Syne proteins anchor muscle nuclei at the neuromuscular junction

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Vertebrate skeletal muscle fibers contain hundreds of nuclei, of which three to six are functionally specialized and stably anchored beneath the postsynaptic membrane at the neuromuscular junction (NMJ). The mechanisms that localize synaptic nuclei and the roles they play in neuromuscular development are unknown. Syne-1 is concentrated at the nuclear envelope of synaptic nuclei; its *Caenorhabditis elegans* orthologue ANC-1 functions to tether nuclei to the cytoskeleton. To test the involvement of Syne proteins in nuclear anchoring, we generated transgenic mice overexpressing the conserved C-terminal Klarsicht/ANC-1/Syne homology domain of Syne-1. The transgene acted in a dominant interfering fashion, displacing endogenous Syne-1 from the nuclear envelope. Muscle nuclei failed to aggregate at the NMJ in transgenic mice, demonstrating that localization and positioning of synaptic nuclei require Syne proteins. We then exploited this phenotype to show that synaptic nuclear aggregates are dispensable for maturation of the NMJ.

acetylcholine receptors | nuclear envelope | nuclear aggregates | synaptic nuclei | Syne-1

In many cells, nuclei occupy a specific asymmetric position within a voluminous cytoplasm, implying the existence of mechanisms that guide nuclei to appropriate sites and anchor them at those sites once they arrive. Processes that require proper functioning of these guidance mechanisms include fertilization, mitosis, migration, and polarization. Genetic studies have identified some of the molecules that regulate nuclear migration and anchoring in organisms as diverse as *Caenorhabditis elegans*, *Drosophila*, and fungi (1–4). Recent studies have also begun to elucidate mechanisms that underlie nuclear migration in vertebrates (4). On the other hand, little is known about molecules that anchor vertebrate nuclei in place once they have reached their destination.

Here, we address the issue of vertebrate nuclear anchoring with reference to the skeletal neuromuscular junction (NMJ). Each mammalian skeletal muscle fiber forms from the fusion of hundreds of myoblasts and therefore contains hundreds of nuclei. Most of these nuclei are relatively evenly spaced along the fiber, but a few (three to six) are functionally specialized and stably anchored beneath the postsynaptic membrane at the NMJ (5–9). Studies *in vitro* suggested that nuclei migrate through the cytoplasm of the newly formed myotube, and that a few become stably anchored beneath the postsynaptic membrane, whereupon they acquire their specialized properties (10). Nothing is known about how the nuclei become anchored.

A candidate anchoring molecule is Syne-1 (11), a mammalian orthologue of *C. elegans* ANC-1, which positions nuclei at specific locales within *C. elegans* somatic cells (12). Mammalian Syne-1 and -2 (also called nesprin, NUANCE, myne, and Enaptin), *C. elegans* ANC-1, and *Drosophila* MSP-300 all contain: (*i*) N-terminal calponin domains that bind filamentous actin, (*ii*) a large central spectrin-like rod domain, and (*iii*) a C-terminal Klarsicht/ANC-1/Syne homology (KASH) domain that spans the outer nuclear membrane (11, 13–18). Syne-1 is associated with envelopes of nuclei in muscle fibers, and its levels are significantly higher in synaptic than in nonsynaptic nuclei (11). Based on its pattern of localization and similarity to ANC-1, we hypothesized that Syne-1 plays a role in anchoring nuclei at the NMJ. We used a dominant negative strategy to test this hypothesis *in vivo*. This strategy also allowed us to ask whether synaptic nuclear aggregates are required for synaptic development and maintenance.

Materials and Methods

Transgenic Mice. To make the dominant negative Syne-1 (DNS) construct pDS115, the cytomegalovirus promoter of pCS2+MT (19, 20) was replaced with a 3.3-kb *Pfu* polymerase (Stratagene) amplified mouse muscle creatine kinase promoter (21) in the *Sal*I and *Cla*I sites of pCS2+MT. The C-terminal 344 amino acids of Syne-1 were amplified by using *Pfu* polymerase, and primers das207 (CGGAATTCAGCCTGGCTAGGAGAGACAGAG) and das208 (GCCGCTCGAGTCAGAGTGGAGGAGGACC). The amplified product was inserted into the *EcoR*I and *Xho*I sites of pCS2+MT downstream and in frame of a 6× myc tag. Finally, an IRES/GFP cassette (from pIRES2-EGFP; Clontech) was cloned downstream of Syne-1. The expression cassette was completely sequenced, separated from the plasmid backbone by *Not*I digestion, and used to generate transgenic mice by standard methods. Mice were genotyped by PCR from genomic tail clips by using primers in the *myc* tag and in the C terminus of Syne-1. Three transgene-positive lines were bred, but only one expressed DNS at levels detectable with the anti-myc antibody.

Electroporation. The DNS-IRES-GFP expression vector was dissolved into normal saline at a concentration of 2 μg/μl. Tibialis anterior muscles in 3-wk-old mice were injected with 50 μg of DNA and electroporated as described (22). Muscles were dissected 7–10 days after transfection and fixed for 30 min in 2% paraformaldehyde. Fiber bundles exhibiting GFP fluorescence were isolated under a fluorescent dissecting microscope and then stained as outlined below. Transfected fibers were identified by using anti-myc antibodies, rhodamine-α-bungarotoxin-(BTX), and DAPI-stained acetylcholine receptors (AChRs) and nuclei, respectively. Nontransfected (myc-negative) fibers within the same transfected muscle served as controls.

Histology. Tibialis anterior and sternomastoid muscles were isolated and either frozen in liquid nitrogen-cooled isopentane

Abbreviations: DNS, dominant negative Syne-1; NMJ, neuromuscular junction; BTX, rhodamine-α-bungarotoxin; AChR, acetylcholine receptor; P*n*, postnatal day *n*; KASH, Klarsicht/ANC-1/Syne homology.

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and sectioned at 8 μm or fixed for 20 min in 2% paraformaldehyde/PBS. Frozen sections were immunostained as described in ref. 11. Fixed muscles were teased into smaller bundles, incubated overnight with primary antibody diluted in PBS/1% BSA/0.4% Triton at 4°C, rinsed in PBS, incubated with diluted secondary antibodies for 1 h at room temperature, rinsed in PBS, dissected into individual fibers, and then mounted with antifading medium. All slides were viewed with either a confocal (Olympus, Melville, NY) or a compound fluorescent microscope (Zeiss). Mouse monoclonal, clone 9E10, and rabbit polyclonal anti-myc antibodies were from Developmental Studies Hybridoma Bank, University of Iowa, and Sigma, respectively; polyclonal rabbit antibodies against emerin and Sun-2 were kind gifts of G. Morris (North East Wales Institute, Wrexham, U.K.) (23) and P. Stahl (Washington University) (24), respectively; mouse monoclonal antilamin A/C and antiutrophin antibodies were from Chemicon and NovoCastra (Newcastle, U.K.), respectively; polyclonal antibodies to Syne-1 and the AChRγ subunit were generated in our laboratory (11, 25). The antibody to Syne-1 recognizes a region that is highly conserved in Syne-2 (71% identical) and may therefore recognize both proteins. Other reagents used were fluorescent goat anti-rabbit Alexa 488 and Alexa 568 (Molecular Probes); fluorescein-conjugated goat anti-mouse-IgG1 (Chemicon, Temecula, CA); BTX (Molecular Probes); and two DNA-binding dyes, DAPI (Sigma) and BOBO-1 (Molecular Probes).

For ultrastructural analysis, muscle was fixed in 4% glutaraldehyde/4% paraformaldehyde in PBS, washed, refixed in 1% OsO4, dehydrated, and embedded in resin. Thin sections were stained with lead citrate and uranyl acetate and scanned in an electron microscope. Densities of active zones and junctional folds were assessed as described (22, 26).

Results and Discussion

Nuclear Architecture in Muscles Expressing Dominant-Interfering Syne-1. A way to test the hypothesis that Syne proteins anchor nuclei to the NMJ was suggested by the finding that the C-terminal KASH domain of ANC-1 acts in a dominant negative fashion; its overexpression in *C. elegans* leads to a nuclear anchorage defect indistinguishable from that observed in *anc-1* null alleles (15). The KASH domains of mammalian Syne proteins appeared likely to act in a similar way: they mediate nuclear envelope localization in transfected tissue culture cells (14) and displace endogenous Syne from nuclei (13). We therefore generated transgenic mice in which the C-terminal 344 residues of Syne-1, including the KASH domain, were expressed under the control of muscle-specific regulatory elements from the muscle creatine kinase gene (Fig. 1*A*) (21). Six tandem copies of a 13-residue fragment of the *myc* gene were added to the N terminus of the construct as an epitope tag. We call the tagged fragment DNS.

In muscles from heterozygous DNS mice, >98% of nuclei within muscle fibers were myc-positive (Fig. 1*C′*), whereas nonmuscle nuclei (in connective tissue, blood vessels, and nerve trunks) were myc-negative (data not shown). Moreover, levels of endogenous Syne-1 at the nuclear envelope, assayed with antibodies to an epitope outside of the DNS region, were greatly decreased in the DNS-positive nuclei (Fig. 1 *B–E*). We conclude that its C terminus not only directs Syne-1 to the nuclear envelope of myotubes *in vivo* but also displaces endogenous Syne-1 from these nuclei. Thus, the DNS fragment acts as a dominant interfering construct.

Muscles express Syne-2 as well as Syne-1 (11). Because of the high degree of identity in the KASH domains of Syne-1 and -2 (11), DNS transgenic nuclei may have reduced levels of both Syne-1 and -2. No antibodies to Syne-2 were available, so we were unable to assess this possibility definitively. However, our antibody to Syne-1 is was generated to a region highly conserved with

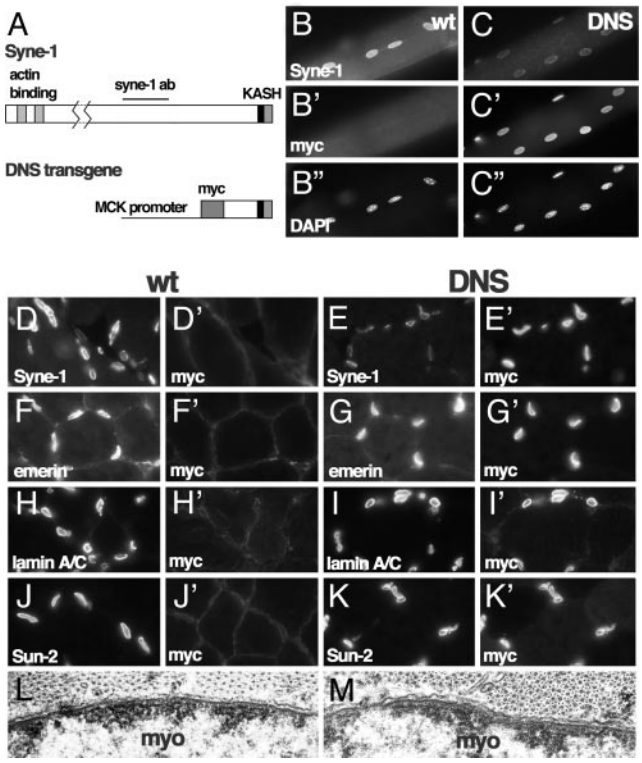

Fig. 1. Transgenic C-terminal domain of Syne-1 displaces endogenous Syne-1 from nuclei. (*A*) Structure of Syne-1 and the DNS transgene. The DNS protein was detected with an antibody to its N-terminal *myc* tag. Endogenous Syne-1 protein was detected by using a polyclonal antibody raised against a domain not included in the DNS transgene (11). (*B* and *C*) Longitudinal sections from wild-type and transgenic muscles, triply stained for Syne-1, myc, and DAPI. The myc-tagged DNS protein localizes to the nuclear envelope of muscle nuclei, and endogenous Syne-1 levels are decreased in myc+ nuclei. The preparations in *B* and *C* were processed in parallel and photographed with identical exposure times. (*D–K*) Cross sections of wild-type (*D*, *F*, *H*, and *J*) or DNS transgenic (*E*, *G*, *I*, and *K*) muscle were stained with antibodies against Syne-1 (*D* and *E*), emerin (*F* and *G*), lamin A/C (*H* and *I*), Sun2 (*J* and *K*), and myc (*D′–K′*). (*L* and *M*) Electron micrographs of nuclear envelopes of myonuclei. No differences were seen between control (*L*) and DNS muscles (*M*).

Syne-2 (71% identical over a 375-residue stretch, including a stretch of 39 of 40 identical residues), so it may recognize Syne-2. If so, our results suggest that neither Syne-1 nor -2 is present at significant levels in myonuclei of DNS mice. Hereafter, we refer to Syne-1 but note that effects documented below might reflect loss of both Syne proteins.

Syne proteins have been proposed to function with UNC-84 homologues Sun1 and -2 to span both membranes of the nuclear envelope, linking the nuclear lamina to the cytoplasmic cytoskeleton (1, 24). To ask whether Syne-1 is required for the localization of Sun and other nuclear envelope proteins, we stained muscles from DNS mice with antibodies to lamin, emerin, and Sun2. All three nuclear envelope markers stained myonuclei as strongly in DNS muscles as in controls, suggesting that most and probably all of Syne-1, including its spectrin repeats and calponin domains, are dispensable for gross organization of the nuclear envelope (Fig. 1 *F–K*). Likewise, the nuclear envelope in DNS transgenic muscles was normal by ultrastructural criteria, including separation of inner and outer membranes, lining by dense lamina, and presence of nuclear pores (Fig. 1 *L* and *M*). Thus, the DNS construct has a selective effect on Syne proteins, and Syne-1 is dispensable for assembly of the nuclear envelope.

Syne-1-Dependent Nuclear Anchoring at the NMJ. To test the role of Syne-1 in the localization of synaptic nuclei, fibers were teased

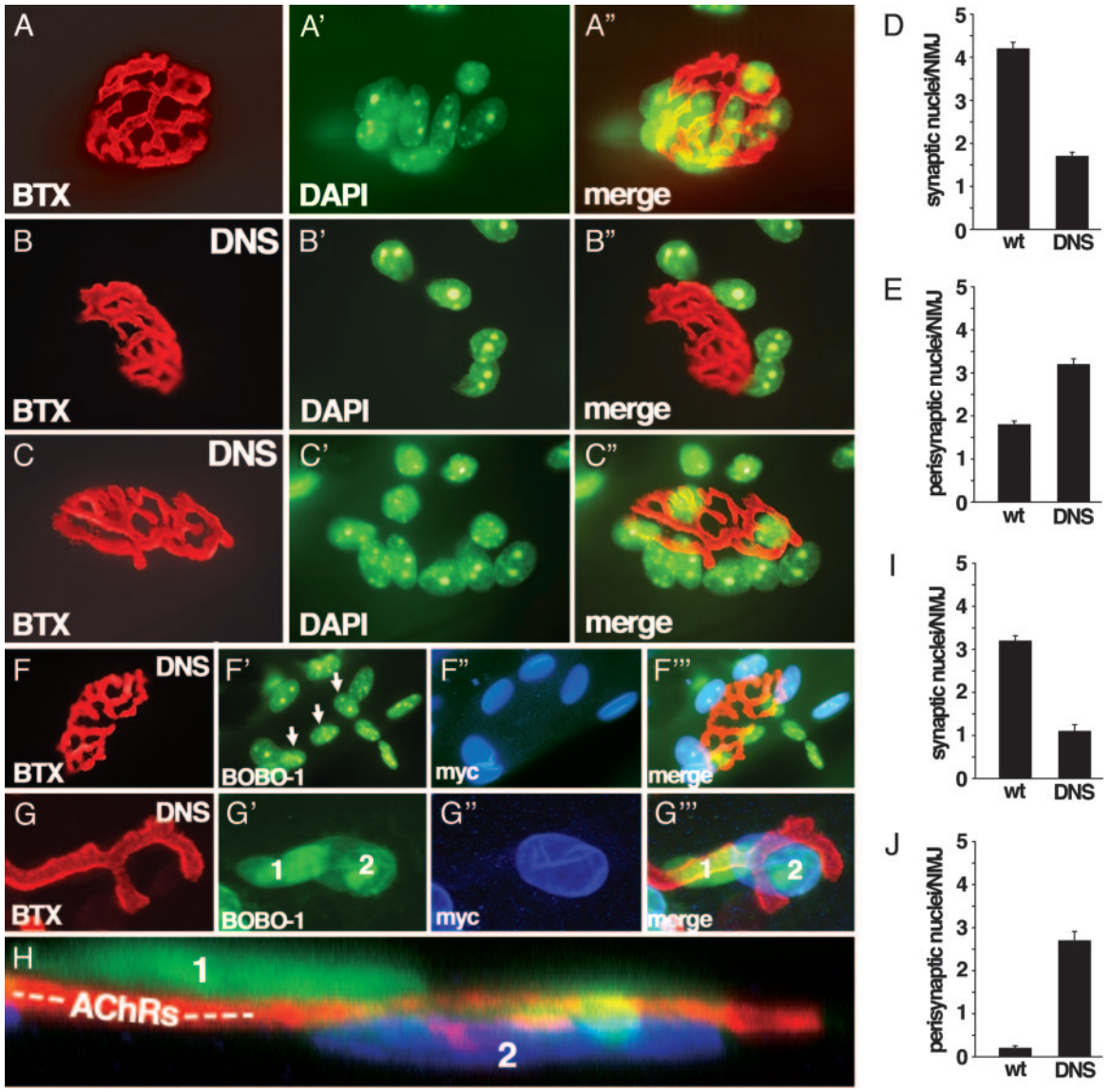

Fig. 2. Decreased numbers of synaptic nuclei and increased numbers of perisynaptic nuclei at NMJs of DNS transgenic mice. (*A–C*) Wild-type (*A*) and transgenic (*B* and *C*) endplates from P30 mice, stained with BTX to mark synapses (red) and DAPI to mark nuclei (green). (*A*) Nuclei are clustered at the wild-type endplate. (*B*) A transgenic NMJ with no synaptic nuclei and multiple perisynaptic nuclei. (*C*) Another transgenic NMJ with two synaptic nuclei and multiple perisynaptic nuclei. (*D* and *E*) Numbers of synaptic (*D*) and perisynaptic nuclei (*E*) in wild-type and DNS transgenic mice (mean ± SEM, $n = 150$ wild-type and 160 transgenic synapses; defined as in text). (*F–H*) Some myc-negative nuclei are associated with the NMJ (*F*, arrows), but confocal rotation (*G* and *H*) and lamin staining (see Fig. 6) show that they are external to the muscle fiber. To assess cellular location of nuclei, a stack of *z* sections (*G*) was rotated and viewed in the *z* plane (*H*). Nucleus 1 is myc-negative and external to the postsynaptic membrane, likely a Schwann cell. Nucleus 2 is myc-positive and within the muscle fiber. (*I* and *J*) The same data set as in *D* and *E* corrected to exclude nonmuscle nuclei.

from the muscles of 1-mo-old DNS mice and stained with a nuclear dye (DAPI) plus BTX, which labels the AChRs of the postsynaptic membrane (8). A nucleus was scored as synaptic if >25% of the DAPI-positive area overlapped with a BTX-positive synaptic site and as perisynaptic if it did not overlap a BTX-positive site but was less than half a nuclear diameter from a site's edge. As shown in Fig. 2 *A–E*, the number of synaptic nuclei was decreased by ≈60%, and the number of extrasynaptic nuclei was increased by ≈180% in DNS mice ($P < 0.0001$ by Student's *t* test). Both synaptic and perisynaptic nuclei were directly adjacent to the muscle membrane. The decrease in synaptic nuclei in DNS muscle nearly matched the increase in perisynaptic nuclei, so that the total number of synapse-associated nuclei (synaptic plus extrasynaptic) differed little between DNS transgenic and control muscle (6.0 vs. 5.0). Likewise, the density of nuclei in nonsynaptic regions of muscle fibers was unaffected by expression of DNS protein (control: 14 ± 0.5 vs. DNS: 14 ± 0.7 per 200-μm myotube; $P > 0.9$ by Student's *t* test). No changes were observed in the location, size, or shape of extrasynaptic nuclei. Thus, the DNS protein acts to shift nuclei from synaptic to perisynaptic sites, implying that endogenous Syne-1 promotes or maintains association of nuclei with synaptic sites.

The presence of supernumerary perisynaptic nuclei in DNS mice suggests that localization of synaptic nuclei involves at least two steps that differ in their dependence on Syne-1. For example, migration of nuclei to the vicinity of synapses could be Syne-independent (or require low levels of Syne), whereas anchoring of the nuclei beneath the postsynaptic membrane could be Syne-dependent (or require higher levels of Syne). This two-step

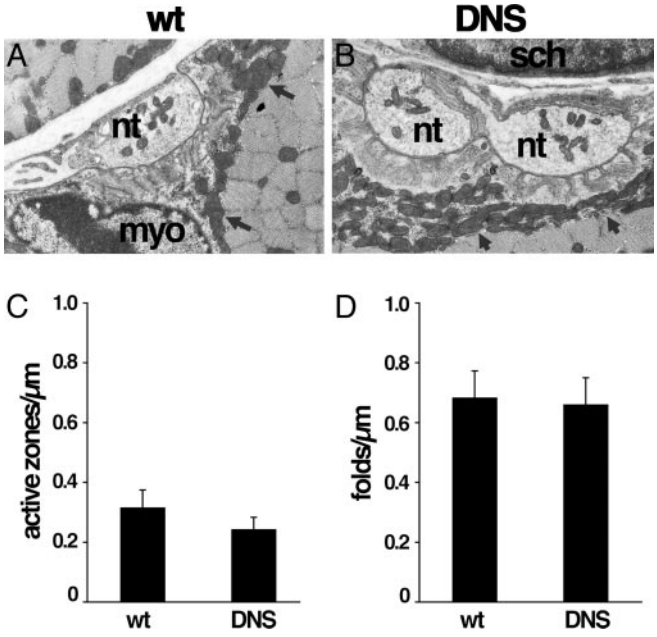

Fig. 3. Ultrastructure of NMJs in DNS mice. Electron micrographs of NMJs from wild-type (*A*) and DNS transgenic (*B*) mice. Ultrastructure of the nerve terminal (nt), infolded postsynaptic membrane, and overlying Schwann cells (sch) is normal. However, aggregates of mitochondria (arrows), that normally flank synaptic nuclei (myo), lie directly beneath the postsynaptic membrane in its absence. Densities of active zones in nerve terminals (*C*) and of junctional folds in the postsynaptic apparatus (*D*) were normal.

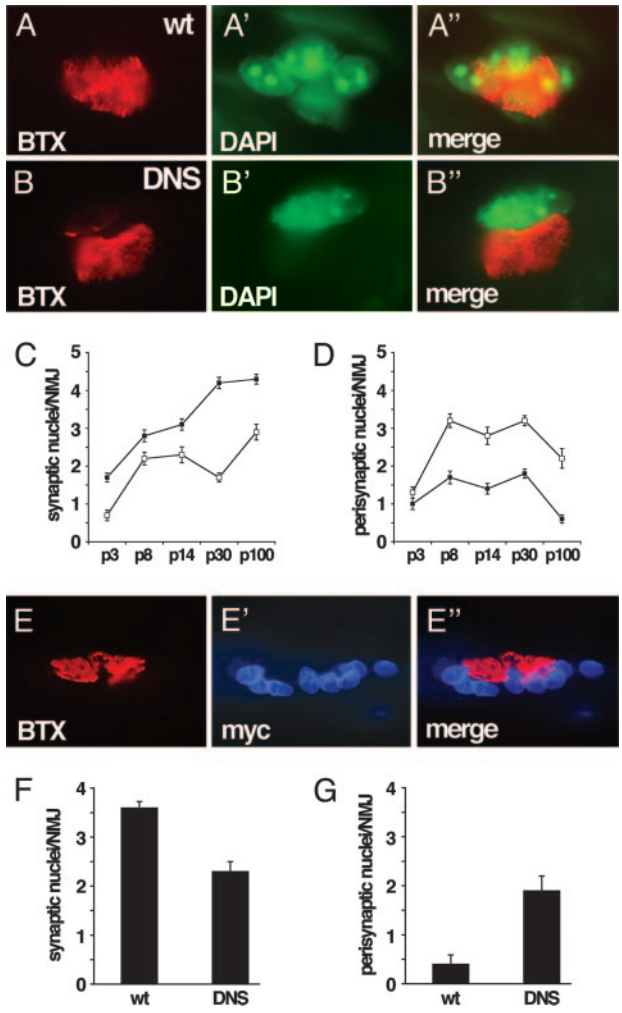

Fig. 4. Syne-1 is required for both formation and maintenance of synaptic nuclear aggregates. (*A* and *B*) Wild-type (*A*) and transgenic (*B*) endplates at P8, stained for BTX (red) and DAPI (green). Nuclei are displaced from the synapse by this stage in transgenic muscle. (*C* and *D*) Numbers of synaptic (*C*) and peripsynaptic (*D*) nuclei in wild-type (filled squares) and DNS transgenic muscles (open squares) as a function of age. (*E*) DNS-expressing fiber from a muscle transfected with DNS at P20, after synaptic nuclear aggregates had formed, then analyzed at P30. (*F* and *G*) Numbers of synaptic (*F*) and perisynaptic (*G*) nuclei in DNS-expressing and nonexpressing (control) fibers of muscles DNS-transfected muscles. (Data in *C*, *D*, *F*, and *G* are means ± SEM; $n \geq 50$ synapses for each point; fibers from four separate mice were used in *F* and *G*.)

model can explain why reduced levels of active Syne lead not only to a loss of synaptic nuclei but also to the acquisition of perisynaptic nuclear aggregates, which are seldom seem in controls.

We further examined nuclei that persisted at NMJs in DNS mice. Triple-staining for chromatin (DAPI), the DNS transgenic fragment (anti-myc), and AChRs (BTX) revealed that most residual synaptic nuclei in the transgenic muscles were DNS-negative (Fig. 2*F*). This result might indicate the presence of two populations of muscle nuclei, one of which lacked DNS. Alternatively, DNS-negative nuclei might be not in muscle fibers but rather within nonmuscle cells associated with the NMJ, including the Schwann (glial) cells that cap nerve terminals and synapse-associated fibroblasts (27, 28). We used two methods to distinguish between muscle from nonmuscle nuclei: (*i*) optical rotation of confocally reconstructed NMJs and (*ii*) staining for lamin, based on the fortuitous observation that nonmuscle nuclei stain more intensely for lamin than muscle nuclei. Both methods revealed that most DNS-negative synapse-associated nuclei were external to the muscle fiber (Fig. 2 *G* and *H* and Fig. 6, which is published as supporting information on the PNAS web site). When only muscle nuclei were counted, the number of synaptic nuclei was 66% less and the number of perisynaptic nuclei 10-fold higher in DNS muscles than in controls (Fig. 2 *I* and *J*). The total number of synaptic plus perisynaptic nuclei did not differ significantly between control and DNS muscles (3.4 vs. 3.8; $P > 0.05$). Interestingly, ≈40% of NMJs in the DNS transgenic muscle bore no synaptic nuclei at all, a pattern seen seldom if ever in controls. This analysis strengthens the conclusion that Syne-1 plays a specific role in the positioning of synaptic nuclei.

Role of Syne Proteins in Mitochondrial Anchoring. In *C. elegans*, ANC-1 is required for positioning of mitochondria as well as nuclei (12). We used electron microscopy to see whether Syne-1 has a similar function at mammalian synapses. In controls, aggregates of mitochondria flanked synaptic nuclei (Fig. 3*A*). Similar aggregates were also present in DNS muscles, suggesting that Syne-1 is dispensable for mitochondrial positioning. In many cases, mitochondrial aggregates in transgenic muscle were directly beneath the postsynaptic membrane (Fig. 3*B*). This result suggests a hierarchy of affinities of organelles for the postsynaptic apparatus, with synaptic nuclei more stably or tightly anchored than mitochondria; when nuclei are absent, mitochondria can take their place.

Role in Syne-1 in Formation and Maintenance of Nuclear Aggregates. The number of nuclei at each NMJ increases 2- to 4-fold during the first 4 postnatal weeks (Fig. 4*C*). Syne-1 could be involved in the formation of these nuclear aggregates or only in their long-term maintenance. To address this issue, we examined younger mice. At all ages examined, from postnatal day (P)3 to P100, DNS muscle

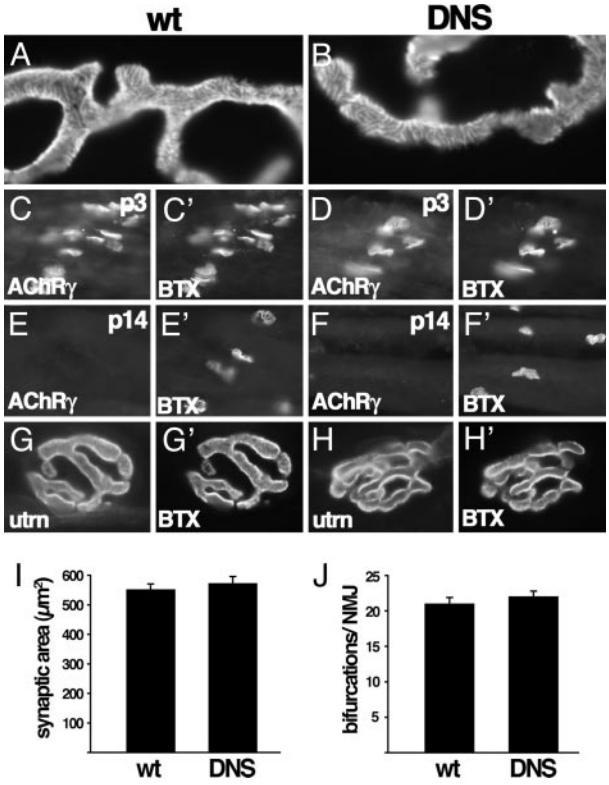

Fig. 5. Normal synaptic architecture in DNS muscles. High-power views of branches from wild-type (*A*) and transgenic (*B*) endplates, stained with BTX, to show striated appearance resulting from junctional folds. (*C–F*) Double staining with BTX plus antibodies to the fetal AChR subunit (γ) at P3 (*C* and *D*) and P14 (*E* and *F*) shows that it is replaced by the adult subunit (ε) on schedule in DNS mice. (*G* and *H*) Normal distribution of the synaptic cytoskeletal protein, utrophin, in DNS muscle. (*I* and *J*) AChR-rich area (*I*) and number of branch points (bifurcations) per synapse (*J*) in NMJs of wild-type and DNS muscles (mean ± SEM, $n = 30$ of each genotype).

fibers bore fewer synaptic nuclei and more perisynaptic nuclei than controls (Fig. 4 *A–D*). These results demonstrate a requirement for Syne-1 in the growth of nuclear clusters.

To ask whether the maintenance of nuclear aggregates also requires Syne, we used an electroporation method (22) to introduce DNS into muscles at P20, after aggregates had formed (Fig. 4*C*), then assessed nuclear distribution at P30. DNS-expressing fibers had fewer synaptic and more perisynaptic nuclei than untransfected fibers in the same muscles (Fig. 4 *E–G*). Thus, Syne is required for both maturation and maintenance of nuclear aggregates. The effect of DNS in these experiments also rules out the possibility that the phenotype documented above for DNS transgenic mice results from sequences at the transgene insertion site or from an effect on some prior aspect of myogenesis.

Effects of Disrupting the Clustering of Postsynaptic Nuclei on the Development of the NMJ. Finally, we used DNS mice to assess the roles of synaptic nuclei in the development and maintenance of the NMJ. These nuclei are transcriptionally specialized: they express genes encoding components of the postsynaptic membrane, such as AChR subunits, at far higher levels than extrasynaptic nuclei. This specialization leads to a local accumulation of mRNA and therefore local synthesis of synaptic components, which is presumed to contribute to synaptic differentiation or stability (6, 7). In addition, synaptic nuclei often occupy spaces between AChR-rich branches, suggesting that they influence synaptic topology. Surprisingly, however, we detected no defects in synaptic structure in DNS transgenic muscle. Normal features included the following: (*i*) AChRs were concentrated at apparently normal density (Fig. 5 *A* and *B*); (*ii*) the fetal γ-AChR subunit was lost on schedule during the first postnatal week, reflecting timely replacement by the adult ε-AChR subunit (Fig. 5 *C–F*); (*iii*) the molecular architecture of the postsynaptic membrane appeared unaffected, with normal colocalization of AChRs with utrophin (Fig. 5 *G* and *H*); (*iv*) synaptic size, branch number, and topology were normal (Figs. 5 *I* and *J*); (*v*) the ultrastructure of the nerve terminal, Schwann cells, and postsynaptic apparatus did not differ detectably between control and DNS mice, except for the loss of synaptic nuclei and their replacement by synaptic mitochondrial aggregates in the latter (Fig. 3 *A* and *B*); and (*vi*) densities of active zones in nerve terminals and of junctional folds in the postsynaptic apparatus were normal (Fig. 3 *C* and *D*). Together, these results suggest that synaptic nuclei are dispensable for normal synaptic maturation. One possible explanation is that perisynaptic nuclei in DNS mice remain transcriptionally specialized and synthesize messages for translation near the NMJ. Indeed, there is evidence for posttranscriptional control of local protein synthesis at the NMJ (29).

Conclusion

We draw five major conclusions from these results. First, nuclei in DNS muscles appear normal by immunohistochemical and ultrastructural criteria, indicating that Syne-1 is dispensable for major aspects of nuclear architecture. This is similar to *C. elegans anc-1* mutants, in which unanchored nuclei are grossly normal in structure (15). Second, the number of nuclei beneath the postsynaptic membrane of the NMJ is substantially reduced in DNS transgenic muscles. Thus, Syne-1 is required for the formation and/or maintenance of nuclear aggregates. No effects were noted on extrasynaptic nuclei, suggesting that the effect is selective. Interestingly, levels of Syne-1 are increased in the central myonuclei that characterize regenerating muscles (11); it will be interesting to determine whether Syne proteins are involved in the positioning of these nuclei. Third, the number of nuclei at the borders of the NMJ is substantially increased in DNS muscles, indicating that nuclear positioning in muscles is a multistep process. One model (see above) is that nuclei migrate to synaptic sites by a mechanism that is Syne-1-independent (or requires only low levels of Syne-1), whereas anchoring of nuclei to the subsynaptic membrane is Syne-1-dependent. In this regard, it is noteworthy that Syne-1 was initially identified by virtue of its ability to interact with the muscle-specific kinase (MuSK), a core component of the postsynaptic membrane at the NMJ (11). Syne-MuSK interactions might contribute to synaptic anchoring of nuclei. Fourth, although Syne-1 are required for clustering of nuclei at the NMJ, they are not required for positioning of mitochondria, further suggesting the existence of multiple mechanisms for organelle positioning. This is different from *C. elegans*, where ANC-1 is required to anchor both nuclei and mitochondria (12, 15). Fifth, we detected no defects in NMJs of DNS mice, despite a substantial reduction in the number of synaptic nuclei. By light microscopic criteria, even those NMJs completely devoid of subsynaptic nuclei appear normal. Thus, synaptic nuclear aggregates do not play an essential role in the maturation or maintenance of NMJs.

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1. Starr, D. A. & Han, M. (2003) *J. Cell Sci.* **116,** 211–216.
2. Reinsch, S. & Gonczy, P. (1998) *J. Cell Sci.* **111,** 2283–2295.
3. Patterson, K., Molofsky, A. B., Robinson, C., Acosta, S., Cater, C. & Fischer, J. A. (2004) *Mol. Biol. Cell* **15,** 600–610.
4. Morris, N. R. (2000) *J. Cell Biol.* **148,** 1097–1101.
5. Couteaux, R. (1973) in *Structure and Function of Muscle*, ed. Bourne, G. H. (Academic, New York), Vol. 2, pp. 483–530.
6. Schaeffer, L., de Kerchove d'Exaerde, A. & Changeux, J. P. (2001) *Neuron* **31,** 15–22.
7. Merlie, J. P. & Sanes, J. R. (1985) *Nature* **317,** 66–68.
8. Sanes, J. R. & Lichtman, J. W. (2001) *Nat. Rev. Neurosci.* **2,** 791–805.
9. Bruusgaard, J. C., Liestol, K., Ekmark, M., Kollstad, K. & Gundersen, K. (2003) *J. Physiol.* **551,** 467–478.
10. Englander, L. L. & Rubin, L. L. (1987) *J. Cell Biol.* **104,** 87–95.
11. Apel, E. D., Lewis, R. M., Grady, R. M. & Sanes, J. R. (2000) *J. Biol. Chem.* **275,** 31986–31995.
12. Hedgecock, E. M. & Thomson, J. N. (1982) *Cell* **30,** 321–330.
13. Zhen, Y. Y., Libotte, T., Munck, M., Noegel, A. A. & Korenbaum, E. (2002) *J. Cell Sci.* **115,** 3207–3222.
14. Zhang, Q., Skepper, J. N., Yang, F., Davies, J. D., Hegyi, L., Roberts, R. G., Weissberg, P. L., Ellis, J. A. & Shanahan, C. M. (2001) *J. Cell Sci.* **114,** 4485–4498.
15. Starr, D. A. & Han, M. (2002) *Science* **298,** 406–409.
16. Mislow, J. M., Kim, M. S., Davis, D. B. & McNally, E. M. (2002) *J. Cell Sci.* **115,** 61–70.
17. Rosenberg-Hasson, Y., Renert-Pasca, M. & Volk, T. (1996) *Mech. Dev.* **60,** 83–94.
18. Padmakumar, V. C., Abraham, S., Braune, S., Noegel, A. A., Tunggal, B., Karakesisoglou, I. & Korenbaum, E. (2004) *Exp. Cell Res.* **295,** 330–339.
19. Turner, D. L. & Weintraub, H. (1994) *Genes Dev.* **8,** 1434–1447.
20. Rupp, R. A., Snider, L. & Weintraub, H. (1994) *Genes Dev.* **8,** 1311–1323.
21. Jaynes, J. B., Johnson, J. E., Buskin, J. N., Gartside, C. L. & Hauschka, S. D. (1988) *Mol. Cell. Biol.* **8,** 62–70.
22. Grady, R. M., Akaaboune, M., Cohen, A. L., Maimone, M. M., Lichtman, J. W. & Sanes, J. R. (2003) *J. Cell Biol.* **160,** 741–752.
23. Manilal, S., Nguyen, T. M., Sewry, C. A. & Morris, G. E. (1996) *Hum. Mol. Genet.* **5,** 801–808.
24. Hodzic, D. M., Yeater, D. B., Bengtsson, L., Otto, H. & Stahl, P. D. (2004) *J. Biol. Chem.* **279,** 25805–25812.
25. Missias, A. C., Chu, G. C., Klocke, B. J., Sanes, J. R. & Merlie, J. P. (1996) *Dev. Biol.* **179,** 223–238.
26. Patton, B. L., Cunningham, J. M., Thyboll, J., Kortesmaa, J., Westerblad, H., Edstrom, L., Tryggvason, K. & Sanes, J. R. (2001) *Nat. Neurosci.* **4,** 597–604.
27. Koirala, S., Reddy, L. V. & Ko, C. P. (2003) *J. Neurocytol.* **32,** 987–1002.
28. Gatchalian, C. L., Schachner, M. & Sanes, J. R. (1989) *J. Cell Biol.* **108,** 1873–1890.
29. Chakkalakal, J. V. & Jasmin, B. J. (2003) *BioEssays* **25,** 25–31.