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GA-Binding Protein Is Dispensable for Neuromuscular Synapse Formation and Synapse-Specific Gene Expression▽

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The mRNAs encoding postsynaptic components at the neuromuscular junction are concentrated in the synaptic region of muscle fibers. Accumulation of these RNAs in the synaptic region is mediated, at least in part, by selective transcription of the corresponding genes in synaptic myofiber nuclei. The transcriptional mechanisms that are responsible for synapse-specific gene expression are largely unknown, but an Ets site in the promoter regions of *acetylcholine receptor* (*AChR*) subunit genes and other "synaptic" genes is required for synapse-specific transcription. The Ets domain transcription factor GA-binding protein (GABP) has been implicated to mediate synapse-specific gene expression. Inactivation of GABPα, the DNA-binding subunit of GABP, leads to early embryonic lethality, preventing analysis of synapse formation in *gabpα* mutant mice. To study the role of GABP at neuromuscular synapses, we conditionally inactivated *gabpα* in skeletal muscle and studied synaptic differentiation and muscle gene expression. Although expression of *rb*, a target of GABP, is elevated in muscle tissue deficient in GABPα, clustering of synaptic AChRs at synapses and synapse-specific gene expression are normal in these mice. These data indicate that GABP is dispensable for synapse-specific transcription and maintenance of normal AChR expression at synapses.

During neuromuscular synapse formation, postsynaptic proteins, including acetylcholine receptors (AChRs) and the muscle-specific receptor tyrosine kinase, MuSK, become localized to the synaptic membrane of skeletal muscle fibers. Similarly, the mRNAs encoding many postsynaptic components become concentrated in the synaptic region of myofibers, because their corresponding genes are transcribed selectively in subsynaptic muscle nuclei (4, 28). The transcription factor GA-binding protein (GABP) has been implicated to mediate synapse-specific gene expression (32).

GABP is a dimer of GABPα, which contains an Ets domain that binds DNA, and GABPβ, which contributes a nuclear localization sequence and the transcriptional activation domain to GABP (18, 31). Dimerization of GABPα and GABPβ is mediated by interactions between four amino-terminal ankyrin repeats in GABPβ and the Ets domain, plus a short α-helix adjacent to the Ets domain, in GABPα (1, 39). While there is one known *gabpα* gene, two *gabpβ* genes, *gabpβ1* and *gabpβ2*, have been found in mammals, and *gabpβ1* gives rise to at least four distinct splice isoforms (7, 10, 18, 41). Certain GABPβ isoforms mediate formation of a heterotetramer, composed of two GABPα/β dimers, which binds to paired Ets sites, whereas other GABPβ isoforms are recruited to a single Ets site as part of a GABPα/β dimer (7).

Synapse-specific transcription of the *AChR* δ gene is dependent upon a single Ets site in its promoter region (16). GABP is the major Ets protein in myotube nuclear extracts that binds this Ets site (9, 33). The *AChR* ε promoter also contains an Ets site that binds GABP, and mutations in this Ets site are responsible for certain congenital myasthenic syndromes in humans (22, 23, 35). While the importance of Ets sites in synapse-specific gene expression is widely accepted, the role of GABP in synapse formation is less clear. Transfection of a dominant-negative form of GABPβ, lacking sequences required for transcriptional activation, inhibits AChR cluster formation and induction of an *AChR* ε reporter construct by ectopic agrin expression in adult skeletal muscle (2). Transfection of a dominant-negative form of GABPβ also attenuates the activation of a *MuSK* reporter construct by agrin in vitro (17). Furthermore, transfection of a mutant form of GABPα that cannot be phosphorylated at threonine 280 interferes with the induction of *AChR* ε gene expression by neuregulin 1 (Nrg-1) in cultured muscle cells (38). Forced expression of the DNA-binding domain of Ets2, another Ets domain-containing transcription factor, in skeletal muscle leads to defects in the organization and size of primary gutters and secondary folds at neuromuscular synapses and reduces the expression levels of "synaptic" genes (6). These results indicate that interfering with the function of multiple members of the Ets family of transcription factors by transfection of dominant-negative constructs affects the expression of genes that are preferentially transcribed in subsynaptic nuclei at the neuromuscular synapse. These studies, however, do not address whether GABP is required for neuromuscular synapse formation or synapse-specific gene expression.

Deletion of GABPα in mice results in embryonic lethality prior to embryonic day 7.5 (E7.5) of development (26), preventing an analysis of a potential role for GABP in synapse formation, which begins at E13. Here we conditionally inactivated *gabpα* specifically in skeletal muscle and analyzed syn-

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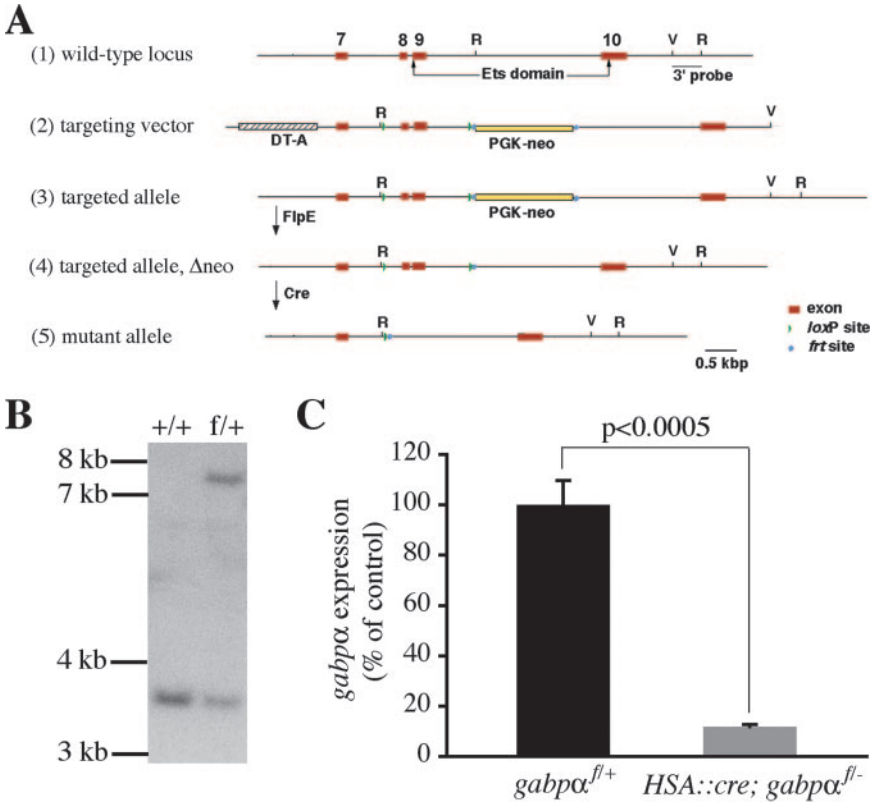

FIG. 1. Generation of null and loxP-flanked *gabp*α alleles and deletion of *gabp*α in skeletal muscle. (A) The cartoon shows the targeting strategy to generate the *gabp*α^{f} and *gabp*α^{-} alleles. The following are depicted: (1) the normal *gabp*α locus (exons are numbered, and the sequence encoding the Ets domain is indicated by arrows); (2) the targeting vector, which includes a frt-flanked neomycin resistance cassette (PGK-neo) and a diphtheria toxin A (DT-A) cassette; (3) the loxP-flanked, targeted allele following homologous recombination; (4) the targeted allele after removal of PGK-neo by FlpE; and (5) the mutant allele after Cre recombinase-mediated removal of exons 8 and 9, which encode the bulk of the Ets domain. EcoRI (R) and EcoRV (V) restriction sites and the position of the probe used for Southern blot hybridization are indicated. (B) Southern blot analysis of EcoRI-digested DNA from ES cells carrying two wild-type alleles (+/+) or one loxP-flanked allele and one wild-type allele (f/+) of *gabp*α. (C) RNA isolated from gastrocnemius muscles from P21 *gabp*$\alpha^{f/+}$ ($n = 3$) or *HSA*::*cre*; *gabp*$\alpha^{f/-}$ mice ($n = 4$) was reverse transcribed into cDNA, and expression of the wild-type *gabp*α transcript was measured by quantitative PCR using primers in exons that are deleted after Cre-mediated recombination of loxP sites (see Materials and Methods). *gabp*α expression in muscle tissue is reduced by 88% for *gabp*α mutant mice compared to levels for control littermates ($P < 0.0005$). Error bars in panel C indicate standard errors of the means.

aptic development. We find that GABPα is dispensable for synapse-specific gene expression and clustering of synaptic AChRs during synapse formation. Furthermore, postnatal synaptic maturation is normal in conditional *gabp*α mutant mice. These findings suggest that additional proteins bind the Ets site in *AChR* genes and stimulate their expression in synaptic nuclei.

MATERIALS AND METHODS

Generation of mice carrying a loxP-flanked allele of *gabp*α. To generate the loxP-flanked *gabp*α allele (*gabp*α^{f}), we introduced loxP sites into introns 7 and 9 of a *gabp*α genomic DNA fragment encompassing exons 7 through 10; furthermore, we introduced a frt-flanked neomycin resistance cassette into intron 9 and a diphtheria toxin A cassette at the 5′ end of the targeting vector (Fig. 1A). Because exons 8 and 9 encode the majority of the Ets domain and sequences immediately amino-terminal to the Ets domain, which are required for GABP function, deletion of these exons is likely to result in a null allele (*gabp*α^{-}). Indeed, *gabp*$\alpha^{-/-}$ mice died during embryogenesis, whereas *gabp*$\alpha^{+/-}$ mice survived as adults (see below). 129S6/SvEvTac-derived W4 embryonic stem (ES) cells were electroporated with the targeting vector and selected with neomycin, and surviving clones were screened for homologous recombination by Southern blotting using a 3′ EcoRV/EcoRI fragment as a probe (Fig. 1A and B). One *gabp*$\alpha^{f/+}$ ES cell clone was chosen for blastocyst injections, and resulting chimeras were crossed to C57BL/6 mice. Phenotypic analyses were carried out in a mixed background.

Gabp$\alpha^{f/+}$ mice were crossed to FlpE-expressing mice (8) to remove the neomycin resistance cassette from intron 9 (Fig. 1A). We generated *gabp*$\alpha^{+/-}$ mice by crossing *gabp*$\alpha^{f/f}$ mice with *CMV*::*cre* mice, which express Cre recombinase in the germ line (42).

Mouse strains and genotyping. *HSA*::*cre* mice have been described previously (20) and were genotyped as reported previously (13). loxP-flanked and wild-type *gabp*α alleles were detected by PCR using primers that hybridize to sequences in *gabp*α (CTTACAATTTTGAGGTGCATAGACC and CCAAAGGAATTAGG GGAATCTTTCC). The null allele was detected using a separate pair of primers (GGCCAGCCAAGAGCAACA and TCCACCCTTGGACAGATCCTGCAT GGC).

Immunohistochemistry. Motor axons and nerve terminals were visualized by staining with antibodies against neurofilament and synaptophysin, respectively; muscle fibers and AChR clusters were stained with Alexa660-phalloidin and Alexa594-α-bungaratoxin (Alexa594-α-Bgt), as described previously (13).

To determine the branch point number of postsynaptic AChR clusters in P21 diaphragm muscles, we analyzed 10 synapses per animal for 4 animals per genotype. We determined the means for each genotype and compared them in a two-tailed Student *t* test.

Quantitation of synaptic AChRs. The number and density of synaptic AChRs were determined by measuring Alexa594-α-Bgt binding, as described previously (13). We analyzed at least 62 synapses in each P0 diaphragm muscle, at least 27 synapses in each P21 gastrocnemius muscle, and a minimum of 23 synapses in

each P21 diaphragm muscle. The mean AChR level and density from multiple mice (numbers of mice are indicated in the figure legends) with the same genotype were determined. Because the density and level of synaptic AChRs were not significantly different in P21 *gabpα*$^{f/-}$ and *gabpα*$^{f/+}$ mice (data not shown), the data from these two genotypes were grouped.

In situ hybridization. Intercostal and diaphragm muscles were processed for in situ hybridization and hybridized with digoxigenin-labeled riboprobes that recognize the *AChR* α-subunit (5), *AChR* δ-subunit (36), *AChR* ε-subunit (13), or *MuSK* mRNA (11) as described elsewhere (13). Labeling with sense probes resulted in weak, uniform staining for each gene (data not shown).

The width of the in situ hybridization signal was measured relative to the distance between two adjacent ribs using ImageJ (NIH). Means were determined from four tissue samples (from two mice) of the same genotype, and genotypes were compared in a two-tailed Student *t* test.

Quantitative RT-PCR. RNA isolation from gastrocnemius muscle, reverse transcription (RT), and quantitative PCR were carried out essentially as described previously (13). Relative expression levels of *gabpα* and the *AChR* ε-subunit were normalized to *muscle creatine kinase* (*mck*) expression. *retinoblastoma* (*rb*) and cytochrome *c* oxidase subunit IV (*coxIV*) RNA levels were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) expression. The primers used for PCR amplification were TGCATCCTGCACCACCAACT and ATGC CTGCTTCACCACCTTC for *gapdh*, CGTGTCACCTCTGCTGCT and CCTT CATATTGCCTCCCTTCT for *mck*, AATGGGGACAACGTAAGAACA and GTACACAAATCTCTTGCCTTGAAC for wild-type *gabpα*, TGCTAGCCCA GACTGTCTTCTT and GTCGTTGGCGTCCTCAAAG for *AChR* ε, CTTGG CTAACTTGGGAGAAAG and GCTCAGTAAAAGTGAATGGCAT for *rb*, and GGGAGTGTTGTGAAGAGTGAAG and CCTTCTCCTTCTCCTT CAGC for *coxIV*.

RESULTS

Generation of *gabpα* mutant alleles and conditional inactivation of GABPα in skeletal muscle. GABP has been implicated in the regulation of a wide variety of genes, including but not limited to nuclear genes encoding mitochondrial proteins, *rb*, and *AChR* subunit genes (14, 27, 32). To determine the function of GABP in vivo, we generated mice carrying a loxP-flanked allele of *gabpα* (*gabpα*^{f}; Fig. 1A and B). In these mice, exons 8 and 9, which encode the majority of the GABPα Ets domain, are flanked by loxP sites, allowing Cre-mediated deletion of these sequences (Fig. 1A). We also generated mice with a *gabpα* allele lacking exons 8 and 9 (*gabpα*$^{-}$) by crossing *gabpα*$^{f/+}$ mice to *CMV*::*cre* mice, which express Cre in all cell types, including the germ line (Fig. 1A) (see Materials and Methods). We intercrossed *gabpα*$^{+/-}$ mice and failed to obtain *gabpα*$^{-/-}$ newborn mice (data not shown). At E8.5, the earliest stage we examined, we failed to recover homozygous mutant embryos; however, genotyping of extraembryonic membrane tissue from empty deciduas showed that this tissue contained *gabpα*$^{-/-}$ embryos, which had apparently resorbed prior to E8.5 (data not shown). These results indicate that GABPα is required for survival during early embryonic development, as reported previously (26, 43), preventing analysis of its function at later stages of development.

To determine the role of GABP in skeletal muscle, we inactivated GABPα specifically in skeletal muscle. To this end, we generated mice carrying a *human skeletal actin* (*HSA*)::*cre* transgene and null and loxP-flanked alleles of *gabpα*. *HSA*::*cre*; *gabpα*$^{f/-}$ mice were born at the expected Mendelian frequency. Because mice die at birth if the diaphragm and intercostal muscles fail to form or function, these findings suggest that GABP is not essential for the formation of skeletal muscles. To measure the extent of *gabpα* inactivation in skeletal muscle tissue, we isolated RNA from P21 *HSA*::*cre*; *gabpα*$^{f/-}$ mice and *gabpα*$^{f/+}$ littermates and used a quantitative, real-time PCR assay to measure the level of *gabpα* expression (Materials and Methods). We found that the expression of RNA encoding wild-type GABPα is reduced nearly 10-fold in muscle tissue from *HSA*::*cre*; *gabpα*$^{f/-}$ mice (11.8% ± 0.9% of the level found in muscle tissue from *gabpα*$^{f/+}$ mice; $P < 0.0005$) (Fig. 1C). Because GABPα is expressed in most if not all cell types (19, 24, 26) and because muscle tissue contains fibroblasts, smooth muscle cells, Schwann cells, and endothelial cells in addition to muscle fibers, these data establish a minimal reduction of GABPα expression within skeletal myofibers. Residual expression of GABPα in vascular, interstitial, and neural cell types is likely to contribute to most if not all of the remaining expression of GABPα in skeletal muscle tissue. Thus, our data indicate that GABPα expression is substantially reduced in skeletal myofibers of *HSA*::*cre*; *gabpα*$^{f/-}$ mice.

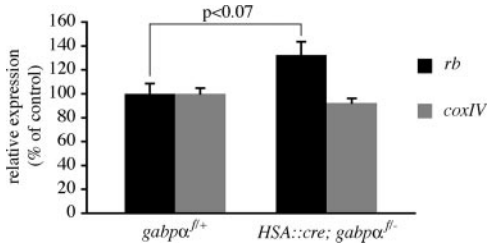

FIG. 2. *rb* gene expression is elevated in skeletal muscle of *HSA*::*cre*; *gabpα*$^{f/-}$ mice. RNA from gastrocnemius muscle of P21 control mice ($n = 3$) or *gabpα* mutant littermates ($n = 5$) was reverse transcribed, and gene expression was measured by quantitative PCR. *rb* mRNA levels are increased by 32% ($P < 0.07$), whereas *coxIV* mRNA levels are unchanged in muscle from *gabpα* mutant mice ($P > 0.2$). Error bars indicate standard errors of the means.

***rb* gene expression is regulated by GABP.** GABP has been implicated in the regulation of nuclear genes encoding mitochondrial proteins, including cytochrome *c* oxidase subunits and the *rb* gene (14, 27). GABP binds the promoter regions of these target genes, and GABP overexpression induces their transcription; conversely, their transcription is attenuated by expression of dominant-negative forms of GABP (25, 30, 37, 40).

To determine whether GABP regulates the expression of *rb* and *coxIV*, we measured their expression in gastrocnemius muscles from P21 *HSA*::*cre*; *gabpα*$^{f/-}$ mice and *gabpα*$^{f/+}$ littermates by quantitative RT-PCR. We found that *coxIV* mRNA levels are normal in muscle from *HSA*::*cre*; *gabpα*$^{f/-}$ mice (92.2% ± 3.9% of control; $P > 0.2$) (Fig. 2). *rb* expression, on the other hand, was elevated in muscle from *HSA*::*cre*; *gabpα*$^{f/-}$ mice (132.5% ± 11.1% of control; $P < 0.07$) (Fig. 2). These findings indicate that *coxIV* expression in muscle is not dependent upon GABP and suggest that *rb* is negatively regulated by GABP.

Skeletal muscle GABPα is not required for synapse formation and synaptic gene expression during embryonic development. GABP has been implicated in synapse-specific gene expression and neuromuscular synapse formation (32). To study presynaptic and postsynaptic differentiation in *HSA*::*cre*; *gabpα*$^{f/-}$ mice, we stained whole mounts of diaphragm muscle from P0 *HSA*::*cre*; *gabpα*$^{f/-}$ mice and control littermates with probes that allowed us to visualize motor axons, nerve terminals, muscle fibers, and AChRs (Materials and Methods).

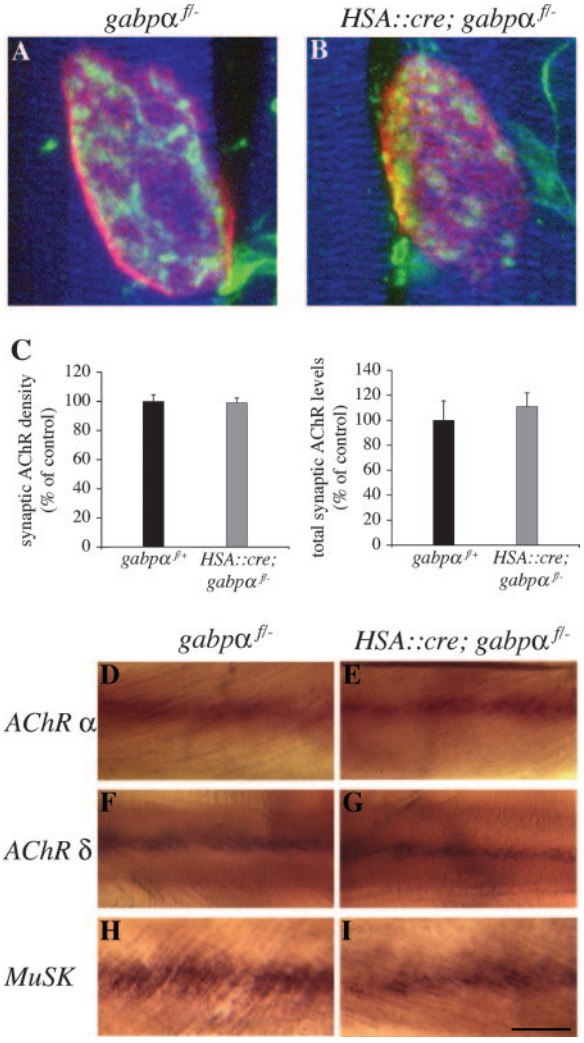

FIG. 3. GABPα is dispensable for AChR clustering and synapse-specific gene expression. (A and B) Whole mounts of diaphragm muscles from P0 *HSA*::*cre*; *gabpα*$^{f/-}$ mice (B) or control littermates (A) were stained with Alexa594-α-Bgt to visualize postsynaptic AChRs (red), antibodies against neurofilament and synaptophysin to visualize motor axons and nerve terminals (green), respectively, and Alexa660-phalloidin to visualize muscle fibers (blue). AChR clusters are of similar sizes and shapes in control mice and *gabpα* mutant mice. (C) Synaptic AChR density and total synaptic AChR levels are indistinguishable in conditional *gabpα* mutant mice ($n = 2$) and control littermates ($n = 4$). (D to I) Whole mounts of intercostal muscles from newborn control mice (D, F, and H) or *HSA*::*cre*; *gabpα*$^{f/-}$ mice (E, G, and I) were processed for in situ hybridization. *AChR* α-subunit (D and E), *AChR* δ-subunit (F and G), and *MuSK* (H and I) mRNAs are concentrated in the central region of muscle from *HSA*::*cre*; *gabpα*$^{f/-}$ mice, as with control mice. Scale bar = 5 μm for panels A and B or 120 μm for panels D to I. Error bars in panel C indicate standard errors of the means.

Muscle fibers in *HSA*::*cre*; *gabpα*$^{f/-}$ mice are of normal arrangement and size, and the positions of the main intramuscular nerve and synaptic sites appear normal (Fig. 3A and B) (data not shown). Moreover, in *HSA*::*cre*; *gabpα*$^{f/-}$ mice, as in wild-type mice, AChRs are clustered at synaptic sites, and the size and shape of presynaptic nerve terminals and postsynaptic AChR clusters appear normal (Fig. 3A and B). Similar results were obtained for gastrocnemius muscle (data not shown). These results indicate that expression of GABPα in skeletal muscle is not essential for the formation of muscle fibers, growth of motor axons to muscle, or the formation of neuromuscular synapses.

To determine whether the number and density of synaptic AChRs are normal in the absence of skeletal muscle GABPα, we measured synaptic AChR protein expression by quantitating the amount of Alexa594-α-Bgt bound to synaptic AChRs in diaphragm muscles from P0 *HSA*::*cre*; *gabpα*$^{f/-}$ mice and control mice (Fig. 3C) (Materials and Methods). We found no significant difference in the density or number of synaptic AChRs in *gabpα* mutant mice and control littermates (density, 99% ± 3.3% of control, $P > 0.2$; total number, 111% ± 10.9% of control, $P > 0.2$). These findings indicate that the number and density of AChRs at developing synapses do not depend upon GABPα expression in muscle.

To analyze the role of GABP in synaptic transcription, we examined the pattern of *AChR* gene expression in intercostal muscles from P0 mice by in situ hybridization. We found that *AChR* α-subunit and *AChR* δ-subunit mRNAs are concentrated in the central region of muscle from *HSA*::*cre*; *gabpα*$^{f/-}$ mice, as with control mice (Fig. 3D to G). We measured the width of the *AChR* α and *AChR* δ expression domains relative to the distance between individual ribs and found no significant difference ($P > 0.2$) between control mice (*AChR* α, 16% ± 1.1%, $n = 4$; *AChR* δ, 16% ± 2.2%, $n = 4$) and *HSA*::*cre*; *gabpα*$^{f/-}$ littermates (*AChR* α, 17% ± 2.2%, $n = 4$; *AChR* δ, 17% ± 1.9%, $n = 4$). We also analyzed the pattern of *MuSK* gene expression and found that *MuSK* mRNA is patterned normally in *HSA*::*cre*; *gabpα*$^{f/-}$ mice (Fig. 3H and I) and that there is no significant difference ($P > 0.2$) in the width of the *MuSK* expression domain between control mice (21% ± 2.5%; $n = 4$) and conditional *gabpα* mutant mice (17% ± 3.7%; $n = 4$). These findings indicate that GABP function in skeletal muscle is not required to establish the pattern of synapse-specific gene expression during development.

GABPα expression in muscle is dispensable for postnatal maturation of neuromuscular synapses. Postnatally, neuromuscular synapses undergo extensive remodeling and maturation. Hence, we analyzed whether GABP is required for the maturation or maintenance of neuromuscular synapses after birth. Postnatal *HSA*::*cre*; *gabpα*$^{f/-}$ mice appear healthy and do not display any overt phenotype (data not shown). We visualized motor axons, nerve terminals, muscle fibers, and AChRs in whole mounts of diaphragm muscle from P21 mice. We found that AChRs are concentrated at synaptic sites in *gabpα* mutant mice; these AChR clusters are apposed by nerve terminals that have the same size and shape as nerve terminals in wild-type mice (Fig. 4A and B). Similar results were obtained for gastrocnemius muscle (data not shown). To quantitatively compare the morphology of neuromuscular synapses in diaphragms of *HSA*::*cre*; *gabpα*$^{f/-}$ mice and *gabpα*$^{f/+}$ mice, we determined the number of branch points in postsynaptic AChR clusters (see Materials and Methods). We found no significant difference ($P > 0.2$) in branch point number between control mice (5.5 ± 0.58) and conditional *gabpα* mutant mice (5.1 ± 0.30). Thus, GABPα is not essential to maintaining the normal arrangement of AChRs and nerve terminals at neuromuscular synapses in postnatal mice.

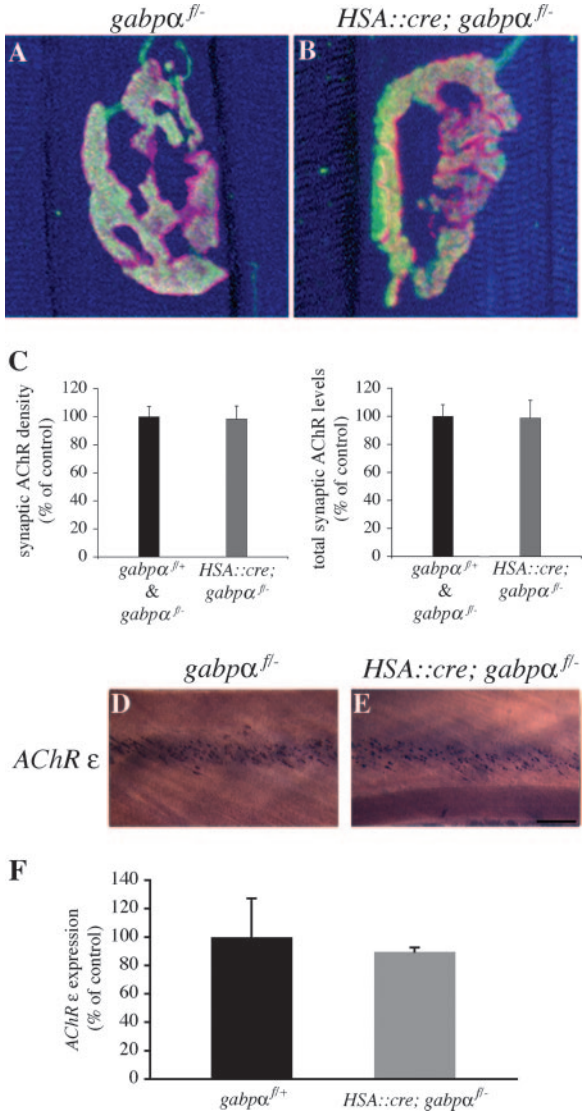

FIG. 4. Neuromuscular synapses mature normally in *HSA*::*cre*; *gabpα*$^{f/-}$ mice. (A and B) Whole mounts of diaphragm muscles from P21 control mice and *HSA*::*cre*; *gabpα*$^{f/-}$ mice were stained as described in the legend to Fig. 3. Neuromuscular synapses exhibit a branched, pretzel-like structure in control mice (A) or *gabpα* mutant mice (B). Furthermore, the sizes of myofibers are similar in *gabpα* mutant mice and control mice (A and B). (C) The density and total number of synaptic AChRs are similar in muscle from control mice ($n = 4$) and *HSA*::*cre*; *gabpα*$^{f/-}$ mice ($n = 6$). (D and E) Whole mounts of intercostal muscles from P21 control mice (D) and *HSA*::*cre*; *gabpα*$^{f/-}$ mice (E) were processed for in situ hybridization. *AChR* ε mRNA is concentrated in the central region of muscle from *gabpα* mutant P21 mice, similar to the case with control mice. (F) RNA from gastrocnemius muscles of P21 control mice ($n = 3$) or *gabpα* mutant littermates ($n = 5$) was reverse transcribed, and *AChR* ε gene expression was measured by quantitative PCR. Expression of *AChR* ε is similar in *HSA*::*cre*; *gabpα*$^{f/-}$ mice and control mice ($P > 0.2$). Scale bar = 10 μm for panels A and B or 200 μm for panels D and E. Error bars in C and F indicate standard errors of the means.

To determine whether GABPα is required to maintain AChR expression postnatally, we measured the densities and numbers of synaptic AChRs in diaphragm muscles from P21 *HSA*::*cre*; *gabpα*$^{f/-}$ mice and control mice (Fig. 4C). We found no significant difference in the density or number of synaptic AChRs in *gabpα* mutant mice and control littermates (density, 98% ± 9.3% of control, $P > 0.2$; total number, 99% ± 12.7% of control, $P > 0.2$). Similar results were obtained for gastrocnemius muscles (density, 109% ± 3.1% of control, $P > 0.2$; total number, 93% ± 4.2% of control, $P > 0.2$; $n = 3$ for control mice; $n = 5$ for mutant mice). These findings indicate that the number and density of AChRs at mature synapses do not depend upon GABPα expression in muscle.

During the first week of postnatal life, expression of the AChR γ-subunit is down-regulated and expression of the AChR ε-subunit is induced; this postnatal switch in AChR subunit expression is critically responsible for a change in the kinetics and conductance of synaptic AChRs (21). Because GABPα has been proposed to play a key role in inducing AChR ε-subunit expression (2, 22, 23, 35), we analyzed AChR ε expression in *HSA*::*cre*; *gabpα*$^{f/-}$ mice. We examined the expression pattern of the *AChR* ε-subunit gene in intercostal muscles of P21 *gabpα* mutant mice by in situ hybridization and found that *AChR* ε-subunit mRNA is concentrated in the central region of muscle from *HSA*::*cre*; *gabpα*$^{f/-}$ mice, as with control littermates (Fig. 4D and E; width of *AChR* ε expression domain, 13% ± 1.5% for controls, $n = 4$; 14% ± 1.1% for mutants, $n = 4$; $P > 0.2$). Similar results were obtained with diaphragm muscle (data not shown). These results indicate that skeletal muscle GABPα is not required for activating *AChR* ε-subunit transcription in subsynaptic nuclei and patterning synapse-specific gene expression postnatally.

To determine whether GABP regulates the level of *AChR* ε-subunit gene expression, we measured *AChR* ε mRNA in gastrocnemius muscle from P21 *HSA*::*cre*; *gabpα*$^{f/-}$ mice and control mice by quantitative RT-PCR. We found that *AChR* ε mRNA levels are normal in *HSA*::*cre*; *gabpα*$^{f/-}$ mice (89.6% ± 2.9% of control; $P > 0.2$) (Fig. 4F). Thus, the *AChR* ε expression level is not dependent upon GABP.

DISCUSSION

The genes encoding several postsynaptic proteins, including AChRs, AChE, and MuSK, are transcribed specifically in myofiber nuclei positioned near the synaptic site. GABP has been implicated as a key transcription factor regulating synapse-specific gene expression, largely because it can bind the *cis*-regulatory element required for synaptic transcription of *AChR* and *AChE* genes. We conditionally inactivated the DNA-binding subunit of GABP, GABPα, in skeletal muscle and found that the pattern of synapse-specific gene expression is normal in these mutant mice. Synapses develop and mature normally in these mice, forming an elaborate, branched shape that contains normal numbers of postsynaptic AChRs. Moreover, *AChR* ε-subunit gene expression is induced postnatally and patterned normally in *gabpα* conditionally mutant mice. These results provide strong evidence against an essential role for GABP in neuromuscular synapse formation and synapse-specific transcription. O'leary et al. have recently described their analysis of mice that are deficient in skeletal muscle GABPα (24a). Although they report that the arborization of nerve terminals is simplified at a subset of synapses and that *AChR* ε gene expression is reduced in the diaphragm muscle, similar to our findings, they report that skeletal muscle

GABPα is not essential for viability, growth, muscle development, or neuromuscular synapse formation.

GABP has been implicated in the induction of the *rb* gene, since the *rb* promoter region contains a binding site for GABP, and overexpression of GABP stimulates expression of a reporter gene controlled by the *rb* promoter in cultured cells (30, 37). Surprisingly, we find that the expression of *rb* is elevated (1.3-fold) in muscle of *HSA*::*cre*; *gabpα*$^{f/-}$ mice, suggesting that GABP suppresses *rb* gene expression, possibly directly, in skeletal muscle. These data demonstrate that deletion of *gabpα* in skeletal muscle of *HSA*::*cre*; *gabpα*$^{f/-}$ mice causes misregulation of at least one proposed GABP target gene, while neuromuscular synapse formation and synapse-specific transcription are unaffected. We cannot exclude the possibility that these mutant mice still express a low level of GABPα, insufficient to repress *rb* expression but fully capable of stimulating synapse-specific gene expression.

We used *HSA*::*cre* mice to conditionally inactivate *gabpα* in skeletal muscle. Previously we demonstrated that the *HSA*::*cre* transgene mediates efficient (>95%) deletion of loxP-flanked target sequences in muscle fibers (13). Here we show that the levels of wild-type *gabpα* transcript are reduced approximately 10-fold in muscle tissue of *HSA*::*cre*; *gabpα*$^{f/-}$ mice compared to those for *gabpα*$^{f/+}$ mice. Because *gabpα* is likely to be expressed in nonmuscle cells within muscle tissue (see Results) and because fibroblasts, smooth muscle cells, Schwann cells, and endothelial cells constitute approximately 50% of the nuclei within muscle tissue (34), the reduction of *gabpα* within muscle fibers of *HSA*::*cre*; *gabpα*$^{f/-}$ mice is likely greater than 10-fold.

The *gabpα*$^{-}$ allele described here is likely to be a null allele, because it lacks the sequences in *gabpα* that encode the DNA-binding domain. Consistent with this idea, *gabpα*$^{-/-}$ mice die before E8.5 (data not shown), as do mice that are homozygous for a *gabpα* allele lacking the first protein-coding exon (26). Because the *gabpα* gene encodes the DNA-binding portion of GABP and GABPβ does not bind directly to DNA (3), deletion of *gabpα* in *HSA*::*cre*; *gabpα*$^{f/-}$ mice abolishes the ability of GABP to stimulate transcription in skeletal muscle.

Expression of a dominant-negative form of GABPβ, which can interact with GABPα but lacks the transcriptional activation domain, inhibits ectopic induction of *AChR* ε gene expression by agrin in adult skeletal muscle, suggesting that GABP can regulate synaptic *AChR* gene expression (2). These findings and our results would be reconciled if a complex of GABPα and transcriptionally defective GABPβ remained bound to the Ets site in the *AChR* ε gene, preventing other, "compensating" Ets proteins from binding to the Ets site and substituting for GABPα. In contrast, removal of GABPα in *HSA*::*cre*; *gabpα*$^{f/-}$ mice may allow for other Ets proteins to occupy the Ets site in *AChR* genes and compensate for the absence of GABP. Consistent with this idea, the DNA-binding specificities of different members of the family of Ets domain-containing transcription factors are very similar (3), and multiple Ets proteins are expressed in muscle (12, 29). Thus, although it is possible that Ets domain-containing transcription factors other than GABP normally confer synapse-specific gene expression, other Ets domain proteins may only compensate for the loss of GABPα. Notably, one Ets family member, Erm, is a particularly attractive candidate for regulating synapse-specific transcription, since *erm* RNA is highly concentrated at synaptic sites in skeletal muscle (12, 15). Further analysis of *erm* mutant mice should reveal whether Erm alone or Erm together with GABP has a role in synapse-specific gene expression.

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