Increased Thalamocortical Synaptic Response and Decreased Layer IV Innervation in GAP-43 Knockout Mice

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INTRODUCTION

The mouse primary somatosensory cortex provides an excellent model system for investigating the cellular and molecular mechanisms underlying neural circuit development because of the stereotyped pattern of layer IV “barrels.” Mutant mice lacking expression of the growth-associated protein, GAP-43, have a grossly abnormal whisker representation with a decrease in the number of barrels due to a decrease in the levels of neurogenic differentiation 2 (NeuroD2, a transcription factor) expression (Inan et al. 2006; Lu et al. 2003). Recent data from our lab also suggest that differences in the probability of release at thalamocortical synapses may impair development of the barrel map (Lu et al. 2006). Thus both pre- and postsynaptic deficits in thalamocortical synaptic function have been implicated in malformation of the barrel map.

Normal development of the barrel map is tied to functional maturation of the thalamocortical synapse. One of the developmental changes observed at the thalamocortical synapse is an increase in the ratio of AMPA to N-methyl-D-aspartate (NMDA) receptor-mediated currents (AMPA:NMDAR) (Crair and Malenka 1995; Lu et al. 2001; Wu et al. 1996). The increase in AMPA:NMDAR current ratio is thought to be mediated by the regulation of AMPA trafficking into the synapse, a process that is compromised in several barrel map mutants (Inan et al. 2006; Lu et al. 2003). Recent data from our lab also suggest that differences in the probability of release at thalamocortical synapses may impair development of the barrel map (Lu et al. 2006). Thus both pre- and postsynaptic deficits in thalamocortical synaptic function have been implicated in malformation of the barrel map. We therefore sought to investigate the role of GAP-43 in regulating thalamocortical synaptic function during barrel map development using whole cell recordings of layer IV neurons in a thalamocortical slice preparation and autaptic cultures of thalamic neurons taken from GAP-43−/− and littermate control mice. Examination of presynaptic release in both the in vitro brain slice and autaptic culture preparations indicates the probability of release at GAP-43−/− and littermate control thalamic synapses is similar. Evidence from the autaptic cultures suggests that the level of neurotransmitter in the cleft of thalamic terminals is increased.

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in GAP-43−/− mice. Examination of postsynaptic function shows that GAP-43−/− mice have increased AMPAR: NMDAR current ratios, larger AMPAR miniature EPSC amplitudes but similar AMPAR function in comparison to littermate controls, consistent with higher concentrations of glutamate at the synapse. Finally, anatomical analysis reveals that thalamic innervation of layer IV in barrel cortex is dramatically reduced in GAP-43−/− mice. These results suggest that GAP-43 plays an important role in regulating neurotransmitter release at the thalamocortical synapse, but the severe barrel map phenotype found in GAP-43−/− mice is due to the combination of defects in the initial guidance of thalamocortical axons and functional impairments in thalamocortical synaptic transmission.

METHODS

Animals and PCR analysis

GAP-43 homozygous knockout (GAP-43−/−) mice were obtained from heterozygous (GAP-43+/−) crosses of seventh-generation backcrosses with C57BL/6N (Maier et al. 1999). DNA for genotyping was purified from tails using the DNeasy kit from Qiagen (Valencia, CA) and PCR reactions prepared using the Multiplex PCR kit from Qiagen. Wild-type PCR primers (5′-GGCTCATAAGGCTGCAACCAAAAT-3′, 5′-CCATCTCCCTCTTCTCCACA-3′) produced a 165-bp band, and mutant primers (5′-CCGGCGCTTGGTGAGAC-3′, 5′-TCGGCACAGGCAAAGTGATGAC-3′) produced a 299-bp band. The PCR reaction was carried out with an initial denaturation step at 95°C for 2 min and 32 cycles at 94°C for 45 s, 67°C for 50 s, 72°C for 1 min, and a single final cycle at 72°C for 10 min. PCR products were resolved on a 2% agarose gel and detected with ethidium bromide. Animals were treated in compliance with the U.S. Department of Health and Human Services and Baylor College of Medicine guidelines.

Protein analyses

ANTIBODIES. The following primary antibodies were used: Serotonin transporter (5-HTT; 1:250) from Immunostar (Hudson, WI); VgluT2 (1:500) from Chemicon (Temecula, CA); Actin (1:1000) from Sigma (St. Louis, MO). The following secondary antibodies were used: mouse IgG–Alexa 488 (1:500), rabbit IgG–Alexa 488 (1:500) from Invitrogen (San Diego, CA) and guinea pig IgG-Alexa 488 (1:500) for IHC; mouse IgG–HRP (1:20,000) and rabbit IgG–HRP (1:12,500) from Pierce (Rockford, IL) for Western blot analysis.

Immunoblotting

Barrel cortex was isolated from P7 mice and homogenized in homogenization buffer consisting of 0.32 M sucrose, 10 mM HEPES, pH 7.4, with protease and phosphatase inhibitor mixture (Sigma). Synaptosomes were prepared as previously described (Lu et al. 2003). Briefly, the homogenates were centrifuged at 800 g for 15 min to yield pellet P1, which is enriched with cell bodies. The supernatant was then centrifuged at 7,100 g for 15 min to yield supernatant P2, the synaptosome enriched fraction. Protein concentrations were measured with Bradford assay using Bio-Rad protein assay kit II (Bio-Rad Laboratories, Hercules, CA). Total protein (20 μg) was loaded into 5–20% gradient SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes (Criterion system from Bio-Rad). Primary antibodies against the protein in question were applied and the appropriate HRP-conjugated secondary antibodies. Immunoreactivity was quantified with ECL pico (Pierce) and densitometric quantification was carried out with Optiquant software (Packard Bioscience, Meriden, CT) by background subtracting and normalizing band intensities to the wild-type band in each set. Each sample was quantified relative to Actin from the same blot.

Immunofluorescence

Mice (P7) were perfused transcardially with PBS followed by 4% PFA, and the brain was removed and postfixed overnight at 4°C in 4% PFA. One-hundred-micrometer sections in the thalamocortical plane were cut with a vibratome (Leica VT100S), and sections remained free-floating for all incubations and washes. Briefly, tissues were permeabilized with 0.7% Triton X-100 in PBS for 20 min, incubated with 0.1 M glycine for 30 min, and then blocked with 1% normal goat serum (NGS) and 2 mg/ml BSA in PBS containing 0.01% Triton X-100 (PBST). Sections were then transferred to the primary antibody for incubation overnight at 4°C with appropriate dilution in PBST with 1% NGS. After washing six times in PBST, sections were incubated in the secondary antibody for 2 h at room temperature then washed again three times. Sections were then wet mounted on slides and coverslipped. Immunolabeling was visualized using epifluorescence and a CCD camera and associated software (Epix, Houston, TX), and images were collected with either this or a Leica DM confocal scanning microscope at ×10. Each image was acquired with the laser intensity adjusted to prevent oversaturation. Camera and illumination settings were optimized for both knockout and wild-type brain sections on slides, and the same settings were preserved across groups of slides. While preserving imaging settings, several images of both knockout and wild-type were obtained and then analyzed together, keeping the number of images from each genotype even. Each of these groups constitutes a single session, and the number of these sessions represents the N used for statistical analysis. Images were analyzed with ImageJ (National Institutes of Health, Bethesda, MD). A polygon was drawn around layer IV on collapsed image stacks, and the same polygon was subsequently moved to layer II/III. Pixel brightness measurements within the polygon at each of these locations were made and measurements from LIV were normalized to LII/III. Averages of these comparisons were made for each genotype from each group. The ratio of staining was then determined between wild-type and knockout images for each group.

Slice electrophysiology

Acute TC slices were prepared as previously described (Lu et al. 2001). The artificial cerebral spinal fluid [ACSF, in mM, 124 NaCl, 5 KCl, 1.25 NaHPO4, 1.3 MgSO4, 2 CaCl2, 26 NaHCO3, and 11 glucose (pH = 7.2, 290–300 mOsmi)] was saturated with 95% O2–5% CO2. The whole cell recording solution containing (in mM) 117.5 cesium gluconate, 17.5 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, 0.3 GTP, 7 phosphocreatine, and 10 bis-(o-aminophenoxo)-N,N,N′,N′′-tetraacetic acid (BAPTA). BAPTA was included in the pipette to prevent inadvertent potentiation of the postsynaptic neuron. Stimuli (100-μs duration) were applied to the ventrobasal thalamus through bipolar sharpened and insulated stainless steel microelectrodes (FHC, Bowdoinham, ME). Data were collected and analyzed on-line using a computer-driven acquisition system (National Instruments, Austin, TX) and software written under the Igor (WaveMetrics, Lake Oswego, OR) programming environment.

Input-output analysis of synaptic transmission using field potential electrodes filled with ACSF was conducted with a range of stimulus intensities (0–850 μA with 50 μA intervals). The nonspecific glutamate receptor antagonist kynurenic acid (10 mM) (Sigma) was occasionally added to the perfusate at the end of the recording to ensure correct identification of fiber volley and synaptic response. An average of 20 sweeps was used to calculate the fiber volley amplitude and field excitatory postsynaptic potential (fEPSP) slope for each stimulus intensity. P6–P10 mice were used for field potential recordings.
Excitatory postsynaptic currents (EPSCs) were measured in voltage-clamp mode using in vitro whole cell voltage-clamp recording techniques in P8–P11 mice following published protocols (Lu et al. 2001). To evaluate and monitor the health of the cell, input and series resistances were continuously monitored with cells that had <300 MΩ input resistance or drifted >20% discarded. Only responses that exhibited short and constant latencies (5–10 ms) that did not change with increasing stimulus intensity were considered monosynaptic. Stimulus strengths with a relatively low stimulation frequency (10- to 15-s interval) that elicited responses half of the saturating response amplitude were used to evoke stable EPSCs. Before any experimental manipulation, 10–15 min of stable baseline response was acquired. EPSC amplitudes were calculated by subtracting the mean current during a fixed 3- to 4-ms window before the stimulus artifact from the mean current during a similar window at the peak of the EPSC. P8-11 mice were used for whole cell electrophysiology.

To measure the AMPAR:NMDAR current ratio, which is a measure of the relative contribution of AMPAR- and NMDAR-mediated currents across a population of synapses, we first isolated the AMPA response by voltage clamping the cell at hyperpolarized membrane potentials (~70 mV) while stimulating the thalamus. We then depolarized the cell to +40 mV to relieve the Mg²⁺ block of the NMDAR and added 2,3-dihydroxy-6-nitro-7-sulfonylbenzofuranoxaline (NBQX; 10 μM, Tocris) to the perfusate to block AMPA and kainate receptors, leaving a pure NMDAR response. To eliminate inhibitory currents these experiments were done in the presence of GABA_A antagonists: 50 μM picrotoxin or 10 μM bicuculline (Tocris Cookson, Ellisville, MO). Cesium ions in the whole cell solution block GABA_B responses.

For analysis of AMPA “evoked miniature” events, stable whole cell voltage-clamp recordings were established at ~70-mV holding potential, and the Ca²⁺ in the ACSF was exchanged for Sr²⁺. Sr²⁺-based ACSF desynchronizes release, allowing isolated evoked miniature currents to be analyzed (Xu-Friedman and Regehr 1999). Evoked miniature events were recorded in 1-s epochs every 2 s in Igor Pro using ACSF (2 mM Sr²⁺) containing 100 μM picrotoxin and 50 μM d-AP5 (d-(-)-2-amino-5 phosphophentonic acid, Tocris) to eliminate inhibitory currents and possible NMDAR current contamination, respectively. Data were analyzed with a purpose-built program written in Matlab (Natick, MA), and the first 100 events were identified and used for analysis in each cell.

For (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801) experiments, NMDA current was isolated, and only cells responding with a significant amount of NMDA current (~25 pA) were used. After acquiring a stable baseline response (15–20 min), the stimulation was stopped and ACSF containing (+)-MK801 (10 μM; Tocris Cookson) was perfused onto the slice (1 ml/min flow rate). After 10 min (to allow enough time for MK801 to equilibrate in the recording chamber), we resumed stimulation and recorded until no NMDA current could be detected for ~5 min. The time course of MK801-dependent blockade was analyzed by normalizing all of the responses to the amplitude of the first NMDA response after MK801 was applied, and the blockade rates were estimated by fitting a double exponential function \( A_1 e^{-t/τ_1} + A_2 e^{-t/τ_2} \) to the decay and calculating a weighted time constant, \( τ_{weighted} = τ_1 \times A_1/(A_1 + A_2) + τ_2 \times A_2/(A_1 + A_2) \), from the fit parameters.

Paired-pulse measurements were done by isolating the NMDAR-mediated current as described in the preceding text and then eliciting pairs of stimuli at interstimulus intervals (ISIs) of 50, 75, 100, and 500 ms. Each condition was presented in pseudorandom order. Ratios were then computed as the mean amplitude response to the second stimulus over the response to the first stimulus.

All summary data are presented as means ± SE. The Student’s t-test was used to determine statistical significance except where otherwise specified.

Primary neuronal cell culture and electrophysiology

Standard procedures were taken to culture thalamic neurons from P1–P2 mouse pups (Moechars et al. 2006). Cultures were plated on astrocytes derived from neonatal cortex tissue. Neurons were plated on island cultures at a density of 3,000 neurons/35-mm dish. Recordings were performed from 10- to 13-day-old neurons that were isolated on astrocyte microislands. Standard extracellular solution contained (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 MgCl₂, and 4 CaCl₂; pH 7.3, 305 mosM. Internal solution contained (in mM) 135 KCl, 18 HEPES, 1 EGTA, 4.6 MgCl₂, 4 ATP, 0.3 GTP, 15 creatine phosphate, and 20 U/ml phosphocreatine kinase. Patch pipettes were fabricated from thick-walled borosilicate glass and had resistances of 2.5–3.5 MΩ. Series resistance was compensated by ~75% and was usually <12 MΩ before compensation. The readily releasable vesicle pool was estimated using application of 500 mM sucrose according to published procedures (Rosenmund and Stevens 1996). Vesicular release probability was calculated by dividing the integral of evoked EPSCs by the integral of the current response to 500 mM sucrose application.

Miniature EPSCs were detected for 60–80 s with 3 mM kynurenic acid applied for 2 s of every 10 s for background noise subtraction. For each cell, data were filtered at 1 kHz and analyzed using template based mEPSC detection algorithms implemented in analysis software Axograph 4.9 (Molecular Devices, Union City, CA). Threshold for detection was set at three times the baseline SD from a template of 0.5-ms rise time and 3-ms decay. mEPSCs were captured from the time during the trace without kynurenic acid, and those outside allowable ranges for amplitude, rise time, and half-width were rejected. The remaining number of events were counted and averaged to determine frequency and mean amplitude. The same procedure was then repeated for the time during the trace when kynurenic acid was applied. The frequency and amplitude of these captured events was considered the rate and amplitudes of false positives. It was subtracted from the original frequency and the average amplitude was adjusted accordingly.

For competitive antagonist experiments, 800 μM d-gluuroxymylycine (g-DGG) and 100 mM NBQX were applied with a fast perfusion system, and EPSCs were recorded after a stable baseline was established. Three to six EPSCs were then averaged for each condition, and the percentage of block was calculated as one minus the g-DGG or NBQX peak amplitude divided by control amplitude.

RESULTS

Reduced fiber response but normal postsynaptic EPSPs in GAP-43-/- barrel cortex

We first assayed thalamocortical synaptic transmission in GAP-43-/- mice and littermate controls using field potential recordings in a thalamocortical brain slice (Fig. 1A). Thalamocortical synapses are glutamatergic, containing a mixture of AMPARs, kainate receptors, and NMDARs (Agmon and O’Dowd 1992; Crair and Malenka 1995; Kidd and Isaac 1999; Lu et al. 2001). Typical fEPSP response in layer IV of barrel cortex as a result of ventral posteriomedial thalamic (VPM) stimulation is composed of a short-latency fiber volley and a longer-latency synaptic response (Agmon and Connors 1991) (Fig. 1B). The thalamic afferent fiber volley and thalamocortical synaptic response are easy to distinguish based on the short latency of the fiber volley and its insensitivity to glutamate receptor antagonists (Fig. 1, B and C). We delivered stimuli of varying strength in the thalamus and measured the amplitude of the fiber volley response as well as the initial slope of the postsynaptic fEPSP with an electrode placed in layer IV. The initial slope of the fEPSP is a good measure of...
monosynaptic thalamocortical synaptic response (Agmon and Connors 1991), whereas the fiber volley amplitude reflects the number of thalamocortical afferents stimulated. We measured the slope of the fEPSP and plotted the response as a function of thalamic stimulation strength. This was indistinguishable in controls and GAP-43/H11002/H11002 mice (n = 8 and n = 4, respectively; Fig. 1D). To assay the level of input to the cortex in GAP-43/H11002/H11002 mice, we examined the fiber volley amplitude as a function of stimulation strength (Fig. 1E). In contrast to the slope of the fEPSP, thalamocortical fiber volley amplitude as a function of stimulation strength was noticeably smaller in GAP-43/H11002/H11002 mice compared with wild-type littermate controls. This suggests that in GAP-43/H11002/H11002 mice fewer thalamic afferents invade layer IV or they are disorganized, leading to smaller fiber volley amplitudes. Plots of the slope of the fEPSP as a function of stimulation strength is often used as a gross measure of synaptic function. For GAP-43/H11002/H11002 mice, this plot (supplemental Fig. 1) is of limited utility because the fiber volley amplitude varies over only a very narrow range. Despite the small fiber volley amplitudes, synaptic response remains quite robust in GAP-43/H11002/H11002 mice, suggesting that existing thalamocortical synapses in GAP-43/H11002/H11002 mutants are functionally intact.

FIG. 1. Reduced fiber volley amplitude, but normal field excitatory postsynaptic potential (EPSP)s in GAP-43/H11002/H11002 mice. A: image of a typical P8 wild-type thalamocortical slice recording preparation showing the recording pipette in the barrels in layer IV, and the bipolar stimulating electrode in the ventrobasal thalamus. B: averaged field potential traces recorded in layer IV of a GAP-43/H11002 thalamocortical slice at P11. Traces are averages of 20 sweeps each taken from a recording before (orange), during (purple), and after (green) washing on the glutamate receptor blocker kynurenic acid. Kynurenic acid blocks the response due to postsynaptic receptor activation (arrow) without affecting the response generated by firing of afferent thalamocortical fibers to the cortex (arrowhead). C: plot of the initial slope of the field EPSP (fEPSP) vs. time (top) and the fiber volley amplitude vs. time (bottom) for the experiment shown in B. During the kynurenic acid wash (purple window), the slope of the fEPSP can be seen to decrease and then recover on washout while the fiber volley amplitude is unaffected by the glutamate receptor blocker. Points in orange, purple, and green represent time points averaged together for corresponding traces in B. D: plot of the slope of the fEPSP vs. stimulation strength, revealing that the GAP-43/H11002 slices exhibit comparable postsynaptic responses to their wild-type littermates at all stimulation strengths. E: plot of the fiber volley amplitude vs. stimulation strength. The fiber volley amplitude in the GAP-43/H11002 slices is much smaller on average, than their wild-type littermates at nearly all stimulation strengths tested.

The online version of this article contains supplemental data.
Reduced thalamocortical afferent innervation of layer IV in GAP-43−/− mice

The small fiber volley amplitudes associated with GAP-43−/− mice suggest that thalamic innervation of barrel cortex is sparse. We quantitatively examined the density of thalamic afferent innervation of layer IV in barrel cortex using two complimentary methods. First, we used immunolabeling for serotonin transporter (5HTT), which is specifically expressed in thalamic afferents (Lebrand et al. 1996). In thalamocortical sections from wild-type littermate controls, 5HTT immunohistochemistry densely labels thalamic afferent terminals in a barrel pattern in layer IV (Fig. 2A). In contrast, in GAP-43−/− mice, 5HTT stain is much weaker with occasional patches of dense staining (Fig. 2B). Quantification of the level of 5HTT label in layer IV relative to layer II/III in the GAP-43−/− mice and littermate controls confirms this visual impression with significantly less stain visible in the GAP-43−/− mice (fraction of WT: 0.54 ± 0.10, n = 4; P < 0.05, paired t-test), corresponding to more sparse thalamic innervation of barrel cortex. To confirm that the reduction in 5HTT staining was in fact due to a reduction in thalamocortical innervation and not due to a direct interaction between GAP-43 and 5HTT, we used immunohistochemistry of another protein, vesicular glutamate transporter 2 (VGluT2), that also serves as a marker of thalamocortical afferents (TCAs) (Nahmani and Erisir 2005). Once again we found reduced staining of VGluT2 in layer IV of GAP-43−/− mice compared with layer IV staining in wild-type littermates (Fig. 2, D—F; fraction of WT: 0.62 ± 0.07, n = 10; P < 0.005, paired t-test). We also measured the amount of VGluT2 using Western blots of synaptosomes prepared from primary somatosensory cortex of GAP-43−/− mice and wild-type littermate controls. We found a significant decrease in VGluT2 protein in the somatosensory cortex of GAP-43−/− mice when compared with wild-type littermates (Fig. 2, G and H; WT: 0.96 ± 0.13, n = 4; KO: 0.49 ± 0.03, n = 4; P < 0.05). These data all suggest a decrease in the density of thalamocortical innervation of somatosensory cortex in GAP-43−/− mice compared with wild-type littermate controls.

High AMPAR:NMDAR ratio at GAP-43−/− thalamocortical synapses

Postsynaptic fEPSPs are normal in GAP-43−/− (Fig. 1) despite reduced thalamocortical innervation. This suggests a greater functional response per input in GAP-43−/− mice such that any given increase in activation of the thalamocortical afferents generates a greater synaptic response in the layer IV cortical cells. We checked this possibility by examining the ratio of AMPAR:NMDAR currents at thalamocortical synapses in GAP-43−/− and wild-type mice. In wild-type mice, the ratio of AMPAR:NMDAR currents is 1.2 ± 0.10, n = 5; in GAP-43−/− mice, the ratio is 0.8 ± 0.20, n = 6; P < 0.03, paired t-test). This suggests a greater functional response per input in GAP-43−/− mice compared with wild-type mice.
AMPAR:NMDAR ratio increases during the first week after birth, which is typical of glutamatergic synaptic development at central synapses (Crair and Malenka 1995; Lu et al. 2001; Wu et al. 1996). In several strains of mutant mice with barrel map defects, however, the AMPAR:NMDAR current ratio remains low (Inan et al. 2006; Ince-Dunn et al. 2006; Lu et al. 2003), which suggests that interfering with activity-dependent signals thought to be responsible for glutamatergic synapse development also perturbs barrel development. We found that AMPAR:NMDAR current ratios were actually larger in GAP-43−/− mice (2.16 ± 0.43; n = 16) than littermate controls (1.06 ± 0.15; n = 13; P < 0.05) (Fig. 3). These results show that thalamocortical synaptic maturation is not impaired in GAP-43−/− mice. Instead, the increased AMPAR:NMDAR current ratio suggests that thalamocortical synapses in GAP-43−/− mice are stronger, exhibiting a greater synaptic response to a given level of thalamic activation at resting membrane potentials.

We next examined if EPSC amplitudes were affected at thalamocortical synapses in GAP-43−/− mice by measuring miniature AMPAR-mediated synaptic currents (AMPA minis) in GAP-43−/− mice and wild-type littermate controls. Substitution of Ca2+ with Sr2+ in the extracellular ACSF leads to a reduction in synchronous evoked responses and the appearance of delayed “miniature” responses due to the persistence of asynchronous quantal release (Xu-Friedman and Regehr 1999) (Fig. 4, A and B). This makes Sr2+ a useful tool to analyze evoked miniature excitatory postsynaptic currents (evoked mini EPSCs) in the slice preparation, where contamination from polysynaptic events with standard Ca2+-evoked mini EPSCs is problematic. Comparison of the evoked mini EPSC amplitude histograms shows a shift in the distribution, with the histogram for GAP-43−/− mice showing more large-amplitude events than wild-type littermate controls (Fig. 4C).

FIG. 3. Increased AMPA receptor (AMPAR):N-methyl-D-aspartate receptor (NMDAR) ratio in GAP-43−/− mice. A–C: sample AMPAR-mediated (arrows) and NMDAR-mediated (arrowheads) thalamocortical excitatory postsynaptic currents (EPSCs, average of 20 sweeps each) from a P10 wild-type littermate control (A) and P9 GAP-43−/− (B) mouse. C: traces in A and B overlaid and scaled so that the NMDAR currents are the same amplitude, revealing dramatic increase in AMPAR-mediated EPSC in GAP-43−/− thalamocortical synapses. D: quantification of AMPAR:NMDAR ratios for wild-type (1.06 ± 0.15; n = 13) and GAP-43−/− neurons (2.16 ± 0.43; n = 16) showing the significant difference in AMPAR:NMDAR ratio at the thalamocortical synapse between genotypes (P < 0.05).
The mean cumulative probability distributions also revealed a shift in evoked mini EPSCs in GAP-43−/− mice toward larger amplitudes compared with wild-type littermate controls (Fig. 4D, means of 12.01 ± 0.91 vs. 9.97 ± 0.83 pA; respectively, \( P < 0.001 \), Kolmogorov-Smirnov test). The difference between genotypes was not due to an intrinsic difference in RMS noise levels (2.29 ± 0.22 vs. 2.73 ± 0.18 pA). The large AMPAR mini EPSC amplitudes observed in GAP-43−/− mice suggests that at least part of the increased AMPAR:NMDAR ratio observed in GAP-43−/− mice is due to an increase in the level of activation of postsynaptic AMPA receptors.

**Decreased paired-pulse ratio in GAP-43−/− mice**

Because GAP-43 is predominantly a presynaptic molecule (Gispen et al. 1985; Ramakers et al. 1991, 1992; Van Lookeren Campagne et al. 1989) and has been specifically implicated in neurotransmitter release (Dekker et al. 1989; Gamby et al. 1996), we next examined a measure of presynaptic neurotransmitter release efficacy. The paired-pulse (PP) ratio is a standard measure of presynaptic function that is typically inversely related to the probability of neurotransmitter release (Zucker and Regehr 2002). PP ratios are determined by measuring the relative response amplitude of two closely spaced stimuli. We used NMDAR-mediated excitatory postsynaptic currents (NMDA-EPSCs) to examine PP ratios at TC synapses with a range of ISIs (50, 75, 100, and 500 ms). NMDAR currents have been used in other studies to assay presynaptic release (Chen et al. 2002; Gipson and Yeckel 2007; Kielland and Heggelund 2002; Liu et al. 2006; Lu et al. 2006; Yanagisawa et al. 2004) and are preferred over AMPAR currents if long-latency polysynaptic contamination is a significant concern since the pharmacological isolation of NMDAR-mediated currents removes polysynaptic elements from the thalamocor-
tical response. At the Ca\(^{2+}\) concentration typically used for brain slice preparations (2 mM), paired stimuli produce a strong paired pulse depression (PPD) (Fig. 5, A–C), consistent with the high \(P_t\) typically found at TC synapses in vitro (Gil et al. 1999; Yanagisawa et al. 2004). The PP ratios were no different in wild-type littermate control or GAP-43\(^{-/-}\) mice at any of the ISIs tested. However, if the absence of GAP-43 in mutants led to an increased probability of release, it may be difficult to elicit a greater PPD because probability of release is already high at the thalamocortical synapse, masking the difference between GAP-43\(^{-/-}\) and control mice. To circumvent this, we lowered the Ca\(^{2+}\) concentration in the bath to 1 mM, which also more closely mimics the Ca\(^{2+}\) concentration observed in the brain in vivo (Sanchez-Vives and McCormick 2000; Yamaguchi 1986; Zhang et al. 1990). At 1 mM Ca\(^{2+}\) we observed a larger increase in PP ratio in wild-type littermate mice than GAP-43\(^{-/-}\) mice, revealing a significant difference in PP ratio between genotypes (Fig. 5, D–F). Significantly lower PP ratios were evoked in GAP-43\(^{-/-}\) TC synapses (Fig. 5F, \(P < 0.001\) for difference between GAP-43\(^{-/-}\) and wild-type littermate control, 2-way ANOVA). Lower PP ratios are usually an indication of higher presynaptic release probability. However, to see if this was indeed the case we next performed a more direct test of release probability, MK-801 block of NMDAR-mediated currents.

**Fast block of NMDA-EPSCs by MK-801 in GAP-43\(^{-/-}\) TC synapses similar to controls**

MK-801 is an irreversible open channel blocker of NMDA receptors (Huetterlin and Bean 1988). The rate at which the amplitude of NMDAR-mediated EPSCs are attenuated by MK-801 during repetitive stimulation is directly related to presynaptic release probability (Hessler et al. 1993; Manabe and Nicoll 1994; Rosenmund et al. 1993). NMDAR-EPSCs decay quickly in the presence of MK-801 in wild-type neurons at typical (2 mM) Ca\(^{2+}\) concentrations (Fig. 6, A and B), consistent with high probability of release at TC synapses. As seen with the PP ratio, blockade by MK-801 in GAP-43\(^{-/-}\) neurons in 2 mM Ca\(^{2+}\) was similar to wild-type littermate neurons (Fig. 6C). Switching from 2 mM external Ca\(^{2+}\) to 1 mM Ca\(^{2+}\) significantly slowed the rate of block by MK-801 in wild-type cells as expected. However, in 1 mM Ca\(^{2+}\), blockade was still not different between GAP-43\(^{-/-}\) neurons and wild-type littermates (Fig. 6D). Fitting the decay of the NMDA current in MK-801 by a double exponential reflects this with a weighted time constant of 21 ± 5 stimuli in GAP-43\(^{-/-}\) neurons (\(n = 4\)) versus 16 ± 2 stimuli for wild-type neurons in 1 mM Ca\(^{2+}\) (\(n = 6\) and 5 ± 1 stimuli for wild-type neurons in 2 mM Ca\(^{2+}\) (Fig. 6F). The same rate of NMDA-EPSC blockade by MK-801 in wild-type and GAP-43\(^{-/-}\) neurons at both Ca\(^{2+}\) concentrations (2 and 1 mM) suggests there is no
difference in probability of release between thalamocortical synapses in GAP-43−/− mice and wild-type littermate controls. It is possible that intrinsic variability in MK-801 block rates could preclude the observation of any difference between GAP-43−/− mice and littermate controls. Therefore as an additional positive control, we included low levels of the NMDAR antagonist APV in the bath while examining the rate of NMDA EPSC blockade by MK-801. Adding low concentrations of APV to the bath reduces the number of NMDARs available for block by MK-801 on any given stimulus. In this way, APV “protects” a subset of the NMDARs from MK-801 block during a single release event. Unlike MK-801, however, APV will then dissociate from the NMDAR, and a similar proportion of the remaining NMDARs will be “protected” on the next exposure to glutamate. This results in fewer NMDARs “permanently” blocked by MK-801 during each release event, significantly slowing the rate of MK-801 blockade in wild-type TC synapses, as expected (P < 0.01; Fig. 6, E and F). This is consistent with the significant change in block rate seen between wild-type cells in 2 and 1 mM Ca2+ (P < 0.01), which also changes the probability of release at the synapse. Therefore the MK-801 assay is capable of resolving differences in release probability despite the fact that we see no difference between neurons from GAP-43−/− mice and wild-type littermate controls. To further examine how the absence of GAP-43 affects neurotransmitter release from thalamic cells, we turned to a reduced preparation of thalamic neurons in autaptic cultures. This reduced preparation permits a more careful examination of synaptic function and was previously used in cultures of thalamic neurons to measure subtle differences in synaptic release properties (Moechars et al. 2006).

**Increased glutamate concentration in the cleft of GAP-43−/− autaptic synapses**

With the autaptic culture preparation, we can examine synaptic function with high signal-to-noise in the absence of many potentially confounding effects present in the thalamocortical slice preparation such as the reduced number of thalamocortical synapses in GAP-43−/− mice (Figs. 1 and 2). We first measured the amplitude of the evoked autaptic EPSCs and found no difference between genotypes (littermate controls: 4.06 ± 0.36 nA, n = 38; GAP-43−/−: 4.65 ± 0.59 nA, n = 42; P = 0.41). We also examined the size of the readily releasable

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**FIG. 6.** GAP-43−/− exhibit no difference in release probability. A and B: sample experiment with the NMDAR response amplitude vs. time (A) and sample traces taken at progressively later times after wash on of MK801 (B). A baseline NMDAR current is isolated before switching off stimulation to wash on MK801. With resumption of stimulation, the NMDAR current amplitude decreases steadily with a rate that is proportional to the probability of release. Color of the sample traces (B) corresponds to the points colored on amplitude plot (A). C: comparison of GAP-43−/− and GAP-43−/− cells in 2 mM calcium revealed no difference in the rate of NMDAR block by MK801. D: unlike with paired-pulse stimulation, no difference was seen in 1 mM Ca2+. This indicates that the probability of release is not different between GAP-43−/− and wild-type thalamocortical synapses. E: repeating the experiment in D while including a low concentration of the competitive NMDAR blocker 2-amino-5-phosphonovaleric acid (APV) in the bath reduces the rate of block of the NMDAR by MK801 in wild-type neurons. F: weighted time constants were measured by fitting a double-exponential curve to the NMDAR amplitude decay over time. These reveal a significant difference in the rate of NMDAR block by MK801 between wild-type cells measured with and without APV in the bath (no APV: 16 ± 2; n = 6; with APV: 44 ± 9; n = 6; P < 0.01; GAP-43−/−: 21 ± 5; n = 4) and between wild-type cells in 1 mM and 2 mM Ca2+ (5 ± 1; n = 7; P < 0.01). These results indicate that this experiment is capable of resolving differences in release probability in the slice.
pool by application of 500 mM sucrose (Rosenmund and Stevens 1996) and found no difference between GAP-43<sup>−/−</sup> and wild-type littermate control thalamic neurons (littermate controls: 231 ± 25 pC, n = 27; GAP-43<sup>−/−</sup>: 240 ± 50 pC, n = 28; P = 0.88). The probability of vesicular release in autaptic cultures was then measured by calculating the average percentage of the readily releasable pool that was released during an evoked response. As in the slice preparation, we found no difference in the probability of release between GAP-43<sup>−/−</sup> neurons (0.16 ± 0.01; n = 26) and wild-type littermate control (0.15 ± 0.01; n = 24; P = 0.68) neurons (Fig. 7C). An analysis of spontaneous release events, however, revealed a significant increase in miniature EPSC amplitudes in the GAP-43<sup>−/−</sup> neurons (33.28 ± 2.22 pA; n = 41) compared with

![Image](http://jn.physiology.org/)

**Fig. 7.** GAP-43<sup>−/−</sup> thalamic synapses exhibit no difference in release probability but have larger mini EPSCs despite normal AMPAR function. A: image of recording setup for an autaptic neuron. B, left: sample miniature events from a wild-type (top) and a GAP-43<sup>−/−</sup> (bottom) neuron. Right: average sample traces from a wild-type (top) and a GAP-43<sup>−/−</sup> (bottom) neuron. C: probability of vesicular release as measured at autaptic thalamic synapses is not different between wild-type (0.15 ± 0.01; n = 24) and GAP-43<sup>−/−</sup> (0.16 ± 0.01; n = 26; P = 0.68) neurons. D: amplitude of mini EPSCs measured at the thalamic autaptic synapses is significantly larger for GAP-43<sup>−/−</sup> neurons (33.28 ± 2.22; n = 41) compared with cultures from wild-type neurons (26.40 ± 1.30; n = 40; P < 0.01). E: sample responses to the exogenous application of the glutamate agonist kainate in GAP-43<sup>−/−</sup> (black) and GAP-43<sup>−/−</sup> (gray) neurons. F: amplitude of the kainate-induced response is not different between GAP-43<sup>−/−</sup> (3926 ± 426 pA; n = 17) and GAP-43<sup>−/−</sup> (4660 ± 574 pA; n = 17; P = 0.31) neurons.
neurons from wild-type littermate mice (26.40 ± 1.30 pA; n = 40; P < 0.01; Fig. 7D) but no change in mEPSC frequency (littermate controls 8.5 ± 1.1 Hz, n = 38; GAP-43−/− 11.1 ± 1.1 Hz, n = 40; P = 0.1). Finally, we examined AMPAR response to the exogenous application of kainate, a glutamate receptor agonist that will activate synaptic and extra-synaptic receptors. This analysis revealed no difference between GAP-receptor agonist that will activate synaptic and extra-synaptic response to the exogenous application of kainate, a glutamate function is unchanged in GAP-43

1995), then this analysis shows that postsynaptic AMPAR (Jones and Baughman 1991; Liu et al. 1999; Rosenmund et al. synaptic receptors dominate the kainite-induced response. One possibility is through higher agonist concentrations in the synaptic cleft on thalamic stimulation.

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we used the weak glutamate receptor antagonist g-DGG, which by virtue of its low affinity and fast dissociation will bind and unbind to the receptor during a typical EPSC. Thus a concentration of g-DGG that blocks 50% of control synaptic currents will block less current in the mutant if the concentration of glutamate in the synaptic cleft is higher. In contrast, a similar fractional blockade of the EPSC by a high-affinity antagonist should be independent of glutamate concentration because the antagonist remains bound to the receptor during a typical synaptic transient. We found that blockade of the AMPAR-EPSCs by g-DGG was reduced in GAP-43−/− thalamic neurons (0.34 ± 0.02; n = 18) compared with neurons from wild-type litters (0.41 ± 0.02; n = 17; P < 0.05). In contrast, the effect of the high-affinity glutamate antagonist, NBQX, was not different in GAP-43−/− neurons (0.60 ± 0.03; n = 18) relative to wild-type littermate controls (0.61 ± 0.02; n = 16; P = 0.72). This result shows that the amount of glutamate present in the synaptic cleft is higher in GAP-43−/− neurons. The higher levels of clef glutamate

How can the absence of GAP-43 lead to increased mEPSC amplitudes while leaving AMPAR function apparently intact? One possibility is through higher agonist concentrations in the synaptic cleft, leading to greater activation of glutamate receptors. To investigate this possibility, we examined the affects of competitive glutamate receptor antagonists on AMPAR-EPSCs in GAP-43−/− autapses and wild-type littermate controls (Fig. 8, A and B). We used the weak glutamate receptor antagonist g-DGG, which by virtue of its low affinity and fast dissociation will bind and unbind to the receptor during a typical EPSC. Thus a concentration of g-DGG that blocks 50% of control synaptic currents will block less current in the mutant if the concentration of glutamate in the synaptic cleft is higher. In contrast, a similar fractional blockade of the EPSC by a high-affinity antagonist should be independent of glutamate concentration because the antagonist remains bound to the receptor during a typical synaptic transient. We found that blockade of the AMPAR-EPSCs by g-DGG was reduced in GAP-43−/− thalamic neurons (0.34 ± 0.02; n = 18) compared with neurons from wild-type litters (0.41 ± 0.02; n = 17; P < 0.05). In contrast, the effect of the high-affinity glutamate antagonist, NBQX, was not different in GAP-43−/− neurons (0.60 ± 0.03; n = 18) relative to wild-type littermate controls (0.61 ± 0.02; n = 16; P = 0.72). This result shows that the amount of glutamate present in the synaptic cleft is higher in GAP-43−/− neurons. The higher levels of clef glutamate

FIG. 8. GAP-43−/− thalamic synapses exhibit increased glutamate at the synaptic cleft. A: sample responses from wild-type (top) and GAP-43−/− (bottom) cells. Responses are from before (black; arrow) and after (black; arrowhead) wash on of the glutamate receptor antagonist, b-glutamylglycine (DGG), as well as after wash on of 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline (NBQX, gray). B: GAP-43−/− cells exhibit reduced block by DGG than cells from wild-type (WT) littermate controls [degree of block (% KO]: 34 ± 2; n = 18; WT: 41 ± 2; n = 17; P < 0.05]. This is consistent with increased glutamate present in the synaptic cleft on thalamic stimulation. C: Synaptic block by NBQX was no different between GAP-43−/− cells and WT littersmates [degree of block (% KO]: 60 ± 3; n = 18; WT: 61 ± 2; n = 16; P = 0.72].
concentration in GAP-43<sup>−/−</sup> neurons in comparison to wild-type neurons would also cause the larger mini EPSCs amplitudes observed at mutant synapses (Figs. 4 and 7D).

**Discussion**

Our data indicate that GAP-43 is important for development of neurotransmitter release properties at thalamocortical synapses as well as for proper thalamocortical afferent innervation of the barrel cortex. Using two different in vitro preparations to study properties of synaptic transmission in GAP-43<sup>−/−</sup> mice, we show that thalamic synapses lacking GAP-43 exhibit signs of increased glutamate at the synaptic cleft resulting in a stronger postsynaptic response. We also find that the total innervation of primary somatosensory cortex by thalamocortical afferents is decreased in GAP-43<sup>−/−</sup> mice relative to their littermates.

**GAP-43 regulates synaptic glutamate release**

GAP-43 was previously implicated in regulating synaptic function and plasticity. GAP-43 associates with proteins in the presynaptic terminal important for neurotransmitter release, such as synaptotagmin and SNAP-25 (Haruta et al. 1997; Neve et al. 1998; Witzmann et al. 2005). Several lines of evidence suggest that GAP-43 affects neurotransmitter release with transfection of GAP-43 correlated with increased release of hormonal neurotransmitter or acetylcholine (Gamby et al. 1996; Kumagai-Tohda et al. 1993) and antibodies or antisense proteins directed against GAP-43 inhibiting neurotransmitter release (Dekker et al. 1991; Gamby et al. 1996; 1995; Hens et al. 1993; Ivins et al. 1993; Kumagai-Tohda et al. 1993). GAP-43 may regulate neurotransmitter release by acting in association with PKC via activity-dependent phosphorylation in association with the synaptic core complex (Dekker et al. 1989; Haruta et al. 1997; Routtenberg et al. 2000; Schmidt 2004).

We examined if the absence of GAP-43 significantly alters the formation of synaptic contacts between the thalamus and the cortex. Despite reduced fiber volley amplitudes, our output-input analysis reveals that the postsynaptic field EPSP in the GAP-43<sup>−/−</sup> mice is relatively normal. This suggests a stronger postsynaptic response to a given level of thalamic activation at thalamocortical synapses in the GAP-43<sup>−/−</sup> mice. Whole cell physiology supports this finding at the level of individual cells. We find that the ratio of AMPA to NMDA receptor activation is significantly increased in GAP-43<sup>−/−</sup> thalamocortical synapses, indicating a more efficacious response of the cell at membrane potentials near rest. This is supported by the finding of increased AMPA mini amplitudes in the knockout. Interestingly, an increased synaptic response in GAP-43<sup>−/−</sup> mice is also consistent with behavioral data that reveal a more robust behavioral response to whisker stimulation in GAP-43<sup>−/−</sup> mice than in wild-type animals (Metz and Schwab 2004).

Despite the presynaptic localization of GAP-43 (Gispen et al. 1985; Ramakers et al. 1991, 1992; Van Lookeren Campagne et al. 1989), we did not find an effect of GAP-43 on probability of vesicular release using whole cell recordings either in a thalamocortical slice preparation or in autaptic cultures of thalamic neurons. Instead we find evidence for an increase in concentration of glutamate in the cleft of synapses formed in the absence of GAP-43. Several lines of argument support this contention. First, glutamate antagonists are not as effective at reducing the synaptic response at GAP-43<sup>−/−</sup> synapses as in controls. Second, AMPA mini amplitudes are larger in GAP-43<sup>−/−</sup> mice, despite AMPAR function appearing similar, reflecting the higher concentration of glutamate. Third, higher AMPAR:NMDAR current ratios in GAP-43<sup>−/−</sup> mice are also consistent with higher glutamate concentrations in the cleft because AMPA receptors have a lower affinity for glutamate and are therefore more sensitive to glutamate concentration than NMDA receptors. Finally, the decreased paired-pulse ratio in GAP-43<sup>−/−</sup> thalamocortical synapses may be caused by a higher saturation of receptors in GAP-43<sup>−/−</sup> neurons from the first stimulation, which reduces the number of available receptors able to respond to the subsequent second pulse if delivered too soon after the first pulse (Chen et al. 2002). This interpretation depends on the assumption that AMPA receptors are not normally saturated at the thalamocortical synapse. Although no one has directly tested receptor saturation at the thalamocortical synapse, AMPA receptors are not saturated at many CNS synapses (Bekkers et al. 1990; Forti et al. 1997; Ishikawa et al. 2002; Liu et al. 1999; Liu and Tsien 1995; Mainen et al. 1999; McAllister and Stevens 2000).

We considered the possibility that the synaptic differences observed in the GAP-43<sup>−/−</sup> mice were simply a homeostatic compensation (Turrigiano and Nelson 2004) for decreased thalamocortical innervation of cortex. However, the similar effects on synaptic function observed in GAP-43<sup>−/−</sup> neurons in the autaptic cultures and the acute thalamocortical slice preparation argues against this explanation. Similarly, the greater synaptic response in the GAP-43<sup>−/−</sup> mice could theoretically be due to a decrease in 5HT activation of TCAs because innervation of the barrel field by serotonergic afferents from the raphe is decreased in GAP-43<sup>−/−</sup> mice (Donovan et al. 2002), and serotonin can reduce thalamocortical synaptic responses (Laurent et al. 2002; Rhoades et al. 1994). However, we observe similar effects on synaptic function in both the acute slice preparation as well as the autaptic cultures, which are not subject to serotonergic modulation.

Glutamate concentration in the cleft could be increased in GAP-43<sup>−/−</sup> mutant synapses in several ways, including slowing the rate of glutamate re-uptake, increasing the number or size of vesicles released, increasing the time vesicles spend open and fused to the membrane, or changing the concentration of glutamate packed into each vesicle. It is unlikely that GAP-43 is affecting the number of vesicles released because the frequency of mini events in the slice and autaptic cultures was unchanged. It is possible that GAP-43 affects the concentration of glutamate in the vesicles or vesicle turnover. GAP-43 is known to be involved in endocytosis (Neve et al. 1998), and this can affect filling of the vesicles (Dickman et al. 2005; Zhang et al. 1998). Further experiments are necessary to address these various possibilities.

Here we provide evidence from an intact neural circuit that GAP-43 is necessary for the proper development of functional synaptic properties. Several studies have examined the role of GAP-43 in hippocampal based learning and memory. Only a few have looked at properties of vesicular release directly. Those studies have shown either no difference in release properties or hippocampal anatomy in the absence of GAP-43.
(Capogna et al. 1999) or a decrease in transmitter release in heterologous expression systems (Dekker et al. 1989; Gambry et al. 1996). However, no measure of GAP-43 function at the thalamocortical synapse has been attempted until now despite the gross anatomical defects in barrel formation seen in GAP-43\(^{−/−}\) mice. Our data imply that thalamocortical synapses, unlike synapses in the hippocampus, are dependent on GAP-43 for normal development of their functional properties.

**GAP-43 in axon pathfinding**

Thalamocortical afferents must travel a great distance en route to the cortex to reach their destination, making proper axon pathfinding critical for their proper targeting. A wealth of data demonstrate a role for GAP-43 in axon extension and pathfinding (Aigner and Caroni 1995; Kruger et al. 1998; McIlvain et al. 2003; Meiri et al. 1998; Shen et al. 2002; Strittmatter et al. 1995; Zhang et al. 2000; Zhu and Julien 1999). GAP-43 is a major constituent of the growth cone (de Graan et al. 1985; Katz et al. 1985; Meiri et al. 1986) and can affect axon growth dynamics (Aigner and Caroni 1995; Aigner et al. 1995; Dent and Meiri 1998; Meiri et al. 1998). Overexpression of GAP-43 leads to excessive nerve sprouting (Aigner et al. 1995). Conversely, cells with reduced GAP-43 exhibit decreased neurite branching and extension (Aigner and Caroni 1995; Meiri et al. 1998). Animals with no GAP-43 have defects in commissure crossing (Shen et al. 2002; Strittmatter et al. 1995) and proper navigation of the optic tract (Zhang et al. 2000). Consistent with this evidence for a role of GAP-43 in axon pathfinding, our experiments demonstrate reduced fiber responses in the somatosensory cortex of knockout mice along with reduced expression of the thalamocortical markers 5HTT and VGlut2. We do not think this data can be accounted for by a role of GAP-43 in reducing the expression of VGlut2 because other studies have shown that decreased expression of VGlut2 actually causes a reduction in the amplitude of AMPA receptor minis (Moechars et al. 2006), whereas we observe an increase in the GAP-43\(^{−/−}\) mice.

Analysis of GAP-43 homozygous knockout phenotypes is difficult, which is presumably why no quantified measure of cortical innervation in GAP-43\(^{−/−}\) mice has been previously reported. Few homozygous mutant GAP-43 mice survive to adulthood with most dying within a few days after birth (Maier et al. 1999; Strittmatter et al. 1995). However, severe barrel defects are evident in GAP-43\(^{−/−}\) mice. Cytochrome oxidase histochemistry reveals that the afferents that make it to the cortex form tight clusters that are randomly scattered throughout the primary somatosensory cortex, instead of the regular arrays of barrels usually seen in wild-type mice (Maier et al. 1999). Functional imaging confirms a severely disturbed barrel map in GAP-43\(^{−/−}\) mice with no evidence for somatotopy (Dubroff et al. 2006). This phenotype is more severe than other barrel mutants, such as the “barrelless” (Adenyl Cyclase\(^{1−/−}\)) mice, which lack barrels but still have grossly intact somatotopy (Welker et al. 1996). However, our measurements of reduced thalamic innervation of cortex are consistent with reports from heterozygous GAP-43 mice, which have disturbed thalamocortical axon pathfinding (McIlvain et al. 2003). Overall, this data along with our finding of increased synaptic response in the GAP-43\(^{−/−}\) mice suggest that GAP-43 is involved in both thalamocortical axon innervation of cortex and normal formation of functional thalamocortical synapses.

**Why do GAP-43\(^{−/−}\) mice lack barrel maps?**

Several lines of evidence indicate that neural activity is required for the development and plasticity of somatosensory barrel maps. First, mutant mice lacking functional NMDA (NR1 or NR2B) or metabotropic glutamate (mGluR5) receptors have defects in the whisker-related barrel patterns in the trigeminal pathway (Hannan et al. 2001; Iwasato et al. 1997; Kutsuwada et al. 1996; Li et al. 1994; Mori et al. 1998). Second, NMDAR-mediated activity is required for barrel map rearrangement (Schlaggar et al. 1993) and the refinement of receptive fields in barrel cortex (Fox et al. 1996) although this effect is not mediated through excitatory cortical cells (Datwani et al. 2002) and may therefore involve inhibitory circuitry or some other source of activity mediated signaling. Third, mutant mice with defects in neurotransmitter release (Lu et al. 2003, 2006; Salichon et al. 2001) or the activity-dependent strengthening of the thalamocortical synapse (Inan et al. 2006) have barrel map defects. These data suggest that neuronal activity, specifically through glutamatergic synapses, modulates the precise formation of a whisker-related pattern in rodent somatosensory cortex.

Because the GAP-43 protein has been tied to synaptic function, we hypothesized that a role of GAP-43 in regulating thalamocortical synaptic transmission might explain the barrel map deficits seen in GAP-43\(^{−/−}\) mice (Maier et al. 1999; McIlvain et al. 2003; Rekart et al. 2005). Surprisingly, we found that thalamocortical synapses in GAP-43\(^{−/−}\) mice are actually stronger than wild-type synapses. We also found dramatically reduced innervation of layer IV by TCAs in the GAP-43\(^{−/−}\) mice to go along with evidence for a role of GAP-43 in the proper development of neurotransmitter release properties.

The abnormally strong synapses observed in GAP-43\(^{−/−}\) mice might drive synapse formation at inappropriate locations. This would disrupt mechanisms of synapse addition and elimination that allow neurons to form topographically appropriate connections because proper synapse formation is important for driving neuronal branch dynamics (Meyer and Smith 2006; Ruthazer et al. 2006). The thalamocortical synaptic defects we see in the GAP-43\(^{−/−}\) mice, then, could lead to a failure of TCAs to properly re-sort after entering the cortex. This would result in the disturbed topography observed in GAP-43\(^{−/−}\) mice and could lead to an overpruning of TCAs that results in the reduced TCA innervation of layer IV.

Another possibility is that GAP-43 is disrupting both axon guidance and synaptic function, and these independent effects together produce the severe barrel phenotype observed in GAP-43\(^{−/−}\) mice. The GAP-43 protein is almost certainly essential for mediating the axon guidance or targeting signals that direct thalamic afferents to barrel cortex in a topographic manner. This is, in fact, the hypothesis put forward in the original report of barrel map defects in GAP-43\(^{−/−}\) mice (Maier et al. 1999) and is consistent with a host of literature suggesting that GAP-43 function is related to axon growth cone guidance (McIlvain et al. 2003; Sretavan and Kruger 1998; Strittmatter et al. 1995; Zhu and Julien 1999). If TCAs reach the cortex too widely displaced from their target regions,
then subsequent re-sorting of the afferents may not be possible through normal activity-dependant mechanisms, especially if those mechanisms are disrupted by synaptic differences in the GAP-43+/− mice as our data suggest. It is also possible that many afferents simply get lost along the way and ultimately never reach the primary somatosensory cortex with the remaining afferents forming abnormally strong connections with their misplaced synaptic targets. Further studies are required to differentiate between these alternatives.

The data presented here suggest a dual role for GAP-43 in thalamocortical development, first in axon guidance and second in the development of normal synaptic function. Our data show that GAP-43 plays an important role in regulating glutamate concentration in the synaptic cleft. The specific and unusual synaptic defects we report in GAP-43 homozygous mutant mice provide important insight to GAP-43 function, a protein the purpose of which has been difficult to determine despite its ubiquitous expression and the availability of genetic and biochemical tools to study its function for more than two decades.

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References


Meiri KF, Pfeningher KH, Willard MB. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to gp46, a major polypeptide of a subcellular fraction enriched in growth cones. Proc Natl Acad Sci USA 83: 3537–3541, 1986.


