Comment on "Reelin Promotes Peripheral Synapse Elimination and Maturation"

Individual synaptic sites on rodent skeletal muscle fibers are innervated by two or more axons at birth. All inputs but one are then eliminated during the first two postnatal weeks, leaving each muscle fiber singly innervated (*1*). Quattrocchi *et al*. (*2*) reported that polyneuronal innervation of neuromuscular synapses persists well beyond this period in *reeler* mice (mutant mice lacking Reelin, a protein important for brain development). Their evidence was in large part histological: Axons were labeled with antibodies to neurofilaments and synaptic vesicle proteins, and postsynaptic sites were labeled with α-bungarotoxin, which binds specifically to acetylcholine receptors in the postsynaptic membrane. Neuromuscular junctions were described as showing multiple axon branches converging on an individual postsynaptic site. The finding that Reelin plays a key regulatory role in the much-studied but poorly understood process of synapse elimination has attracted considerable attention (*3*).

Eager to further analyze roles of Reelin in the neuromuscular system, we stained muscles using the protocols outlined in (*2*). In addition, we crossed *reeler* mutants to transgenic mice in which motor axons were labeled by expression of green fluorescent protein (GFP) or its yellow spectral variant, YFP [(*4*); Figs. 1 and 2]. The *reeler* genotype was confirmed by symptomatology (ataxia), polymerase chain reaction (PCR), and brain histology (disruption of cortical and/or cerebellar lamination) (data not shown).

We assessed polyneuronal innervation in muscles from a total of 13 *reeler* mutants. We imaged neuromuscular junctions in the diaphragm, triangularis sterni, sternomastoid, soleus, tibialis anterior, and extensor digitorum longus muscles from at least two mutant animals. This set includes most of the muscles studied in (*2*). Ten of the mutants were studied at ages after synapse elimination is complete in control animals [postnatal day (P)15–39]. We found no evidence of extensive polyneuronal innervation in any of these *reeler* muscles. That is, ≤2% of neuromuscular junctions per muscle were polyneuronally innervated in both mutants and controls. In contrast, Quattrocchi *et al*. reported an incidence of 42.7% polyneuronal innervation at P30 (*2*). In a few cases (<10%), single endplates appeared to be innervated by two axons when viewed in thick confocal stacks. However, examination of single optical sections or rotation of the stack showed that the majority of these represented superimposition of two neighboring endplates, each innervated by a single axon (see insets in Fig. 2A; synapses 8/9 and 24/30).

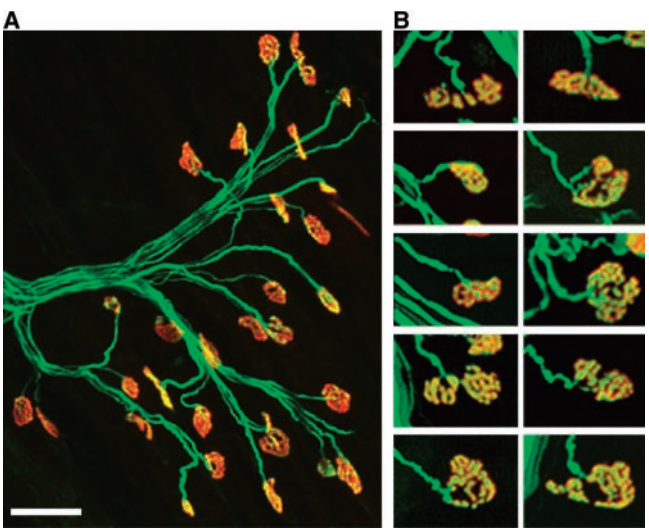

Fig. 1. Single innervation of synaptic sites in *reeler* mice. (**A**) Low power view of a P20 *reeler* muscle stained with antibodies to neurofilaments. (**B**) High power views of individual synaptic sites from triangularis sterni of a P26 *reeler* mouse in which axons were GFP-positive. In each case, postsynaptic acetylcholine receptors were stained with rhodamine-bungarotoxin. Scale bar, 50 μm.

We also studied muscles from three *reeler* mice and three control mice during the final days of naturally occurring synapse elimination, and contrary to (*2*), found no major delay in synapse elimination in the mutant. At P13, for example, we found 5.4% polyneuronal innervation in hindlimb muscles of *reeler* mice (n = 292 fibers) and 4.2% polyneuronal innervation in controls (n = 252).

Quattrocchi *et al*. (*2*) also reported that the number of synaptic sites per muscle was increased by 79.2% in the *reeler* diaphragm, and that the density of synapses in sections was increased 3.9-fold in the *reeler* soleus. Because nearly all fibers bear a single synapse in control mice (*1*), this result indicates that some mutant fibers bear two or more neuromuscular junctions. In our analysis, however, the total number of junctional sites per hemidiaphragm did not differ between *reeler* mice (mean ± SD: 3640 ± 693, n = 3) and littermate controls (3344 ± 463, n = 3, P = 0.57, Student's t test). We also stained diaphragms for acetylcholinesterase as an alternate means of marking synaptic sites (*5*), and then teased fibers from the muscles. Of 134 fibers examined, none had more than one synaptic site (Fig. 3).

Quattrocchi *et al*. (*2*) further stated that synaptic maturation is impaired in the absence of Reelin, reporting a 31 to 57.2% decrease in neuromuscular junction area at P7–P30 [table 1 in (*2*)]. They argued that this decrease could not be accounted for by the muscle atrophy that occurs in *reeler* mutants. We also noted that muscles were atrophic in older *reeler* mice and found a decreased synaptic size (acetylcholine receptor-rich area at P28 in diaphragm: control mean ± SEM: 347 ± 13.5 μm^2, n = 24; *reeler*, 291 ± 13.0 μm^2, n = 23; P < 0.01, Student's t test). However, we believe that atrophy could account for the decreased synaptic size; when we measured synaptic area in the diaphragm at P21, before atrophy is severe, we found no significant difference between control (mean ± SEM: 187 ± 8.4 μm^2, n = 28) and *reeler* mice (177 ± 7.2 μm^2, n = 29, P = 0.39).

As a second measure of maturation, Quattrocchi *et al*. (*2*) took advantage of the fact that the initially plaque-shaped postsynaptic apparatus becomes perforated and branched postnatally (*1*). They determined the percentage of synaptic sites that had >1 perforation in hindlimb muscle and reported that this fraction was 82.8% in controls, but only 35.8% in mutants at P30. We made similar measurements at P26 in triangularis sterni muscle, but found no difference in perforation (mean number of perforations or openings: 4.84 in *reeler*, 4.97 in littermate control; n = 150 synapses measured in each animal; P = 0.54, Student's t test).

Although the same mutant allele sometimes has different phenotypes in different genetic backgrounds, we do not believe that strain differences explain our inability to

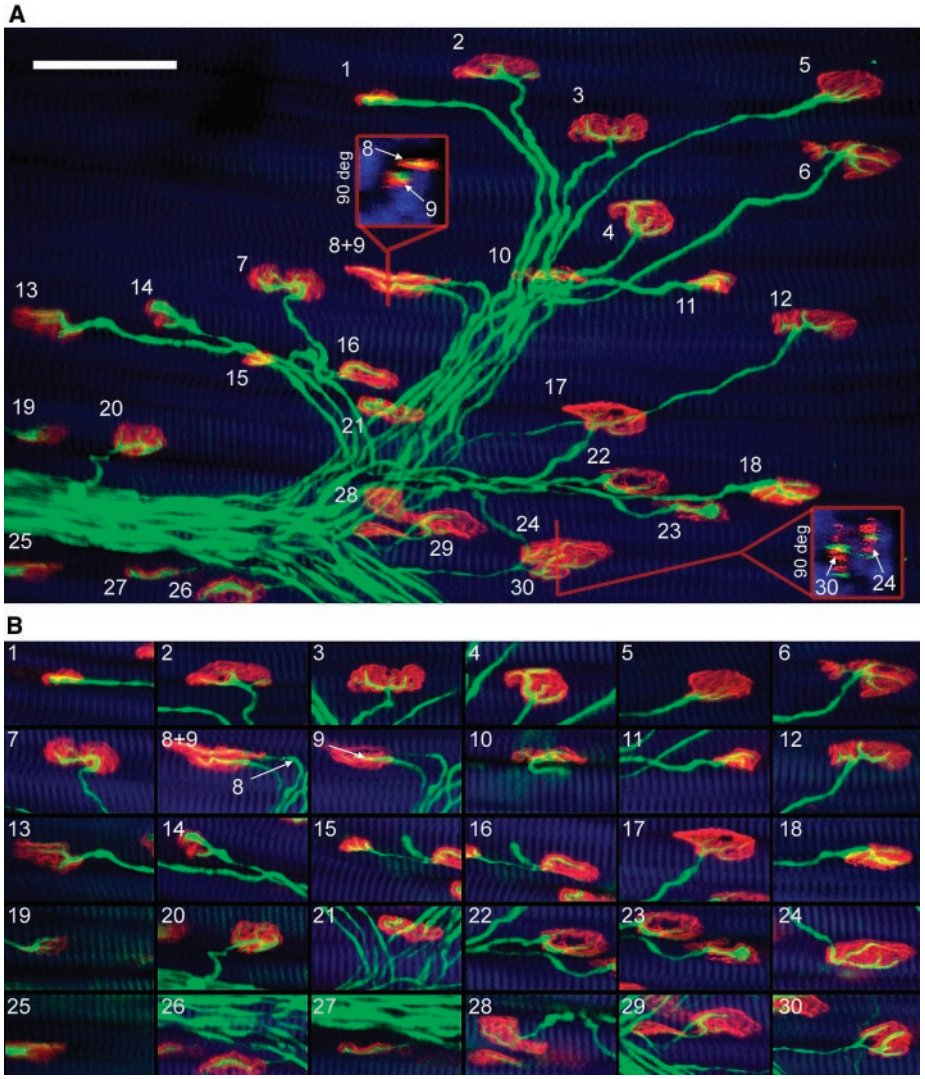

Fig. 2. Analysis of 30 synaptic sites in a single field from the triangularis sterni muscle of a P20 *reeler* mouse. Axons were stained with antibodies to neurofilaments (green), postsynaptic acetylcholine receptors were stained with bungarotoxin (red), and muscle fibers were stained with phalloidin (blue). (**A**) Projection of the entire confocal stack of the field. Insets show digital rotation of two pairs of junctions around the axis indicated by the red line. (**B**) Individual synaptic sites, numbered as in (A), but shown as projections from a few optical sections centered on the site. In the whole stack, synapses 8/9 and 24/30 appear to be single endplates innervated by two axons, but thinner stacks and rotation of the stack showed that they were two superimposed endplates, each innervated by a single axon. Scale bar, 50 μm.

replicate the results of Quattrocchi *et al*. (*2*). Weinzierl and Feng (Durham, NC) used *reeler* mice from Jackson Laboratory (Bar Harbor, ME), as did Quattrocchi *et al*. Bidoia, Buffelli, and Cangiano (Verona, Italy) used *reeler* mice originally obtained from Jackson Laboratories (gift of F. Keller, Universita Campus Bio-Medico, Rome) and subsequently crossed to YFP-16 mice (*4*); *reeler*,YFP-16 mice and littermate controls were analyzed, as well as *reeler* mice from the same litters that were YFP-negative. Misgeld, Lichtman and Sanes (St. Louis, MO) used *reeler* mice originally obtained from Jackson Laboratory and subsequently crossed to GFP-I mice (*4*) for several generations; *reeler*,GFP-I mice and littermate unaffected GFP-I mice were analyzed, as well as *reeler* mice from the same litters that were GFP-negative. The St. Louis group also examined a *reeler* mouse from the colony used in (*2*), kindly provided by G. D'Arcangelo (Baylor College of Medicine, Houston, TX) and illustrated in Fig. 2.

In summary, three groups of investigators, using animals from four different colonies, have been unable to replicate the main findings of Quattrocchi *et al*. (*2*). The Verona and St. Louis groups both assessed polyneuronal innervation. The Durham and Verona groups both assessed synapse number. The Durham and St. Louis groups both assessed synaptic maturity. We have no ready explanation for the difference between our results and those of Quattrocchi *et al*. (*2*).

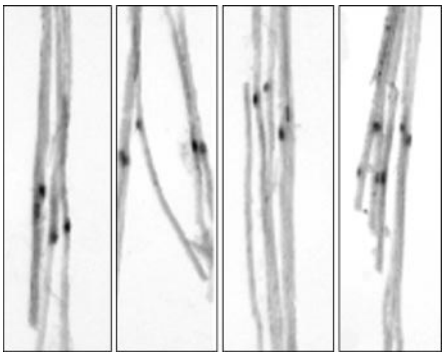

Fig. 3. Single synaptic sites on muscle fibers in *reeler* mice. Muscle fibers were teased from a P70 *reeler* diaphragm that had been stained for acetylcholinesterase.

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1 December 2003; accepted 1 March 2004

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Science **303** (5666), 1977.
DOI: 10.1126/science.1094146