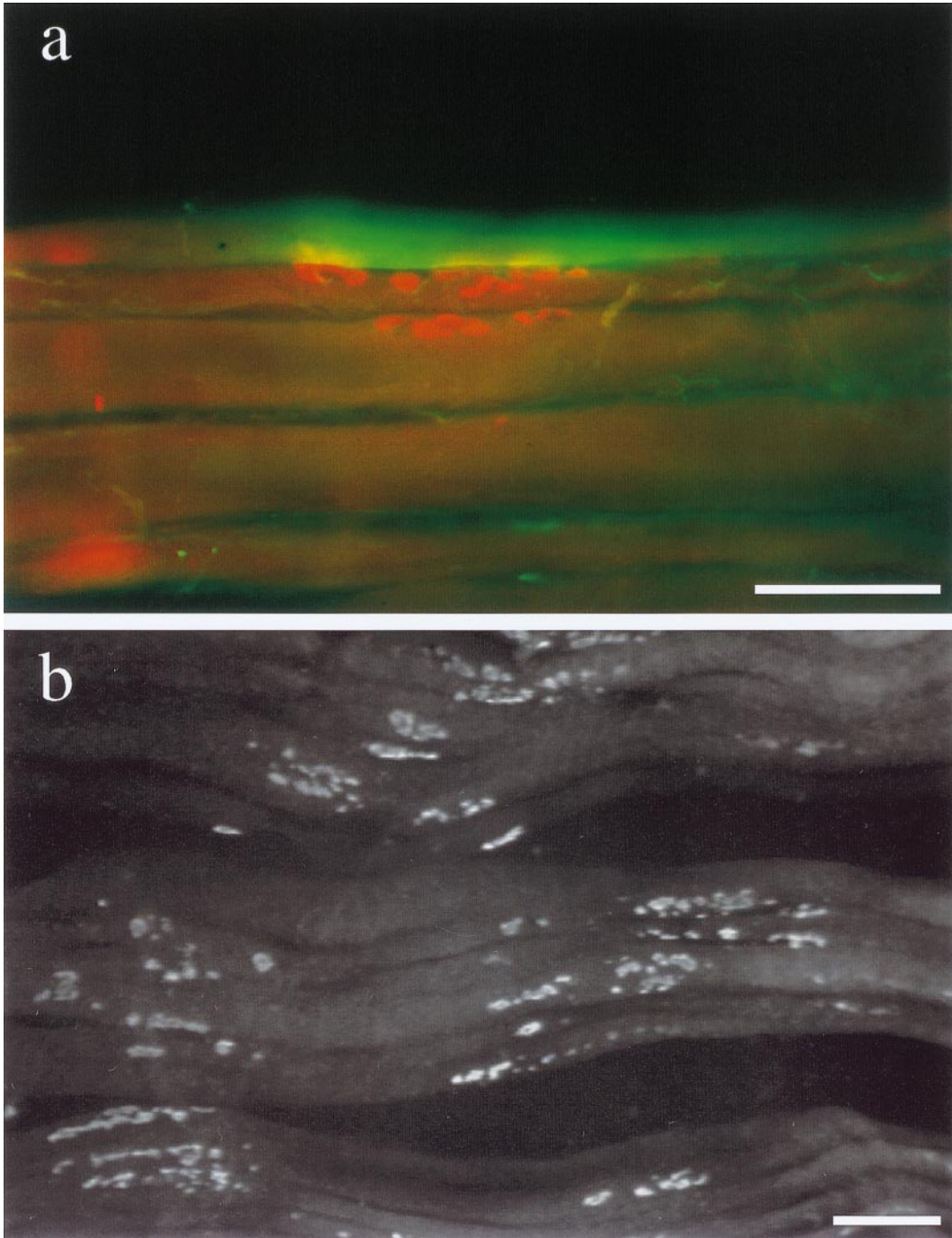
Neural agrin-DNA was also injected into single surface fibers to obtain more precise information on the distribution of AChR aggregates around transfected fibers and hence on the effective range of released agrin. Figure 2 provides an example of such intracellularly injected fibers ( $n = 97$  in 7 muscles). The figure shows cross sections of a soleus muscle in which one injected surface fiber contains neural agrin and the surrounding fibers have several AChR aggregates on their surface. The AChR aggregates were larger on muscle fibers close

to the agrin-positive fiber (arrow) than on muscle fibers further away (Fig. 2a), suggesting that the effective agrin concentration gradually declined with transverse distance from its site of secretion. The AChR aggregates covered only a fraction of the circumference of each fiber and were preferentially located on those parts that faced the agrin-positive fiber. In serial cross sections from the same muscle (Fig. 2b), the label for agrin and the number of AChR aggregates declined towards zero over a longitudinal distance of 50–100  $\mu\text{m}$  in either direction from the site of most intense labeling shown in Fig. 2a. These findings indicate that the number, size, and distribution of AChR aggregates on fibers surrounding an agrin-positive fiber segment depend on the local concentration of secreted agrin and on the capacity of muscle fibers to form AChR aggregates.

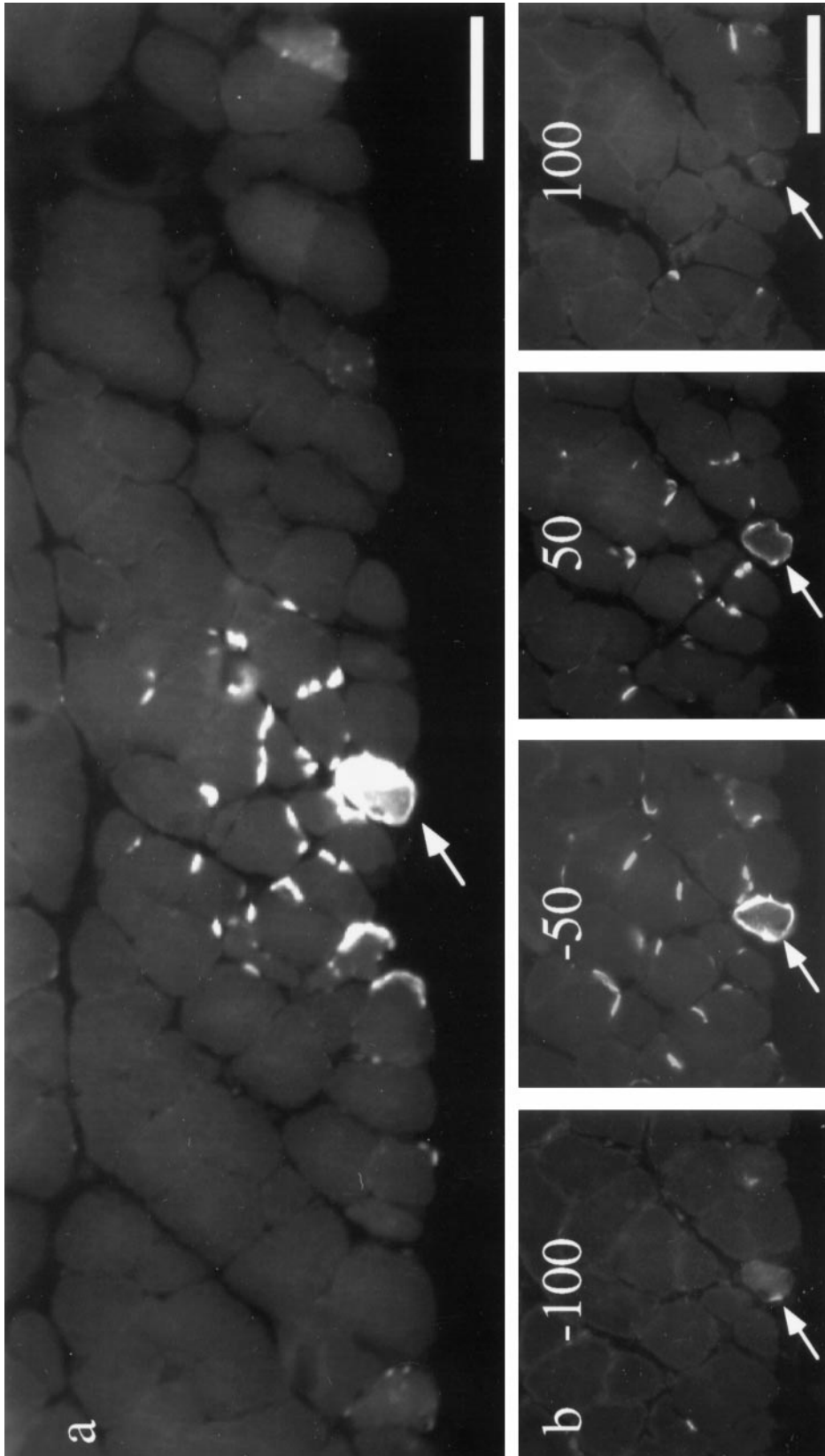
### *Agrin-Induced AChR Aggregates in Electrically Stimulated Muscles*

We then injected agrin-DNA into denervated soleus muscles and implanted electrodes to start electrical stimulation of the muscle at different times after the injection. During the stimulation all the AChR aggregates disappeared except at one site on many fibers where one or a few closely adjacent aggregates survived (Fig. 3). The surviving sites reached their maximum size in less than 20 days of stimulation and then essentially remained at this size as long as the stimulation lasted (up to 10 weeks, Fig. 4). The length of the activity-resistant surviving sites varied nearly symmetrically around a median length of  $32 + 3.5 / - 2.7 \mu\text{m}$  ( $\pm 95\%$  confidence interval,  $n = 44$ , Fig. 5b), whereas the AChR aggregates on unstimulated fibers were numerous, mostly smaller, and distributed along a length of up to 400  $\mu\text{m}$  on each fiber (Figs. 1 and 5a). Usually, DNA was injected 3–4 days after denervation and muscle stimulation started 10–12 days later. However, similar results were obtained when denervation and DNA injection occurred at the same time and stimulation was started as early as 2 days or as late as 18 days after the DNA injection. These results show that electrical muscle stimulation elicited processes in the denervated muscle fibers which caused all the agrin-induced AChR aggregates to disappear except at one site on many fibers. This activity-resistant site then reached and maintained a certain size.

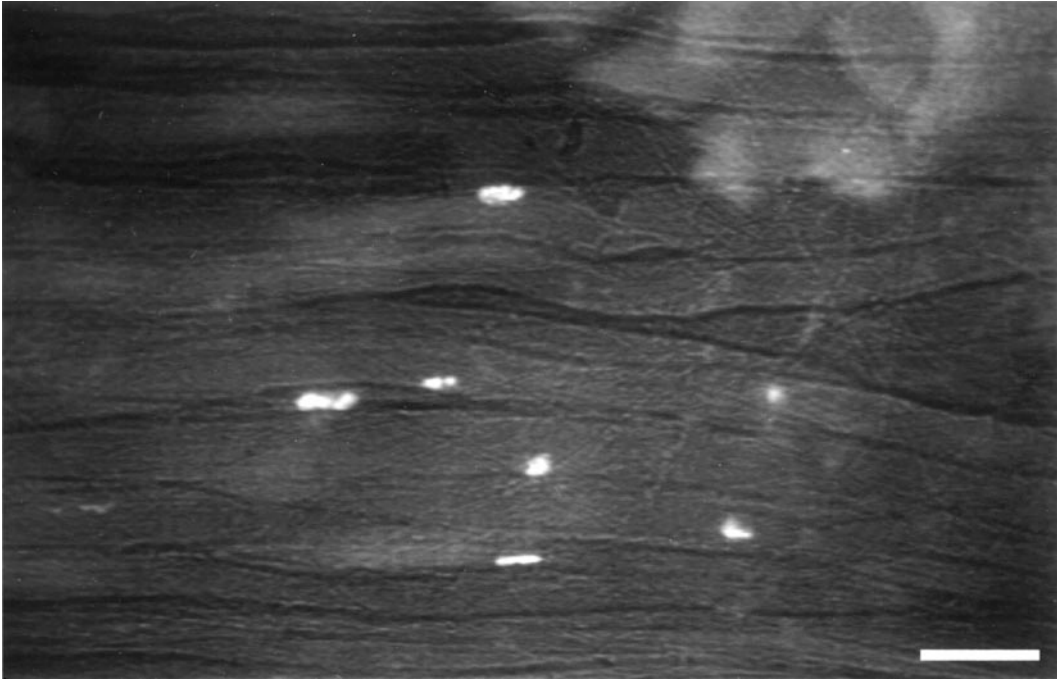
The number of fibers with AChR aggregates was lower in stimulated than in unstimulated muscles, indicating that some stimulated fibers lost all their aggregates (see Fig. 1 and 3 for representative comparison). This loss of AChR aggregates could not be ex-



**FIG. 1.** Distribution of agrin-induced AChR aggregates in the extrajunctional region of denervated soleus muscles transfected with a vector encoding neural agrin. (a) A whole mount of a bundle of muscle fibers examined 13 days after denervation and 10 days after injection of the vector. The muscle was permeabilized and treated with antibody to reveal neural agrin (green) and with TRITC- $\alpha$ -bungarotoxin to reveal AChR aggregates (red). (b) A whole mount of a bundle of muscle fibers examined 32 days after denervation and 28 days after injection of the vector. There are many strings of AChR aggregates along the fibers, indicating the presence of agrin secreting segments in nearby fibers (not stained in this preparation). Scale bar, 100  $\mu$ m.



**FIG. 2.** Agrin-induced AChR aggregates on muscle fibers surrounding a single surface fiber injected with neural agrin-DNA. (a) A cross-section of a muscle at the site of maximum labeling by antibody against neural agrin (arrow), showing many AChR aggregates on surrounding nontransfected fibers. (b) Cross-sections at 50- $\mu$ m intervals from the section in (a) show that both the intensity of agrin labeling and the number of AChR aggregates decline toward zero with distance from the site of maximum agrin content. The muscle was taken for examination 17 days after denervation and intracellular injection of agrin-DNA. Scale bar, 100  $\mu$ m.



**FIG. 3.** Agrin-induced AChR aggregates persist in extrajunctional regions of denervated directly stimulated soleus muscles. A whole mount of a bundle of muscle fibers 32 days after denervation, 28 days after intramuscular injection of neural agrin-cDNA, and 24 days after onset of electrical muscle stimulation. AChR aggregates are stained by TRITC- $\alpha$ -bungarotoxin. The frequency of aggregates is less than that in unstimulated muscles (compare with Fig. 1b). Comparable aggregates were shown in other preparations to be near agrin-positive fiber segments. Scale bar, 100  $\mu$ m.

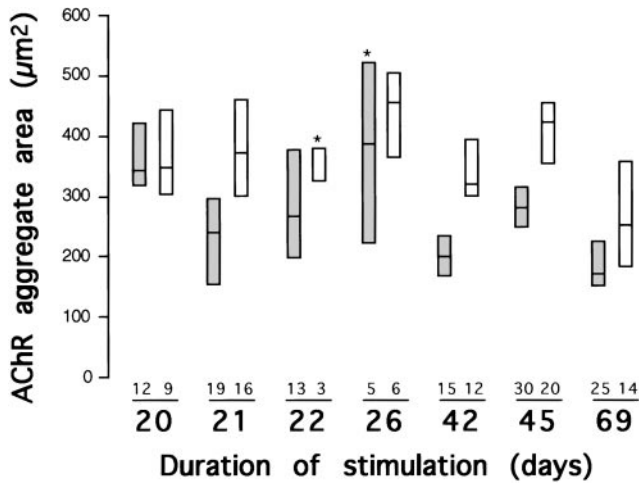
plained by an effect of stimulation on agrin expression because there was no noticeable difference in intensity of agrin labeling or length of agrin-positive segments in stimulated ( $95 + 32 / - 21 \mu\text{m}$ , median  $\pm 95\%$  confidence intervals,  $n = 43$ ) and unstimulated ( $90 + 22 / - 33 \mu\text{m}$ ,  $n = 16$ ,  $P = 0.289$ ) muscles.

Many proteins, normally present in postsynaptic apparatus, become concentrated at the agrin-induced AChR aggregates in the extrajunctional region of soleus muscles (Cohen *et al.*, 1997; Jones *et al.*, 1997; Meier *et al.*, 1997, 1998; Rimer *et al.*, 1998, 1997). These proteins include AChE, neuregulin (NRG), erbB3 receptors, and  $\gamma$ - and  $\epsilon$ -subunit AChRs. We searched for these proteins by immunocytochemistry in muscles stimulated for 25–30 days and found a high concentration of stain for all of them at the surviving AChR aggregation sites (not shown). In cross-sections from such muscles, 17 of 17 AChR aggregates stained for AChE, 18 of 18 for NRG, 23 of 23 for erbB3, while 15 of 15 AChR aggregates stained negative for erbB3 after preincubation with a control peptide. The relative amount of AChE accumulation in stimulated and unstimulated muscles was assessed on isolated bundles of muscle fibers by bathing them in a conventional histochemical staining solution (Buckley and Heaton, 1968) and determining the time between application of

the reaction mixture and the appearance of colored reaction product as seen through a stereo microscope. In stimulated muscles, the mean time for appearance of patches of AChE stain was 3.4 min ( $n = 5$ , 1–5 min), while in unstimulated muscles it was 12.3 min ( $n = 3$ , 7–17 min). At the denervated synaptic sites in the same muscles the corresponding values were 3.1 min ( $n = 5$ , 1–5 min) and 8.3 min ( $n = 3$ , 3–15 min). Thus, electrical stimulation of denervated muscles led to increased AChE activity both at the original postsynaptic apparatus and at agrin-induced AChR aggregation sites. Of 34 agrin-induced AChR aggregates examined with antibodies against  $\epsilon$ - and  $\gamma$ -AChR subunits, 33 aggregates stained strongly with the  $\epsilon$ -antibody. Most of these aggregates stained only for the  $\epsilon$ -subunit, while a few (16%) also showed some staining with antibody against the  $\gamma$ -subunit. These results indicate that agrin alone can act locally on muscle fibers to induce activity-resistant expression of  $\epsilon$ -AChR subunits and accumulations AChE, NRG, and erbB3.

### **Comparisons of Agrin-Induced and Nerve-Induced AChR Aggregates**

Neural agrin and transplanted axons induced the formation of AChR aggregates that were similar in



**FIG. 4.** Agrin-induced and soleus nerve-induced AChR aggregation sites have similar areas in stimulated denervated soleus muscles. Each pair of blocks is from the same soleus muscle and represents the area of AChR aggregates seen *en face* in their entirety at agrin-induced sites (closed blocks) and at original endplates in the same muscle (open blocks). Agrin-DNA was injected intramuscularly 0–4 days after denervation. Stimulation started 2–12 days later and lasted for the number of days indicated by the large numbers below each pair of muscles. The small numbers show the number of agrin-induced and original nerve-induced AChR aggregates examined. Horizontal lines in each block represent median values  $\pm$  95% confidence intervals. The two blocks indicated by \* show the entire range of values.

appearance and responded similarly to electrical muscle activity. Thus, the axon-induced AChR aggregates at early immature stages (Fig. 6a) and at later stages in electrically inactive muscles were numerous and irregularly distributed along the fibers, as were the agrin-induced AChR aggregates on inactive denervated muscle fibers (Fig. 6b). However, the axon-induced aggregates spanned longer distances along the fibers (up to 8 mm, Skorpen *et al.*, 1999) than the agrin-induced aggregates (up to 400  $\mu$ m).

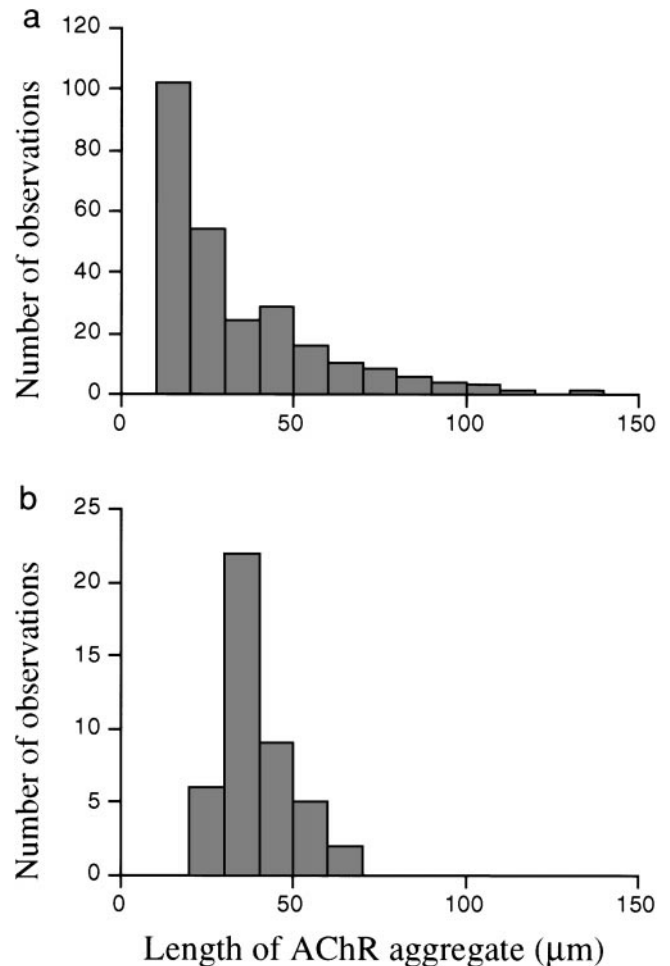
Electrical muscle activity, elicited either by intact axons or by muscle stimulation after cutting the axons at an early stage of synaptogenesis, led, on average, to the elimination of all but one axon-induced AChR aggregation site per 1.5 mm length of fiber (Skorpen *et al.*, 1999). Similarly, electrical muscle stimulation led to the elimination of all but one agrin-induced AChR aggregation site.

The activity-resistant agrin-induced winners were more spotty in appearance than the AChR aggregates at the original soleus endplates in the same muscles but otherwise similar (Figs. 6c and 6d). Many agrin-induced winners were as large as the original soleus junctions, although, on average, they were smaller ( $285 \pm 25.7$

$\mu$ m<sup>2</sup>,  $n = 110$  vs  $391 \pm 28.9 \mu$ m<sup>2</sup>,  $n = 83$ , mean values  $\pm$  95% confidence intervals). The winners induced by transplanted fibular axons were considerably larger ( $604 \pm 51.7 \mu$ m<sup>2</sup>, Skorpen *et al.*, 1999) than both the agrin-induced winners and the original endplates in the soleus.

## DISCUSSION

We have studied the effects of neural agrin on the expression and distribution of AChRs in extrajunctional regions of rat soleus muscles and then compared these



**FIG. 5.** Direct muscle stimulation affects the length of AChR aggregation sites. The histograms represent the lengths of neural agrin-induced AChR aggregation sites in denervated/unstimulated (a) and denervated/stimulated (b) muscles. Unstimulated fibers contain predominantly small aggregation sites, whereas stimulated fibers contain longer aggregation sites distributed nearly symmetrically around a mean value.

effects with those of transplanted axons as they form ectopic neuromuscular junctions in the same regions. The effects were strikingly similar in every respect examined. Both neural agrin and transplanted axons induced on denervated inactive muscles fiber the appearance of numerous AChR aggregates, whose features and distribution along the fibers were similar. The axon-induced aggregates spanned longer stretches of fibers than the agrin-induced aggregates but this difference was expected because axons ramify extensively and often contact single muscle fibers along many mm of their length, while agrin is released from only a few, short fiber segments scattered throughout the muscle.

### ***Selection of Winners and Elimination of Losers***

The agrin- and axon-induced AChR aggregates responded similarly to electrical stimulation. In both cases, only a few aggregates survived the stimulation (the winners), while the rest disappeared (the losers). The elimination of losers occurred in activity-dependent refractory zones on each side of the winners. In ectopically innervated fibers these zones were, on average, 0.75 mm long (Skorpen *et al.*, 1999). In fibers exposed to neural agrin they were at least 0.2 mm long. A measure of the total length of the refractory zone around agrin-induced winners was not obtained because effective concentrations of agrin did not reach adjacent muscle fibers over a length of more than 0.4 mm. Some muscle fibers with agrin-induced AChR aggregates lost all their aggregates after the onset of electrical muscle stimulation. Perhaps the concentration of agrin was below some threshold for induction of stable, activity-resistant AChR aggregates at these sites.

### ***The Size of Winners***

The agrin-induced winners were considerably smaller than the axon-induced winners but similar in size to the denervated original endplates in the same muscles. Why transplanted fibular axons form larger junctions is not clear. Interestingly, when soleus axons are transplanted to extrajunctional regions of their own muscle, they form much smaller junctions than fibular axons transplanted to the same regions (Wærhaug and Lømo, 1994). This result shows that fast and slow types of motoneurons form ectopic junctions of different sizes, which may be related, at least in part, to the different firing patterns of such motoneurons (Hennig and Lømo, 1985). An interesting question for future studies is the extent to which a winner's size may depend on the

amount of agrin acting on it and the pattern of electrical activity in the muscle fibers.

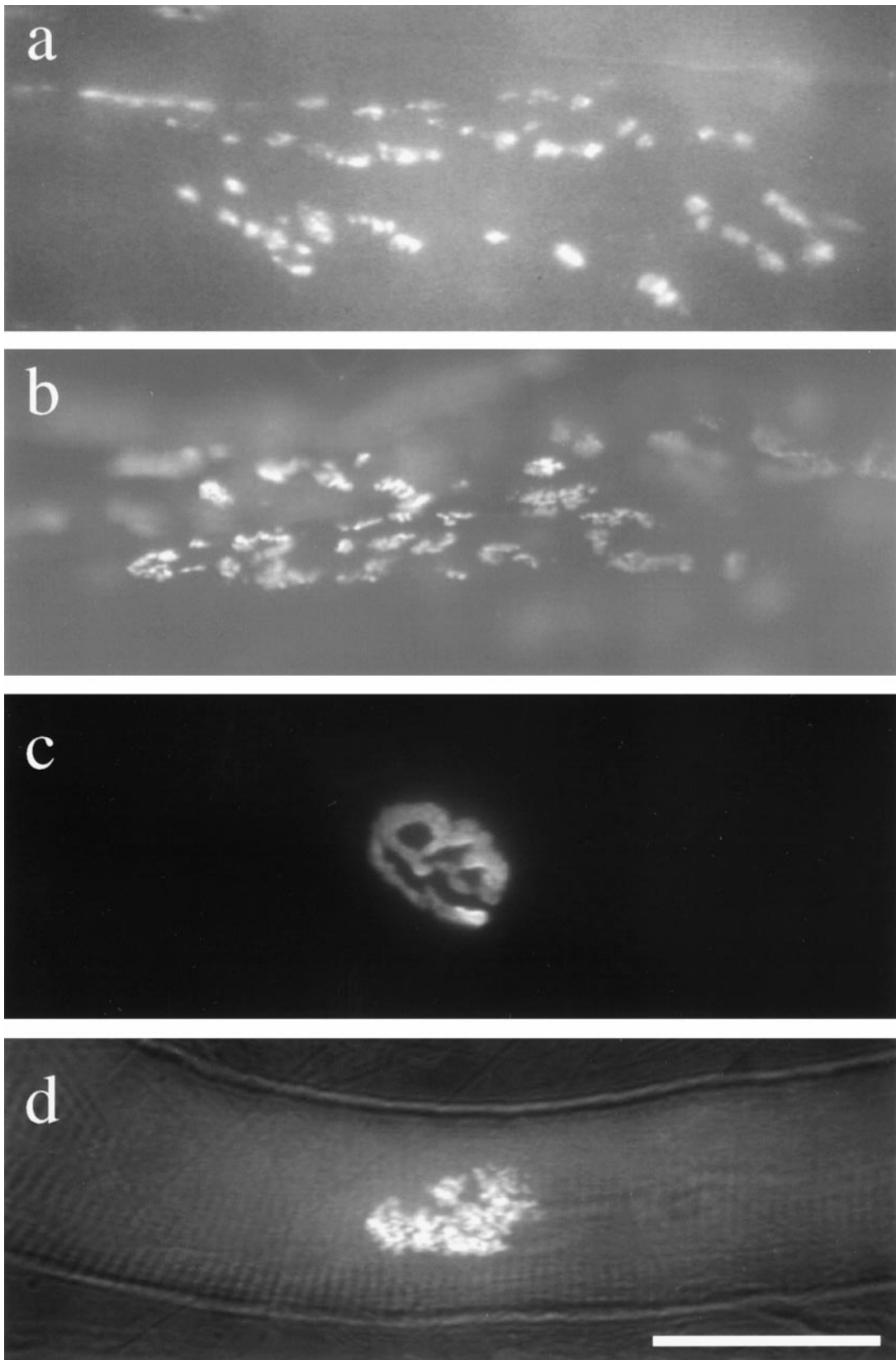
The agrin-induced winners lacked the sharp borders and ribbon-like pattern typical of normal junctions with their regularly arranged postsynaptic folds. Axon-induced winners become similarly disorganized if the axons are cut at an early stage of synaptogenesis and the muscle is stimulated directly (Skorpen *et al.*, 1999). In both cases the lack of orderly organization may be attributed to the absence of presynaptic elements during the activity-dependent maturation of the axon- or agrin-induced sites.

### ***Neural Agrin as Trophic Factor Interacting with Electrical Muscle Activity***

After early denervation, axon-induced winners reach normal size only if electrical muscle stimulation starts soon after the denervation. If the stimulation starts 2 weeks later, the winners become much fewer in number and smaller in size (Skorpen *et al.*, 1999). Similarly, after denervation, the original soleus junctions lose a substantial number of their AChRs, which can be prevented if direct muscle stimulation starts immediately after the denervation but not 2 weeks later (Andreose *et al.*, 1995). These results indicate that axons provide a trophic influence which, after denervation, persists like a trace in electrically active fibers but disappears in electrically inactive fibers. The similarities between agrin- and axon-induced AChR aggregates described above indicate that neural agrin can provide this trophic influence. One difference, however, deserves comment. Whereas muscle stimulation loses its effectiveness at axon-induced AChR aggregates when it starts late after denervation, the stimulation is equally effective at agrin-induced AChR aggregates whether it starts early or late after injection of agrin-DNA. But this difference can be accounted for by noting that nerve-derived agrin will eventually disappear at denervated aggregates, whereas neural agrin continues to be released by transfected fibers. An interesting problem for future studies, is whether agrin is the trace or whether it only induces the trace. A related problem is at which step or steps electrical muscle activity may modify the agrin pathway. The present model appears to be suitable for addressing such problems.

### ***Activity-Resistant Expression of Postjunctional Proteins at Agrin-Induced Winners***

If neural agrin is the trophic factor that induces the formation of the postsynaptic apparatus and, together



**FIG. 6.** Agrin-induced and nerve-induced AChR aggregates are similar in size and disposition. (a and b) AChR aggregates induced by transplanted axons at an immature stage of ectopic neuromuscular junction formation (a) and by neural agrin released from transfected muscle fiber segments (b). (c and d) AChR aggregates at an original soleus junction (c) and at a neural agrin-induced ectopic site that survived electrical muscle stimulation (d).

with electrical muscle activity, causes its maturation, it must make AChR expression in subsynaptic nuclei resistant to electrical muscle activity. Previous work has shown that neural agrin induces ectopic postsynaptic-like sites in innervated soleus muscles (Jones *et al.*, 1997; Meier *et al.*, 1997) and in denervated muscles where some sites survive after reinnervation of the original endplates (Rimer *et al.*, 1997). Such muscles are electrically active but they also contain neuromuscular junctions from which undetermined trophic effects may spread and affect ectopic AChR expression (Kues *et al.*, 1995; Witzemann *et al.*, 1991). Thus, neuron-derived trophic factors other than agrin cannot be rigorously excluded from being the stabilizers in such experiments. It has been shown in other experiments that neural agrin-induced AChR aggregates in denervated muscle survive electrical stimulation for 4–6 days (Jones *et al.*, 1997). But if the stimulation also stabilizes AChRs already in the aggregates, as reported for AChRs at synaptic sites (Caroni *et al.*, 1993), the AChRs labeled at 4–6 days could have been “old” AChRs already in the membrane of the postsynaptic-like apparatus at the time stimulation began rather than new AChRs derived from activity-resistant expression. In the present experiments, some agrin-induced AChR aggregation sites survived at least 10 weeks of electrical muscle stimulation and showed no obvious drop in number or size between 20 and 69 days of stimulation (the longest duration attempted). Assuming a half-life of adult type AChRs of no more than 10–12 days, as found at neuromuscular junctions in normal, denervated/reinnervated, and denervated/stimulated rat soleus muscles (Fumagalli *et al.*, 1990; Salpeter *et al.*, 1993), these results strongly indicate that neural agrin induces activity-resistant AChR expression in the absence of other nerve-derived factors. Our finding that accumulations of NRG and erbB3 persisted at agrin-induced AChR aggregates in long-term denervated and electrically stimulated muscles is consistent with the possibility that neural agrin induces AChR expression through muscle NRG and erbB receptors (Jones *et al.*, 1997; Meier *et al.*, 1998; Moscoso *et al.*, 1995; Rimer *et al.*, 1998).

### Conclusion

Neural agrin and transplanted axons induce on denervated rat soleus muscle fibers AChR aggregates that show similar activity-dependent development. In the

presence of electrical muscle activity, the aggregates undergo similar selection for survival (winners) or elimination (losers), development of winners to an appropriate size, and appearance of refractory zones on each side of the winners. These similarities suggest that neural agrin is the nerve-derived trophic factor, which, in combination with electrical muscle activity, elicits processes in the muscle fibers that regulate the size and distribution of ectopic neuromuscular junctions on adult rat soleus muscle fibers.

## EXPERIMENTAL METHODS

### *Animals, DNA Injections, and Chronic Muscle Stimulation*

Male Wistar rats (200–250 g.b.w.) were used. All operations were done under anesthesia by Equithesin (0.4 ml/100 g.b.w.) injected i.p. Both hind legs were denervated by cutting the sciatic nerve in the thigh, reflecting it and fixing it subcutaneously by a suture. In the same session or 3 or 4 days later, the soleus muscle on the right side was exposed and two multistranded teflon coated wires (AS632, Cooner Sales, Chatsworth, CA) implanted. The uninsulated end of one wire was placed dorsally across the proximal half of the soleus, that of the other wire ventrally across the distal half. From the lateral edge of the soleus, the insulated wires ran under the skin, through attachments to the skull by screws and dental cement into a flexible protective tube that allowed free movements within the animal's cage, to rotating contacts and a stimulator above the cage. The animals did not appear to suffer pain during the experiments. The experiments were conducted in conformity with the laws and regulations controlling experiments and procedures in live animals in Norway, i.e., the Welfare of Animals Act of 20 December 1974, Articles 21 and 22 and the regulations concerning Biological Experiments on Animals of 22 December 1977. The experiments involving chronic muscle stimulation have been inspected and permitted by the Norwegian Experimental Board and Ethical Committee for Animal Experiments and were overseen by the veterinarian responsible for the animal house.

Immediately after implantation of electrodes, 50 µg of agrin DNA (Y4Z8) in 50 µl 0.9% NaCl was injected into

**FIG. 6.** (Continued). The muscle in (a) was examined 20 days after transplantation of foreign axons and 6 days after denervation by section of the soleus axons. The muscle in (b) was examined 21 days after denervation and 17 days after injection of agrin-DNA. The muscle in (c) and (d) was examined 27 days after denervation of the original junctions, 24 days after injection of agrin-DNA, and 21 days after onset of stimulation.

the proximal half of the right SOL as well as the left SOL, using a sharp cannula (25G). Direct stimulation of the right SOL started after another 2–12 days, the pattern being 60 bipolar pulses at 100 Hz every 60 s, each pulse being 0.2 ms long and 8–12 mA strong in each direction. Both SOL muscles were removed after 20–69 days of stimulation and examined for ACh sensitivity (5 animals), presence of agrin and/or extrajunctional AChR aggregates (29 animals).

In other rats ( $n = 7$ ), the soleus was denervated as described above and exposed *in situ*. Micropipettes were filled with agrin-DNA dissolved in a solution of 10 mM NaCl, 0.1 mM EDTA, 100 mM Kalium gluconate, and 10 mM Tris-HCl at pH 7.5. After filling, the pipettes were sharpened on a microbeveler (WPI 48000) and, under a stereo microscope for fluorescence (Leica MZ12), inserted into single surface muscle fibers, while the resting membrane potential was monitored. Pressure of up to 3 bar was applied as pulses or continuously (Picospritzer II, General Valve Corp.) to inject agrin-DNA together with rhodamine labeled dextran (D-3308, MW 3000, Molecular Probes, Europe). Fibers injected with dextran were easily identified *in situ* by their red fluorescence 14–19 days after injection. The method of intracellular injection has been described in detail by Utvik *et al.* (1998).

In other experiments we transplanted the superficial fibular nerve to the proximal half of the soleus, cut the tibial nerve containing soleus axons in the thigh 2 weeks later (day 0), and removed the soleus 6 and 21 days later to examine the ectopic AChR aggregates induced by the fibular nerve. In some animals we also cut the fibular nerve on day 6, implanted stimulating electrodes on the soleus and started stimulation at 100 Hz (60 pulses every 1 min) on day 7, and removed the muscles on day 21. The results confirmed results obtained by Skorpen *et al.* (1999), which should be consulted for further details.

### ACh Sensitivity

To show that the muscle stimulation had been effective we examined the sensitivity of the muscles to iontophoretically applied ACh (Fumagalli *et al.*, 1990). When unstimulated muscles were placed in a bath 3 weeks after denervation, application of 2  $\mu$ M ACh perchlorate caused a prolonged rise in muscle tension to 40–72% of maximum tetanic tension ( $n = 5$ ). In contrast, in stimulated muscles denervated for a similar length of time there was either no rise ( $n = 4$ ) or a 0.5% rise in tension ( $n = 1$ ). Evidently, the stimulation had blocked the appearance of AChRs in extrajunctional regions of the denervated muscles and had therefore been effective.

### Immunohistochemistry

The following antibodies were used: a monoclonal antibody (Agrin-86) against neural agrin (Y4Z8) from StressGen, a polyclonal antibody raised in rabbits against a peptide sequence present in the EGF domain of all known NRG variants (Jo *et al.*, 1995), a polyclonal antibody against erbB-3 and its blocking peptide from Santa Cruz Biotechnology (Santa Cruz, CA), and a polyclonal antibody against rat AChE rabbit.

For labeling erbB-3, NRG, and AChR aggregates in cross-sections, the muscles were frozen in liquid nitrogen-cooled isopentane, stored at  $-80^{\circ}\text{C}$ , and cut transversely into 12- $\mu$ m-thick sections in a cryostat. The sections were then incubated with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA, Sigma) for 20–30 min at  $4^{\circ}\text{C}$  and then overnight at  $4^{\circ}\text{C}$  or for 3 h at room temperature with diluted primary antibody in 0.1% BSA in PBS. The sections were washed with three changes of PBS for 30 min and incubated 1 h at room temperature with goat anti-rabbit fluorescein-conjugated secondary antibody (Cappel, Organon Teknika Corp., Durham, NC) diluted 1/200 in 0.1% BSA in PBS, which, in addition, contained TRITC- $\alpha$ -bungarotoxin at 0.5-1  $\mu\text{g}/\text{ml}$  final concentration. The sections were washed in PBS and mounted with Citifluor (Citifluor Ltd., London, UK). Labeling of  $\epsilon$ - and  $\gamma$ -AChR subunits was performed as described in Rimer *et al.* (1997).

For labeling AChE and neural agrin in cross-sections the procedure was similar to (1) except that the sections were first fixed with 1% paraformaldehyde (PFA) in PBS for 10 min at room temperature, washed 3 times (5 min each time) in PBS containing 0.1% Triton X-100 (Sigma) (PBS-T), and blocked for 10 min with 10% normal goat serum in PBS-T, before the sections were incubated with the primary antibody.

For labeling neural agrin, AChE, and AChR aggregates in whole mounts of muscle fibers, the muscles were fixed by injecting 1% PFA in PBS into the center of the muscles, placing the muscles in 1% PFA for 10 min at room temperature, and washing them in PBS for 1–2 h, while the muscles were dissected into longitudinal bundles, each containing about 50–250 fibers. The bundles were then permeabilized in methanol at  $-20^{\circ}\text{C}$  for 10 min, washed, and incubated with antibodies and TRITC- $\alpha$ -bungarotoxin as described above. After mounting, a small weight was placed on top of the coverslip to flatten the bundles. Later, the coverslip was sometimes removed to allow further dissection into smaller bundles and single fibers. Aggregates of AChRs were digitally stored and analysed, using a Nikon Optiphot microscope, Hamamatsu SIT camera, Pixeltools framegrabber

card, IIfx Macintosh computer and the analysis program Image 1.22/1.33.

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