INTRODUCTION

The safety factor of synaptic transmission at the neuromuscular junction depends on the high density of acetylcholine receptors (ACHRs) in the postsynaptic muscle membrane and on the large amount of transmitter released from the presynaptic nerve terminal with each impulse. In mammalian muscle, an inverse relationship has been observed between quantal size and quantum content. This apparently compensatory relationship has been demonstrated in patients with myasthenia gravis, and quantal content. This apparently compensatory relation-

ship between functional AChR density and ACh release in postsynaptic muscle membranes depends on the high density of acetylcholine receptors (AChRs) in the postsynaptic muscle membrane. In adult mice heterozygous for targeted deletion of type I neuregulins (Ig-NRG+/−), postsynaptic AChR density was decreased and transmitter release was increased. We examined the relationship between functional AChR density and ACh release in postnatal day 7 (P7), P14, and adult NRG-deficient mice. Here we report that changes in postsynaptic sensitivity and transmitter release are not temporally coupled during postnatal development in Ig-NRG−deficient mice. Although miniature endplate potential (MEPP) amplitude was decreased compared with control in P7 Ig-NRG+/− mice, quantum content was not increased. Quantum content was increased in adult heterozygotes despite normal MEPP amplitudes. Thus, during postnatal maturation, both quantal size and quantum content were influenced by decreased Ig-NRG expression, although the effects were dissociated in time.

METHODS

Animals

Mice used in this study were rederived from mice heterozygous for a targeted disruption of the NRG-1, IgG-like domain, which had been maintained on the original mixed background (Kramer et al. 1996). Thus the mice were backcrossed once into one of the parental strains, C57Bl/6. Mice were maintained and bred under standard conditions, consistent with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee.

Electrophysiology

Synaptic transmission was assayed in postnatal day 7, day 14, and adult Ig-NRG heterozygous and wild-type mice. Spontaneous MEPPs and nerve-evoked EPPs were measured with intracellular microelec-
trodes (17–30 MΩ) in isolated diaphragm–phrenic nerve preparations pinned at resting length in specially designed Sylgard (DuPont)-coated Plexiglas perfusion chambers. Electrodes were placed at the edge of each endplate. Motor nerve terminals were labeled with yellow fluorescent protein (YFP) by crossing Ig-NRG<sup>+/−</sup> mice with transgenic mice expressing YFP under the control of neuron-specific elements of the <i>thy1</i> gene (Feng et al. 2000), a kind gift of Joshua Sanes. Phrenic nerves were stimulated through a suction electrode. Extracellular solution [composed (in mM) of NaCl (125), NaHCO<sub>3</sub> (26), NaH<sub>2</sub>PO<sub>4</sub> (1.25), KCl (2.5), CaCl<sub>2</sub> (2.0), and MgCl<sub>2</sub> (1.0)] was bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at 22°C by a specially designed temperature exchange system attached to a circulating water bath (MultiTempIII, Pharmacia Biotech). For focal extracellular recording, microelectrodes (4 MΩ) were filled with 1 M NaCl. To determine endplate size, AChRs were labeled in live whole mounts with Texas red–or Alexa Fluor 594 (AF594)–labeled α-bungarotoxin (Molecular Probes).

MEPPs occur at low frequency at young end plates and thus data were combined from many end plates in several mice in each category. EPPs were recorded in elevated extracellular Mg (12 mM) to decrease the probability of presynaptic ACh release, which eliminates the contribution of MEPPs from those nerve fibers that contain the target muscle. EPPs were recorded in elevated extracellular Mg (12 mM) to decrease the probability of presynaptic ACh release, which eliminates the contribution of MEPPs from those nerve fibers that contain the target muscle. EPPs were recorded in elevated extracellular Mg (12 mM) to decrease the probability of presynaptic ACh release, which eliminates the contribution of MEPPs from those nerve fibers that contain the target muscle.

For real-time PCR standards, PCR fragments generated from genomic DNA using each primer set were amplified and cloned into the PCRII vector using an Invitrogen TA cloning kit.

**RESULTS**

We investigated synaptic transmission in phrenic nerve–diaphragm whole mounts dissected from 7-day-old mice. As summarized in Fig. 1 and Table 1, mean MEPP amplitude was reduced by 20% in Ig-NRG<sup>+/−</sup> mice compared with wild-type (wt) controls. This small but significant effect was observed in normal extracellular magnesium (1 mM) and also in 12 mM Mg (Fig. 1A), even though MEPP amplitude is substantially reduced when recording in high magnesium (Del Castillo and Engbaek 1954; Plomp et al. 1994). The decrease in Ig-NRG<sup>+/−</sup> MEPP amplitude at P (20–25%) is comparable to our previous finding in adult Ig-NRG<sup>+/−</sup> mice (Sandrock et al. 1997).

Despite the decrease in quantal size, mean quantum content was not increased in heterozygous mice at this early stage of development. Rather, the mean stimulus-evoked EPP was reduced, as was mean quantum content (Fig. 1B). There was a

**Quantification of mRNA**

Relative mRNA expression was determined by real-time PCR using the Roche LightCycler. Total RNA was isolated from mouse spinal cord with Trizol reagent (Invitrogen), treated with RNase-free DNase (Promega), extracted with phenol/chloroform, precipitated with NaOAc ET<sub>OH</sub>, and dissolved in DEPC-treated distilled water (dH<sub>2</sub>O). First-strand cDNA was synthesized using reverse transcription (RT) reagent from Invitrogen. Briefly, 3 μg of cleaned total RNA in 10 μl of H<sub>2</sub>O was denatured at 65°C for 5 min, then chilled on ice for 5 min. RT mix (1.5 μl of 150 ng/μl random primer, 1.5 μl of 10 mM dNTPs, 3 μl of 1 M DTT, 6 μl of 5 × buffer, 1 μl of RNase inhibitor, in DEPC-treated H<sub>2</sub>O; total volume 18.5 μl) was added and mixed well, then incubated at 42°C for 2 min. SuperscriptII (1.5 μl, Invitrogen) was added, incubated at 42°C for 1 h, then inactivated at 70°C for 15 min. The 20-μl real-time PCR reaction contained 2 μl cDNA, 2 μl M<sub>g</sub>Cl<sub>2</sub> 0.25 μM each forward and reverse primers, and 2 μl FastStart DNA Master SYBR Green I (Roche) in dH<sub>2</sub>O. Primer sequences were as follows:

- **GAPDH**: 5′ TCA CCA CCA TGG AGA AGG C 3′
- **Ig-NRG**: 5′ ATG AAG AGC CAG GAG TCA GC 3′
- **EGF-NRG**: 5′ CAC ATC TAC ATC CAC GAC TG 3′
- **5′ AGT TTT GGC AAC GAT CAC C 3′

For real-time PCR standards, PCR fragments generated from genomic DNA using each primer set were amplified and cloned into the PCRII vector using an Invitrogen TA cloning kit.

**FIG. 1.** Spontaneous miniature end-plate potentials (MEPPs) are reduced in postnatal day 7 (P7) Ig-NRG<sup>+/−</sup> mice. A: MEPP amplitude is reduced both in 1 mM extracellular Mg and in 12 mM Mg. B: nerve-evoked end-plate potential (EPP) amplitude and quantal content are also reduced in P7 mice. Estimates of quantal content measured by the method of failures (m<sub>0</sub>) or by the ratio of EPP/MEPP (m<sub>0</sub>) are in good agreement. C: superimposed traces show examples of sequential EPPs (n = 20). Scale: 10 ms; 0.5 mV.
close correlation between estimates of quantum content calculated by the method of failures ($m_0$) or by the ratio of mean MEPP to mean EPP ($m_1$). Representative traces are shown in Fig. 1C. At this age, about one half of the end plates are innervated by more than one axon (Sanes and Lichtman 2001). We selected singly innervated fibers by including only those junctions that exhibited smoothly rising EPPs. Nevertheless it is possible that some multiply innervated fibers were included in our sample. Because multiple innervation increases quantal content (Santafe et al. 2001), the reduction at P7 may be greater than we report here. Thus at P7, the decrease in MEPP amplitude was not accompanied by an increase in transmitter release.

Transmitter release clearly was increased in adult Ig-NRG$^{+/−}$ mice. Mean evoked EPP amplitude was increased by 73%, as was mean quantum content (Fig. 2A and Table 1). Based on our earlier studies, we expected the increase in transmitter release to be accompanied by a decrease in quantal size. However, in this series of experiments, MEPP amplitudes recorded in adult Ig-NRG$^{+/−}$ mice were not different from control (Fig. 2B). This was observed both in 1 and in 12 mM Mg. Representative traces are shown in Fig. 2C. We confirmed that Ig-NRG mRNA was reduced in our Ig-NRG$^{+/−}$ mice by quantitative RT-PCR. Comparison with wild-type adults showed that transcripts containing the NRG IgG-like domain were decreased by about 45% (het/wt, 0.54 ± 0.06 in nine pairs). IgG-containing isoforms represent only about 10% of NRG message in motor neurons (Corfas et al. 1995). As expected, total NRG mRNA (messages containing the EGF-like domain) showed little change (het/wt, EGF-NRG, 0.90 ± 0.11). Thus in adult Ig-NRG$^{+/−}$ mice, we found a dissociation between quantal size and quantal content. In contrast to P7, transmitter release was increased despite normal MEPP amplitude.

Embryonic and adult AChRs have different channel properties (Fischbach and Schuetze 1980; Sakmann and Brenner 1978). During the second postnatal week, the embryonic $γ$-subunit characterized by a relatively long mean channel open time (4.5 ms), is replaced by the adult $δ$-subunit characterized by a brief open time (1.4 ms) and a small increase in channel conductance (1.5-fold). Thus if $γ$-subunit expression persisted in adult Ig-NRG$^{+/−}$ mice, it is possible that MEPP amplitude might overestimate receptor density if channel open time was more significant than peak conductance in determining net synaptic current. To test this possibility, we analyzed the decay of miniature end-plate currents (MEPCs) detected by focal extracellular recording. Both Ig-NRG$^{+/−}$ and wt MEPCs showed the rapid decay characteristic of adult receptors (Fig. 3). Representative traces are shown in Fig. 3A and composite histograms of $τ_1$ are shown in Fig. 3B. In adult muscles, there was no difference in AChR $γ$-subunit mRNA levels between wt and Ig-NRG$^{+/−}$ mice (Fig. 3C).

MEPP amplitude and quantal content also are influenced by muscle fiber diameter (a determinant of imput resistance) and
by end-plate size (Harris and Ribchester 1979; Katz and Thesleff 1957). We found no difference in input resistance measured directly in a subset of adult Ig-NRG+/+ and wild-type fibers by injecting 10 nA of current through an electrode placed adjacent to the end plate and recording within 100 μm of the first electrode (1.01 ± 0.04 MΩ, in 2 wt mice, 17 fibers; 1.03 ± 0.04 in 2 het mice, 24 fibers, P = 0.6). Fiber diameters were measured in bright field images obtained near end plates before electrophysiological recording (Fig. 4A). Heterozygous and wild-type fiber diameters were similar both in adult mice and in neonatal mice (Fig. 4B and Table 1). Examples of end plates viewed en-face illustrate a variety of morphologies evident in adult mouse diaphragm (Fig. 4C; Prakash and Sieck 1998). We compared Texas-red α-bungarotoxin–labeled end plates by confocal microscopy in three het (47 fibers) and three wt (44 fibers) mice. No significant difference was observed in total end-plate area (het = 4,205 ± 256; wt = 4,356 ± 314), end-plate length (het = 73.96 ± 2.2; wt = 74.48 ± 2.75), or end-plate width (het = 55.45 ± 1.96; wt = 56.23 ± 2.14). Taken together, these results suggest that the normal MEPP amplitudes recorded in adult Ig-NRG+/+ mice did not arise from differences in receptor subunit composition, muscle fiber diameter, or end-plate size.

We also examined synaptic transmission at an intermediate age, P14. By this time most end plates are singly innervated and the γ- to ε-AChR subunit switch is virtually complete (Missias et al. 1996; Sanes and Lichtman 2001). Mean MEPP amplitude was similar to control in P14 NRG+/− mice (Fig. 5 and Table 1). Evoked EPP amplitudes and mean quantum content were 20% lower than control, although this difference was not statistically significant. The relative increase in Ig-NRG+/− MEPP amplitude compared with wild-type values and the relatively large variance in mean EPP amplitudes suggest that P14 may reflect a transition between the neonatal and adult response to decreased Ig-NRG expression.

**DISCUSSION**

Two observations suggest that evoked transmitter release is not tightly coupled to postsynaptic sensitivity in Ig-NRG–deficient mice. In one week old Ig-NRG+/− mice, MEPP amplitude was decreased relative to control but mean quantum content was not increased. In adult heterozygotes, mean quantum content was increased even though MEPP amplitudes exhibited wild-type values. Thus there was no simple temporal relationship between postsynaptic and presynaptic changes observed in Ig-NRG+/− mice.

**FIG. 3.** Decay of miniature end-plate currents (MEPCs) is the same in wild-type (wt) and Ig-NRG+/− mice. A: representative traces (scale: 5 ms; 0.5 mV). B: histograms of MEPP decay het τ1 = 1.29 ± 0.01 ms; wt τ1 = 1.25 ± 0.01) (het: 2 mice, 15 fibers, 873 events; wt: 2 mice, 32 fibers, 974 events). C: acetylcholine receptor (AChR) γ-subunit mRNA measured by real-time PCR is not increased, and ε-subunit mRNA is not decreased in Ig-NRG–deficient mice.

**FIG. 4.** Muscle fiber diameter and end-plate size. A: bright field images of muscle fibers near yellow fluorescent protein (YFP)–labeled nerve terminals before recording. B: fiber diameters measured in P7, P14, and adult diaphragms show no difference between Ig-NRG−/− and control mice. C: Texas-red α-bungarotoxin–labeled end plates examined by confocal microscopy. No significant difference is evident.

**FIG. 5.** Synaptic transmission at P14. MEPP amplitude is similar to control in P14 Ig-NRG+/− mice. EPP amplitude and quantum content are somewhat reduced but the difference is not statistically significant.
Although dissociated in time, both quantal size and quantum content were influenced by Ig-NRG expression. The simplest interpretation of these results is that type I NRG can influence ACh release at the neuromuscular junction independently of its effects on postsynaptic AChRs. At the nerve terminal, NRG may modulate the surface membrane expression or trafficking of channels that regulate transmitter release, such as voltage-dependent potassium or calcium channels, or muscarinic AChRs (Ford et al. 2003; Rosato Siri and Uchitel 1999; Santafe et al. 2003).

At the postsynaptic membrane, decreased Ig-NRG gene expression results in decreased MEPP amplitude in P7 mice. This is consistent with known actions of NRG1 on AChR transcription in muscle fibers (Buonanno and Fischbach 2001). In the experiments reported here, the appearance of MEPPs of normal amplitude in Ig-NRG+/− adults, rather than the low-amplitude MEPPs observed previously (Sandrock et al. 1997), probably reflects the difference in genetic background in our rederived colony. Several factors may modulate the effects of reduced Ig-NRG expression. During the first 2 wk after birth, the mammalian neuromuscular junction changes dramatically. By P14, motor endplates become singly innervated, subsynaptic nuclei accumulate, embryonic AChRs are replaced by adult receptors, and the postsynaptic membrane develops deep folds with AChRs concentrated in the crests and sodium channels in the troughs (Brenner et al. 1994; Kues et al. 1995; Sanes and Lichtman 2001; Zhu et al. 1995). In addition, NRG protein, diffusely distributed in the basal lamina at birth, becomes concentrated at the endplate in the adult pattern (Loeb et al. 1999; Missias et al. 1997; Moscoso et al. 1995; Sandrock et al. 1995). Thus the efficacy of Ig-NRG signaling may be increased in heterozygous mice, despite reduced expression of Ig-NRG mRNA, by genetic changes that increase the release of NRG from nerve terminals or the accumulation of Ig-NRG isoforms in the synaptic cleft (Loeb 2003). Moreover, AChR expression, turnover, or stabilization may also be affected by the expression of other signaling molecules, including CGRP or NRG-2 (Fontaine et al. 1987; Lai and Ip 2003a,b; Meyer et al. 1997; New and Mudge 1986; Rimer et al. 2004).

It has been shown repeatedly that the phenotype of genetically altered mice, including mice with altered erbB2 receptors, may reflect differences in background genes as well as in the targeted gene (Andrechek et al. 2002; Crawley et al. 1997), and strain-specific differences in gene expression and synaptic properties have been reported (Fernandes et al. 2004; Nguyen et al. 2000). Taken together, our results suggest that in the postsynaptic membrane, MEPP amplitude is not determined simply by the level of Ig-NRG expression, but may be influenced by additional genes. By contrast, the presynaptic increase in transmitter release in Ig-NRG−/− adult mice appears to be more robust since this phenotype was observed in two different genetic backgrounds. The mechanisms responsible, and their developmental regulation, are under investigation.

The observed dissociation between postsynaptic and presynaptic changes in Ig-NRG−/− mice also may be explained by an alternative hypothesis. A slow presynaptic response may be triggered by the decreased postsynaptic transmitter sensitivity observed during the first postnatal week in Ig-NRG−/− mice. A delay in presynaptic compensation for decreased quantal size was seen in TIMG rats chronically treated with α-bungarotoxin (Plomp et al. 1992). Receptor density was decreased within 3 h of the first injection. Transmitter release increased gradually during the first week and plateaued 2 to 3 wk later. This time course is consistent with our results. Whether the increased transmitter release observed in TIMG rats would have been maintained if treatment had stopped and quantal size returned to control levels—as it had in our adult Ig-NRG deficient mice—is not known. However, results in Drosophila neuromuscular junctions suggest a “one-way” mechanism of presynaptic compensation during normal development. When quantal size was experimentally increased in Drosophila end plates there was no homeostatic decrease in transmitter release (Davis et al. 1998; DiAntonio et al. 1999; Petersen et al. 1997). Thus in Ig-NRG−/− adult muscle fibers, as in Drosophila muscle, transmitter release may remain elevated despite an increase in quantal size. One attractive hypothesis is that regulation of synaptic efficacy may be set by early activity-dependent events (Davis and Bezprozvanny 2001; Landmesser 1998).

CaMKII has been identified as a postsynaptic regulator of retrograde signaling in Drosophila muscle with altered postsynaptic receptor density (Haghighi et al. 2003) and is required for the increased transmitter release observed in toxin-induced myasthenic (TIMG) rats (Plomp and Molenaar 1996). The size of the end plate is not altered in either system. It is possible that CaMKII is also involved in the regulation of transmitter release in NRG-deficient mice. Opposite actions of this kinase have been observed: inhibition of CaMKII increases transmitter release in DGlurIIA mutants, but inhibition of CaMKII decreases transmitter release in TIMG rats. Inhibition of CaMKII has no effect on transmitter release in wild-type fly or rat muscle. It will be interesting to determine whether these two actions are evident at different developmental stages in NRG-deficient mice.

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