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Glutamic Acid Decarboxylase 65: A Link Between GABAergic Synaptic Plasticity in the Lateral Amygdala and Conditioned Fear Generalization

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An imbalance of the gamma-aminobutyric acid (GABA) system is considered a major neurobiological pathomechanism of anxiety, and the amygdala is a key brain region involved. Reduced GABA levels have been found in anxiety patients, and genetic variations of glutamic acid decarboxylase (GAD), the rate-limiting enzyme of GABA synthesis, have been associated with anxiety phenotypes in both humans and mice. These findings prompted us to hypothesize that a deficiency of GAD65, the GAD isoform controlling the availability of GABA as a transmitter, affects synaptic transmission and plasticity in the lateral amygdala (LA), and thereby interferes with fear responsiveness. Results indicate that genetically determined GAD65 deficiency in mice is associated with (1) increased synaptic length and release at GABAergic connections, (2) impaired efficacy of GABAergic synaptic transmission and plasticity, and (3) reduced spillover of GABA to presynaptic $GABA_B$ receptors, resulting in a loss of the associative nature of long-term synaptic plasticity at cortical inputs to LA principal neurons. (4) In addition, training with high shock intensities in wild-type mice mimicked the phenotype of GAD65 deficiency at both the behavioral and synaptic level, indicated by generalization of conditioned fear and a loss of the associative nature of synaptic plasticity in the LA. In conclusion, GAD65 is required for efficient GABAergic synaptic transmission and plasticity, and for maintaining extracellular GABA at a level needed for associative plasticity at cortical inputs in the LA, which, if disturbed, results in an impairment of the cue specificity of conditioned fear responses typifying anxiety disorders.
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INTRODUCTION

With a life time prevalence at around 30%, anxiety disorders range among the most prevailing psychiatric disorders (Kessler *et al*, 2005).The pathogenesis of anxiety disorders is deemed to be complex, with an interaction of biological factors and environmental influences (Domschke *et al*, 2013). A failure of the gamma-aminobutyric acid (GABA) system has been suggested as a major neurobiological pathomechanism of anxiety. For instance, subjects with mood and anxiety disorders display abnormally low extracellular GABA levels, in the plasma (Petty and Sherman, 1984; Petty *et al*, 1999) and cerebral spinal fluid (Kasa *et al*, 1982; Kendell *et al*, 2005). The rate of GABA synthesis is controlled through the enzyme glutamic acid decarboxylase (GAD), which catalyzes the conversion of glutamic acid to GABA. The brain contains two isoforms, GAD65 and GAD67, which are encoded by two genes, *GAD2* and *GAD1*, respectively (Erlander *et al*, 1991). The two forms display characteristic differences in localization and activity patterns (Erlander *et al*, 1991; Bowers *et al*, 1998). GAD67 is typically distributed throughout the neuron and almost all of it exists in its active cofactor-bound form, whereas GAD65 is predominantly found in synaptic terminals and much of it is in the form of an inactive apoenzyme (Kaufman *et al*, 1991; Martin and Rimvall, 1993). Both GAD enzymes are highly expressed in the amygdala, where fear training results in a transient decrease in GAD67 and GAD65 mRNA levels in the basolateral complex (Bergado-Acosta *et al*, 2008; Heldt and Ressler, 2007). *Gad65*-null mice show an increase in anxiety and altered responsiveness to anxiolytics (Kash *et al*, 1999), a generalization of learned fear responses (Bergado-Acosta *et al*, 2008), and an impairment of fear extinction (Sangha *et al*, 2009; Sangha *et al*, 2012). In humans, available genetic data indicate an influence of the *GAD2* locus (chromosome 10p12.1) on anxious psychopathology (Smoller *et al*, 2001; Unschuld *et al*, 2009). A substantial body of evidence thus suggests that an interaction between GAD65, GABA availability, and extracellular GABA level may determine fear responsiveness in the amygdala. Therefore, we adopted a genetic mouse model and combined *in vitro* and behavioral approaches to

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characterize the role of GAD65 in the amygdala, and thereby to fill a significant gap in our understanding on how abnormalities in the GABAergic system contribute to anxiety and anxiety disorders.

MATERIALS AND METHODS

Animals

Male mice with a disrupted gene for GAD65 ($Gad65^{-/-}$) and wild-type littermates ($Gad65^{+/+}$) were used, obtained from $Gad65^{+/-}$ x $Gad65^{+/-}$ breeding on a C57BL/6J genetic background (>10 generations of backcross) (Asada *et al* 1996). Animals were kept under a 12 h light/dark cycle with food and water provided *ad libitum*. All experiments were carried out in accordance with the European Committees Council Directive (86/609/EEC) and were approved by local authorities (LANUV NRW, AZ 8.87-50.10.36.09—G 53/2005; AZ 87-51.042010.A218) and the Austrian Animal Experimentation Ethics Board (GZ66.011/28-BrGT/2009).

Electrophysiological Recordings

Mice (8–12 weeks old) were anesthetized with isoflurane (2.5% in O_2) and decapitated. Coronal slices were prepared and whole-cell patch clamp recordings were obtained from principal neurons (PNs) in the lateral amygdala (LA) (Sah *et al*, 2003), as described previously (Sosulina *et al*, 2006; for details, see Supplementary Information). After assessing membrane properties, postsynaptic responses were recorded under current- or voltage-clamp conditions, as previously described (Chauveau *et al*, 2012; for details, see Supplementary Information). Extracellular stimuli (500 μs duration) were delivered every 20 s through a bipolar stainless-steel electrode, placed at thalamic or cortical afferents (Szinyei *et al*, 2000). GABAergic LTP was induced by a theta-burst stimulation (TBS) protocol (four pulses, 50% maximal amplitude, at 100 Hz, repeated 25 times at 2 Hz; sequence repeated four times every 25 s). The stimulation electrode was placed within the LA and thalamic afferents for monosynaptic and disynaptic GABAergic LTP, respectively. TBS at thalamic afferents was used to induce homosynaptic thalamic LTP. For associative cortical LTP, cortical and thalamic afferents were costimulated 45 times at 30 Hz (single train stimulation, STS). Evoked responses were normalized to the mean at baseline (10 min), and LTP was determined from 10 responses at 25–30 min after induction. Cells were held at −5 and −70 mV for measurements of GABAergic and glutamatergic responses, respectively.

Fear Conditioning

Male mice (8–12 weeks old) were fear conditioned using established protocols (Bergado-Acosta *et al*, 2008; Sangha *et al*, 2009). One day after adaptation (presentation of 2.5 kHz tone, 85 dB, 10 s; CS−; 6×), fear was trained by three conditioned stimuli (CS+; 10 kHz tone, 85 dB, 10 s, randomized 10–30 s intervals), each coterminated with a 1-s footshock (US; 0.4 mA, 'low shock intensity'; 0.7 mA 'high shock intensity'). Fear was retrieved 1 day later upon 4× CS+ (and 4×CS−) presentation in a neutral context (for details, see Supplementary Information).

Data Analysis

Data are presented as mean with standard error of the mean (±SEM). The number of experiments is given as (no. of cells/no. of animals). Data were analyzed using Student's *t*-test, one-way analysis of variance (ANOVA), Kruskal–Wallis test, Mann–Whitney test, two-sample Kolmogorov–Smirnov test, or repeated-measurement ANOVA, as applicable. Multiple comparisons were tested with Duncan *post hoc* test. Significance level was set at $p<0.05$. Statistically significant outliers were identified using Grubb's test (significance level $p<0.05$).

Immunocytochemistry and Electron Microscopy Studies

For light (LM) and electron microscopy (EM), mice (11–13 weeks old) were deeply anesthetized (thiopental, 150 mg/kg i.p) and perfused transcardially with phosphate-buffered saline (25 mM, 0.9% NaCl, pH 7.4) followed by ice-cold fixative (composed of 4% w/v PFA and 15% v/v of a saturated solution of picric acid in 0.1 M PB, pH 7.4). For EM, glutaraldehyde (0.05% v/v) was added. Coronal brain slices were cut at 40 and 70 μm for LM and EM, respectively, on a vibratome (Leica VT1000S, Vienna, Austria).

Immunocytochemistry was performed as previously published (Sreepathi and Ferraguti, 2012), using primary antibodies against parvalbumin (PV), calbindin (CB), or calretinin (CR) and biotinylated secondary antibodies (for details, see Supplementary Information). Four sections per mouse were taken between bregma levels −1.6 and −2.0 (Paxinos and Franklin, 2001), and left and right LA were analyzed by a researcher blinded to the genotype, using Neurolucida (MBF Bioscience, Williston, VT). LA–BA boundaries were drawn according to Paxinos and Franklin atlas, and on brain sections labeled for the vesicular glutamate transporter 3, which is selectively enriched in BA. Data are expressed as mean ± SEM of labeled neurons per mm^2 in the LA.

For EM analysis, free floating sections were cryoprotected, freeze–thawed twice, and processed for pre-embedding immunoperoxidase reactions using the avidin–biotin–HRP complex as previously described (Sreepathi and Ferraguti, 2012; for details, see Supplementary Information). GABAergic terminals were identified through vesicular GABA transporter (VGAT) immunoreactivity in serial ultrathin sections (70 nm) of the LA using a Philips CM 120TEM. The density of GABAergic synapses was estimated through dissector analysis on serial ($n=5$) ultrathin sections (Rademacher *et al*, 2010). The length of synapses made by VGAT-immunolabeled axon terminals was estimated from 40–50 randomly selected synapses per mouse. Measurements were taken with the ImageJ software (for details, see Supplementary Information).

RESULTS

Synaptic Transmission in LA Projection Neurons in GAD65-Deficient Mice

Basic GABAergic synaptic properties were analyzed by whole-cell recording of spontaneous inhibitory postsynaptic currents (sIPSCs) from PNs in the LA in the presence of DNQX and AP5 (Figure 1a). The sIPSC frequency in

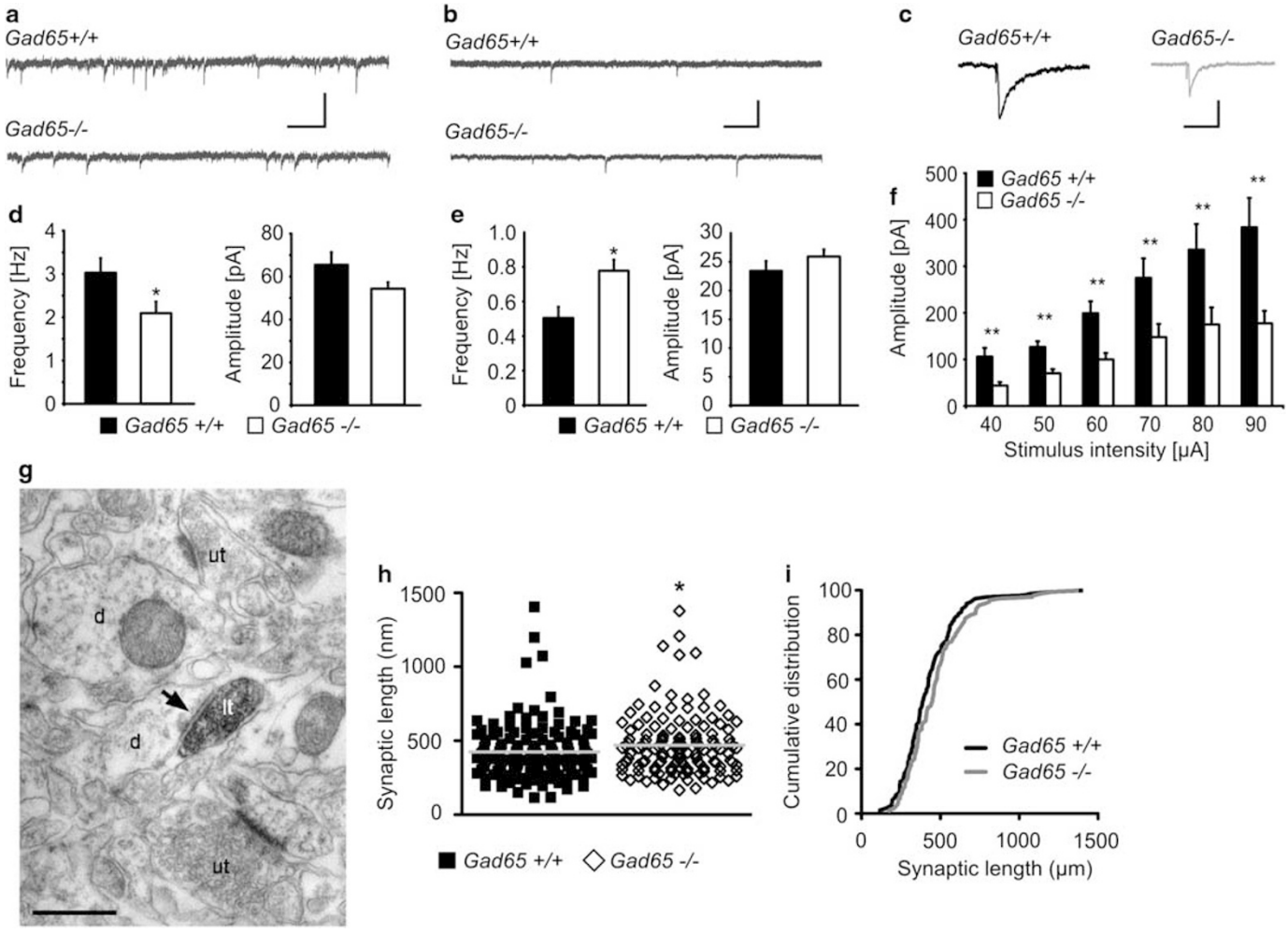

Figure 1 Properties of GABAergic synaptic transmission in the LA of $Gad65^{+/+}$ (filled bars) and $Gad65^{-/-}$ mice (open bars). (a–f) Inhibitory postsynaptic currents (IPSCs) recorded in LA PNs from $Gad65^{+/+}$ and $Gad65^{-/-}$ mice. Example traces show sIPSCs (a), mIPSCs (b) and eIPSCs (c). Scale bars: 100 pA and 500 ms in (a, b), 100 pA and 20 ms in (c). (d) Mean sIPSC frequency(left) and sIPSC amplitude (right) in $Gad65^{+/+}$ ($n = 19/3$) and $Gad65^{-/-}$ ($n = 15/3$). (e) Mean mIPSC frequency and mIPSC amplitude in $Gad65^{+/+}$ ($n = 33/4$) and $Gad65^{-/-}$ ($n = 23/3$). Averages from synaptic activity recorded over 5 min in each neuron. (f) Mean amplitude of eIPSCs at different stimulus intensities. Stimulus intensity was increased stepwise, each intensity was tested five times in a given neuron, and eIPSCs at each intensity were averaged. Original traces depict examples of eIPSC evoked at 70 µA in the two genotypes. The monosynaptic nature of recorded eIPSCs was ascertained by short latencies and resistance to bath-applied DNQX (10 µM). Note the significant reduction of eIPSCs at all tested stimulation intensities in $Gad65^{-/-}$ ($n = 11/3$) compared with $Gad65^{+/+}$ ($n = 12/3$). Data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$. (g–i) Synaptic length of GABAergic synapses in the LA. (g) Electron micrograph of a typical GABAergic synapse in the LA of $Gad65^{-/-}$ mice. Arrow indicates a symmetric synapse, d = dendrite, ut = unlabeled terminal, lt = VGAT labeled terminal. (h) Scattered dot plot (mean shown in grey) and (i) cumulative frequency distribution of the synaptic length of GABAergic synapses in LA of $Gad65^{+/+}$ (sample size, $n = 133$) and $Gad65^{-/-}$ ($n = 135$) mice. Scale bar: 500 nm.

$Gad65^{-/-}$ (2.2 ± 0.3 Hz; $n = 15/3$) was significantly decreased as compared with $Gad65^{+/+}$ (3 ± 0.3 Hz; $n = 19/3$; $p < 0.05$), whereas the sIPSC amplitude was not significantly different (Figure 1d). As the frequency of sIPSCs depends on the overall network activity, miniature IPSCs (mIPSCs) were recorded in the presence of tetrodotoxin (TTX 1 µM; Figure 1b). The mean frequency of mIPSCs in $Gad65^{-/-}$ was significantly increased (0.8 ± 0.06 Hz, $n = 23/3$ *vs* 0.5 ± 0.06, $n = 33/4$ in $Gad65^{+/+}$; $p < 0.02$; Figure 1e), whereas mean mIPSC amplitudes were not different between genotypes (26 ± 1.3 pA, $n = 23/3$; 23 ± 1.7 pA, $n = 33/4$; $p = 0.7$). Next, GABAergic synaptic transmission was evoked by electrical microstimulation within the LA (Figure 1c). The mean amplitude of evoked IPSCs (eIPSCs) was significantly decreased at the tested stimulation intensities in $Gad65^{-/-}$ ($n = 11/3$; $n = 12/3$ in $Gad65^{+/+}$; Figure 1f). As the inhibitory GABAergic and the excitatory glutamatergic systems are known to interact, pharmacologically isolated excitatory postsynaptic currents (eEPSCs) were analyzed (Supplementary Figure S1A–C). No significant differences in the glutamatergic system were detected between $Gad65^{-/-}$ and $Gad65^{+/+}$ (Supplementary Figure S1). In addition, GAD65 deficiency had no effect on passive and active membrane properties in PNs (Supplementary Table S1).

Neuroanatomical Characteristics of Inhibitory Interneurons and Synapses

To establish whether the number of interneurons in the LA differed between *Gad65* genotypes, we analyzed the density of cells expressing the calcium-binding proteins PV, CB, or

CR, which are known to be expressed by the main LA interneuron types. *Gad65*$^{-/-}$ ($n=3$) and *Gad65*$^{+/+}$ ($n=3$) animals showed a similar density for both CB− (*Gad65*$^{-/-}$: 112.0 ± 11.4, *Gad65*$^{+/+}$: 128.0 ± 7.6 neurons per mm^2; $p=0.1$) and CR− (*Gad65*$^{-/-}$: 104.3 ± 5.8, *Gad65*$^{+/+}$: 100.2 ± 9.9; $p=0.35$) labeled neurons in the LA. Likewise, no difference was detected between a different group of *Gad65*$^{-/-}$ and *Gad65*$^{+/+}$ mice in the density of PV immunopositive interneurons (*Gad65*$^{-/-}$: 64.6 ± 4.5, *Gad65*$^{+/+}$: 67.0 ± 5.8 neurons per mm^2; $p=0.4$). To assess the density of GABAergic terminals and synapses in the LA of *Gad65*$^{-/-}$ ($n=3$) and littermate *Gad65*$^{+/+}$ ($n=3$), we performed pre-embedding immuno-electron microscopy using VGAT as a marker for GABAergic synapses (Supplementary Figure S2), and we examined the number of asymmetric (putative glutamatergic) and unlabeled symmetric synapses. Data obtained from the three animals in each group were pooled, as no difference was found among them (Kruskal–Wallis test). The density of each synaptic type, expressed as synapses per μm^3, was similar in *Gad65*$^{-/-}$ and *Gad65*$^{+/+}$ mice ($p>0.1$) (Supplementary Figure S2A and B). Likewise, the number of GABAergic terminals was similar ($p=0.48$), and there was no difference in the ratio between symmetric and asymmetric synapses (Supplementary Figure S2C). Measuring the synaptic length of GABAergic synapses (Figure 1g) revealed a small but significant increase ($p=0.04$; Mann–Whitney test) in *Gad65*$^{-/-}$ mice (Figure 1h), and a shift in the cumulative frequency distribution of GABAergic synapses towards larger synaptic length ($p=0.043$; two-sample Kolmogorov–Smirnov test, Figure 1i).

Alteration of Synaptic Plasticity in the LA of GAD65-Deficient Mice

Besides acting as an inhibitory transmitter via postsynaptic receptors, GABA controls both short- and long-term plasticity in the amygdala via presynaptic $GABA_B$ receptors (Ehrlich *et al*, 2009). Short-term plasticity was examined through application of priming stimuli at either cortical or thalamic afferents, and probing glutamatergic responses to subsequent single test stimuli at the respective other input. Priming has been shown to depress glutamate release through heterosynaptic activation of presynaptic $GABA_B$ receptors (Szinyei *et al*, 2000; Pan *et al*, 2009). In *Gad65*$^{+/+}$, the priming of cortical afferents inhibited thalamically evoked EPSCs in PNs by 30 ± 5% ($n=13/2$) (Figure 2 c, d, g). Similarly, priming of thalamic afferents inhibited cortically evoked EPSCs in *Gad65*$^{+/+}$ by 30 ± 3% ($n=23/3$)

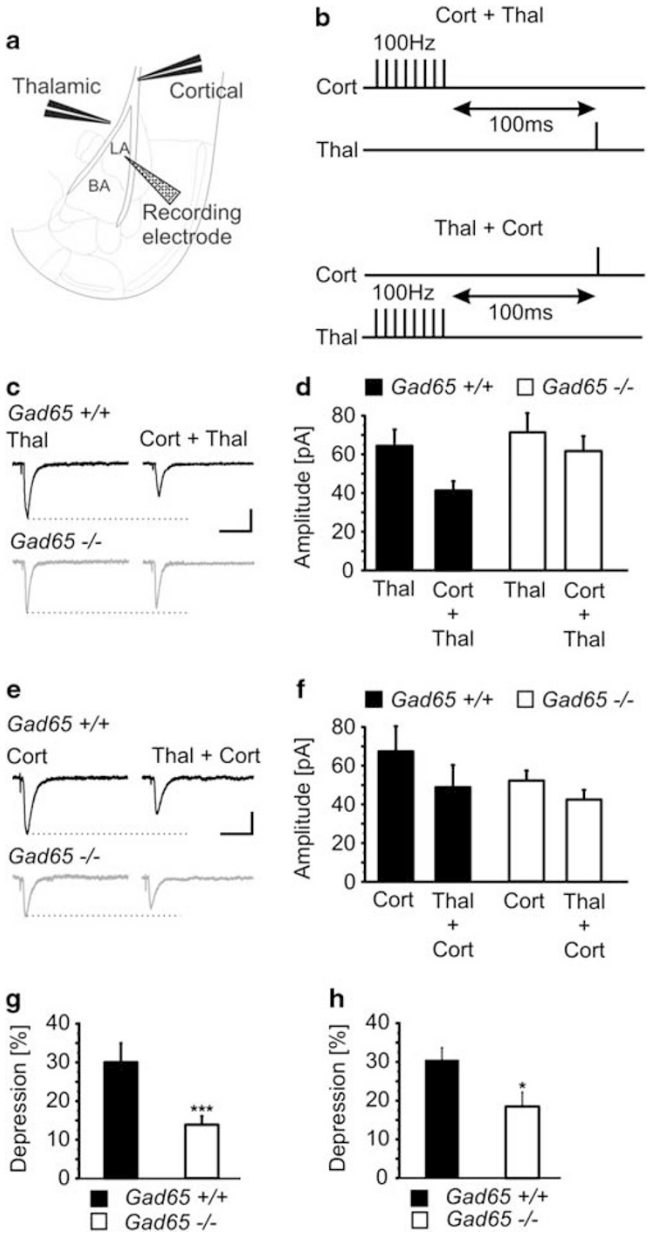

Figure 2 Primed-pulse depression at thalamic and cortical afferents to LA PNs of *Gad65*$^{+/+}$ and *Gad65*$^{-/-}$ mice. (a) Placement of stimulation electrodes at thalamic and cortical afferents, and of the recording electrode in the LA. (b) Stimulus protocol for cortical priming of thalamic inputs (upper; Cort + Thal) and for thalamic priming of cortical inputs (lower; Thal + Cort); single pulse stimulation of one afferent input preceded (100 ms delay) by priming stimulation (eight pulses at 100 Hz) of the other input. Stimulus strength set to 50% maximal. (c, d) Cortical priming of thalamic inputs. (c) Original traces of eEPSCs at thalamic inputs evoked by single stimulation alone and after cortical priming (right) in *Gad65*$^{+/+}$ (upper) and *Gad65*$^{-/-}$ (lower trace). (d) Mean amplitudes of eEPSCs upon single thalamic stimulation (Thal) and after cortical priming (Cort + Thal) in *Gad65*$^{+/+}$ and *Gad65*$^{-/-}$. Single and priming stimulation was tested five times in a pseudo-randomized fashion in each neuron, and data were averaged from recordings in different neurons; *Gad65*$^{+/+}$ ($n=13/2$), *Gad65*$^{-/-}$ ($n=17/3$). (e, f) Thalamic priming of cortical inputs. (e) Original traces of eEPSCs at cortical inputs evoked by single stimulation alone (left) and after thalamic priming (right) in *Gad65*$^{+/+}$ (upper) and *Gad65*$^{-/-}$ (lower trace). (f) Mean amplitudes of eEPSCs upon single cortical stimulation (Cort) and after thalamic priming (Thal + Cort) in *Gad65*$^{+/+}$ and *Gad65*$^{-/-}$. Averaging procedure as in (d); *Gad65*$^{+/+}$, $n=23/3$; *Gad65*$^{-/-}$, $n=27/5$. (g) Percentage of depression (reduction in amplitude of primed response relative to amplitude of non-primed response) induced by cortical priming at thalamic inputs, calculated from data in (d). (h) Percentage of depression induced by thalamic priming at cortical inputs, calculated from data in (f). Note the depression of eEPSCs at both thalamic and cortical afferents upon priming in *Gad65*$^{+/+}$, and reduction of this depression in *Gad65*$^{-/-}$. Scale bars: 20 pA and 50 ms; *$p<0.05$, ***$p<0.001$.

(Figure 2e, f, h). In *Gad65*$^{-/-}$, EPSCs were inhibited by only 14 ± 2% ($n = 17/3$) and 18 ± 3% ($n = 27/5$) at thalamic and cortical afferents upon priming (Figure 2c–h). The priming effect was significant at both thalamic ($F = 36.46$; $p < 0.001$) and cortical afferents ($F = 41.86$; $p < 0.001$). Priming × genotype interaction was significant, in that priming-induced depression was less in *Gad65*$^{-/-}$ compared with *Gad65*$^{+/+}$ (thalamic: $F = 6.33$; $p = 0.017$; cortical: $F = 4.19$; $p = 0.046$).

In a next series of experiments, we tested the consequence of GAD65 deficiency on two forms of LTP, homosynaptic LTP at thalamo-LA afferents (Bauer *et al*, 2002) and associative, heterosynaptic LTP at cortico-LA afferents (Humeau *et al*, 2003). Homosynaptic thalamic LTP is controlled by presynaptic $GABA_B$ receptors, and this influence was significantly reduced in GAD65-deficient mice (Supplementary Figure S3). Associative, heterosynaptic LTP at cortical inputs to LA PNs was examined through stimulation of cortical and thalamic afferents. Costimulation of these two inputs resulted in a significant and sustained increase in EPSP amplitudes in *Gad65*$^{+/+}$ (164 ± 19% of baseline at 25 min post STS; $n = 4/3$; $p < 0.02$; Figure 3b), whereas stimulation of cortical afferents alone did not induce LTP (98 ± 6% of baseline; $n = 10/3$; $p > 0.74$; Figure 3d). By contrast, in *Gad65*$^{-/-}$, stimulation of cortical afferents alone induced robust non-associative homosynaptic LTP, as indicated by a significant increase in EPSP amplitude (141 ± 12% of baseline; $n = 14/4$; $p < 0.002$; Figure 3d). In the presence of the $GABA_B$ receptor-specific antagonist CGP55845, cortical afferent stimulation alone was sufficient to induce LTP in *Gad65*$^{+/+}$ (161 ± 22% of baseline; $n = 7/2$; $p < 0.02$; Figure 3e). These data indicate that GAD65 deficiency results in a shift from a heterosynaptic, associative form to a homosynaptic form of LTP at cortical inputs to LA PNs, which can be mimicked by pharmacological interference with $GABA_B$ receptor signaling. In the presence of the GABA reuptake inhibitor NNC711 (70 μM) in *Gad65*$^{-/-}$, STS applied to cortical afferents alone was insufficient to induce LTP, in that the mean EPSP amplitudes were not significantly different from those during baseline recordings (95 ± 11% of baseline; $n = 10/5$; $p > 0.7$; Figure 3f). Finally, to test the presynaptic nature of homosynaptic LTP observed in *Gad65*$^{-/-}$, the Ca^{2+} chelator BAPTA (20 mM) was added to the intracellular solution. Under these conditions, robust homosynaptic LTP was induced in *Gad65*$^{-/-}$ upon STS of cortical afferents (138 ± 18% of baseline; $n = 11/3$; $p < 0.02$; Supplementary Figure S4B), indicating presynaptic mediation.

Next, we addressed the question whether GAD65 deficiency affects plasticity at GABAergic synapses on PNs in the LA. First, a disynaptic form of inhibitory LTP in PNs was tested, mediated upon thalamic afferent stimulation via interneurons to PNs (Szinyei *et al*, 2007). Disynaptic LTP was induced by TBS (Figure 4a), resulting in an increase in IPSC amplitudes as compared with baseline (*Gad65*$^{+/+}$: 129 ± 13%; $n = 12/4$; $p < 0.04$; Figure 4b). By contrast, in *Gad65*$^{-/-}$, TBS induced a significant reduction of the IPSC amplitudes to 79 ± 5% of baseline ($n = 15/4$; $p < 0.0002$; Figure 4b). Bath application of DNQX at the end of the experiment blocked the IPSCs in both genotypes, demonstrating their disynaptic nature. To exclude the possibility that the failed disynaptic LTP induction in *Gad65*$^{-/-}$ is a result of subthreshold depolarization of local interneurons, the stimulation intensity was increased to 100 and 150%. Whereas the normalized IPSC amplitudes increased with stimulation intensity in *Gad65*$^{+/+}$ PNs (50%: 149 ± 8% of baseline $p < 0.01$; 100%: 173 ± 21%; $p < 0.01$; 150%: 179 ± 24%; $p < 0.01$; $n = 4/2$; Figure 4c), no such increase was observed in *Gad65*$^{-/-}$ PNs (50%: 100 ± 9% of baseline; 100%: 100 ± 9%; 150%: 86 ± 13%; $n = 6/2$; Figure 4c). These data indicate that the impairment of the disynaptic LTP in PNs is not dependent on the intensity of thalamic afferent stimulation.

Possible sites of impaired disynaptic LTP are the glutamatergic inputs to interneurons and/or the GABAergic synapses on the recorded PN. To distinguish between these possibilities, we analyzed inhibitory GABAergic LTP at monosynaptic connections (Lange *et al*, 2012). To analyze this type of inhibitory LTP, the stimulation electrode was placed within the LA (Figure 4d). In both genotypes, LTP could be induced by TBS and a significant increase of the mean normalized IPSC amplitudes was detected as compared with baseline (*Gad65*$^{-/-}$: 140 ± 15%; $n = 10/5$; $p < 0.02$; *Gad65*$^{+/+}$: 253 ± 44%; $n = 10/4$; $p < 0.003$; Figure 4e). Interestingly, the inhibitory LTP was significantly less prominent in *Gad65*$^{-/-}$ than in *Gad65*$^{+/+}$ ($p < 0.05$). To control for the monosynaptic nature of the eIPSCs, DNQX was bath-applied at the end of the experiments (Figure 4f).

Ablation of the *Gad65* Gene and Training with High Shock Intensity: Consequences for Conditioned Fear Responses and Synaptic Plasticity in the LA

Ablation of the *Gad65* gene in mice results in a pronounced context-independent, generalization of conditioned fear responses during long-term memory retrieval (Bergado-Acosta *et al*, 2008). Fear generalization in mice is also observed upon Pavlovian training with high shock intensities (Laxmi *et al*, 2003). In an attempt to relate behavioral and synaptic phenotypes of GAD65 deficiency, we reasoned that generalized fear responses obtained upon standard fear training in *Gad65*$^{-/-}$ mice, and upon training with high shock intensities in *Gad65*$^{+/+}$ littermates, might be associated with a similar set of synaptic alterations in the LA. The *Gad65*$^{-/-}$ mice and their *Gad65*$^{+/+}$ littermates were fear conditioned using an established auditory conditioning paradigm (Sangha *et al*, 2009). Fear was assessed as percentage of freezing to the conditioned (CS+) and non-conditioned (CS−) stimulus. In line with our earlier findings (Bergado-Acosta *et al*, 2008), both genotypes exhibited high freezing to the CS+ during retrieval of fear memory (*Gad65*$^{+/+}$ 68 ± 6%; $n = 3$; *Gad65*$^{-/-}$ 66 ± 5%; $n = 3$; Figure 5a). During CS− presentation, *Gad65*$^{-/-}$ showed a significantly increased freezing response (44 ± 6%; $n = 3$) compared with their *Gad65*$^{+/+}$ littermates (12 ± 4%; $n = 3$). Thus, while *Gad65*$^{+/+}$ differentiated between the CS+ and CS− ($p < 0.05$), the GAD65-deficient mutants displayed similar levels of freezing during presentation of the two stimuli ($p = 0.15$), indicating fear generalization. A similar generalization of fear responsiveness was observed upon high shock intensity training of *Gad65*$^{+/+}$ using an increased intensity of the

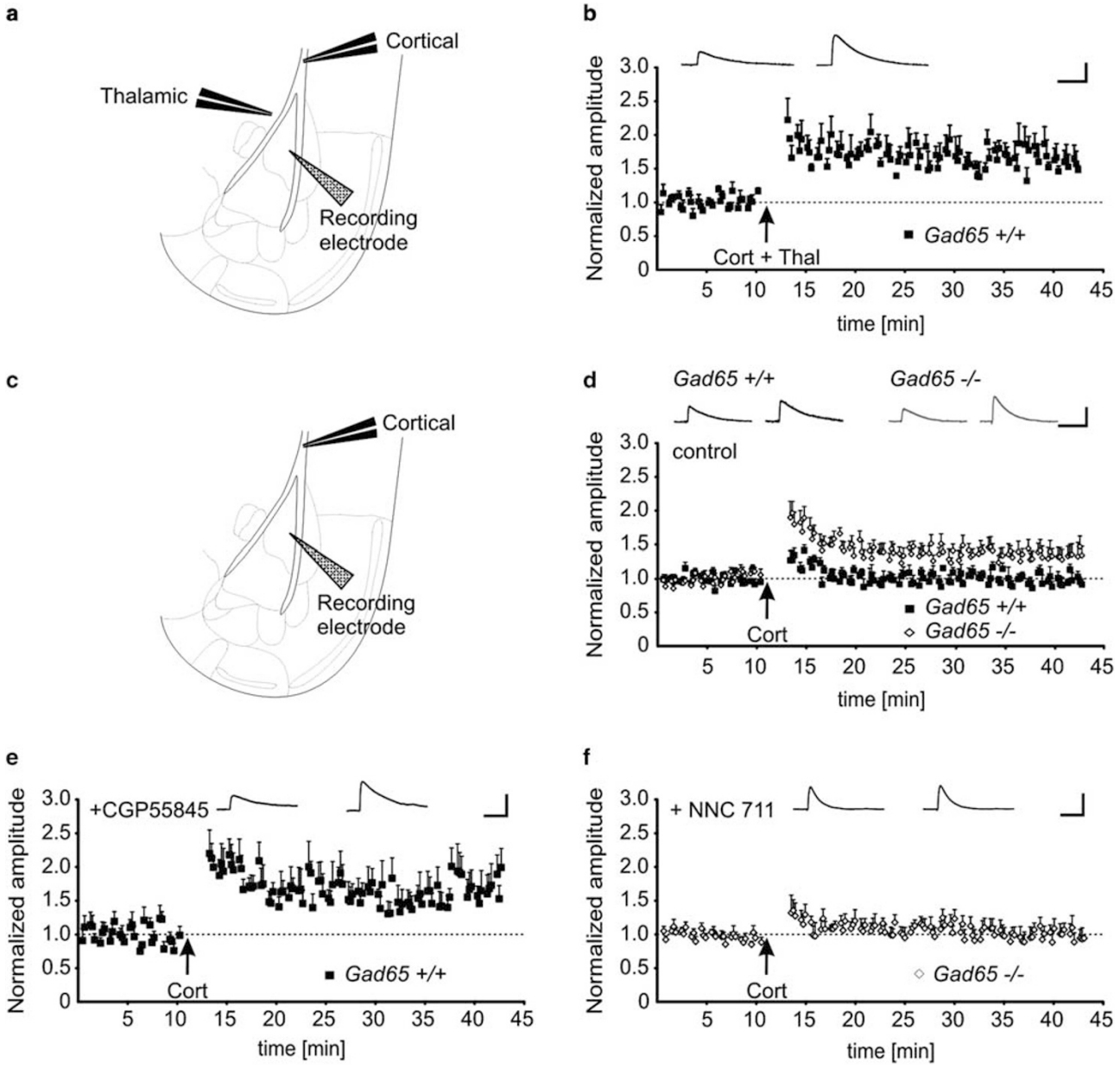

Figure 3 Properties of LTP at cortical afferents to LA PNs of *Gad65*$^{+/+}$ and *Gad65*$^{-/-}$ mice. (a, b) Heterosynaptic, associative LTP. (a) Scheme of placement of electrodes for costimulation of cortical and thalamic afferents, and of electrode for recording eEPSPs in LA PNs. (b) Time course of synaptic changes upon costimulation of cortical and thalamic afferents (45 stimuli at 30 Hz each; marked by arrow) in *Gad65*$^{+/+}$ ($n = 4/3$); example traces of recorded eEPSPs before and 25 min after LTP induction. (c, d) Homosynaptic, non-associative LTP. (c) Placement of stimulation electrode at cortical afferents and of the recording electrode in the LA. (d) Time course of synaptic changes upon stimulation of cortical afferents alone (45 stimuli at 30 Hz; marked by arrow) in *Gad65*$^{-/-}$ ($n = 14/4$; open squares) and *Gad65*$^{+/+}$ ($n = 10/3$; filled squares); original traces exemplify eEPSPs of *Gad65*$^{+/+}$ (black) and *Gad65*$^{-/-}$ (gray) before and 25 min after LTP induction. Note that only costimulation of cortical and thalamic inputs induced LTP at cortical afferents in *Gad65*$^{+/+}$, whereas stimulation of cortical afferents induced LTP in *Gad65*$^{-/-}$. (e) Homosynaptic, non-associative LTP in the presence of a $GABA_B$ receptor antagonist in *Gad65*$^{+/+}$. Time course of synaptic changes after stimulation of cortical afferents alone (paradigm as in d) in the presence of CGP55845 (2 μM), a $GABA_B$ receptor antagonist, in *Gad65*$^{+/+}$ ($n = 7/2$). Original recordings of eEPSPs depicted before and after LTP induction. (f) Lack of homosynaptic LTP in the presence of a GABA reuptake blocker in *Gad65*$^{-/-}$. Time courses of synaptic changes upon stimulation of cortical afferents alone (paradigm as in d) and in the presence of the GABA reuptake blocker NNC711 (70 μM) in *Gad65*$^{-/-}$ ($n = 10/5$); original traces of eEPSPs depicted before and after LTP induction. Scale bars: 6 mV and 100 ms.

unconditioned stimulus (Albrecht *et al*, 2010). Under these conditions, CS+ and CS− evoked similar levels of freezing during fear memory retrieval (CS−: 53 ± 5%; CS+: 46 ± 2; $n = 6$; $p > 0.21$; Figure 5b).

Next, *ex vivo* slices were prepared from the trained animals 24 h after fear retrieval, and non-associative LTP was probed using STS at cortical afferents to LA PNs as before. The PNs recorded after fear training in *Gad65*$^{-/-}$

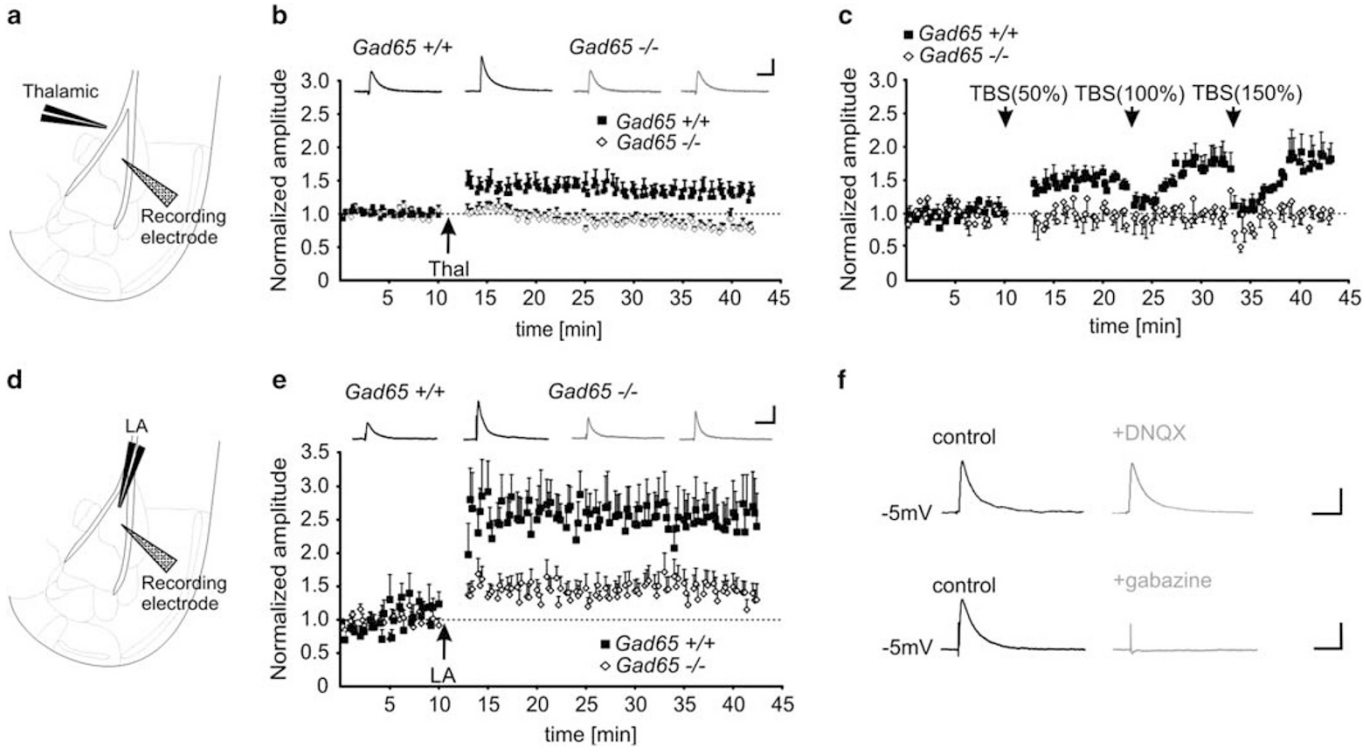

Figure 4 Di- and monosynaptic GABAergic LTP in the LA of *Gad65*$^{+/+}$ and *Gad65*$^{-/-}$ mice. (a-c) Disynaptic GABAergic LTP. (a) Scheme of placement of stimulation electrode at thalamic afferents and of electrode for recording eIPSCs in the LA. (b) Time course of synaptic changes upon TBS (marked by arrow) at thalamic afferents in *Gad65*$^{+/+}$ ($n=12/4$) and *Gad65*$^{-/-}$ ($n=15/4$). Original traces exemplify recorded eIPSCs before and 25 min after LTP induction. (c) Saturation of disynaptic GABAergic LTP in *Gad65*$^{+/+}$ ($n=4/2$) and *Gad65*$^{-/-}$ ($n=6/2$). Percentage refers to maximal stimulation intensity. Note the lack of disynaptic GABAergic LTP in *Gad65*$^{-/-}$ at all stimulation intensities. (d–f) Monosynaptic GABAergic LTP. (d) Placement of stimulation electrode and recording electrode within the LA. (e) Time course of synaptic changes after TBS (arrow) delivered within the LA in *Gad65*$^{+/+}$ ($n=10/4$) and *Gad65*$^{-/-}$ ($n=10/5$), with example traces of eIPSCs depicted before and 25 min after LTP induction. (f) eIPSCs recorded in LA PNs of *Gad65*$^{+/+}$ in the presence of DNQX or gabazine, indicating the monosynaptic GABAergic nature of recorded eIPSCs. Scale bars: 200 pA and 50 ms.

displayed a robust increase of EPSP amplitudes upon STS to $164\pm12\%$ of the baseline ($n=11/3$; $p<0.0001$; Figure 5c), similar to the non-associative LTP obtained in non-trained *Gad65*$^{-/-}$ ($p=0.2$; Figure 3d). In *Gad65*$^{+/+}$, the same stimulation protocol did not result in an increase in EPSPs above baseline values ($87\pm7\%$ of baseline; $n=12/3$; Figure 5c). By contrast, in slices prepared from *Gad65*$^{+/+}$ after high shock intensity training with generalized conditioned fear, STS at cortical afferents induced robust non-associative LTP ($144\pm13\%$ of the baseline, $n=14/3$; $p<0.001$; Figure 5d). This form of LTP was indistinguishable from that in *Gad65*$^{-/-}$ ($p=0.9$). Finally, inclusion of the Ca^{2+} chelator BAPTA (20 mM) in the intracellular solution did not prevent this LTP in LA PNs of *Gad65*$^{+/+}$ after high shock intensity training ($156\pm15\%$ of baseline; $n=10/3$; $p<0.002$; Supplementary Figure S5), supporting the view of presynaptic mechanisms similar to that in homosynaptic LTP in PNs of *Gad65*$^{-/-}$.

DISCUSSION

In this study, we present evidence for an involvement of the 65-kDa isoform of the GABA-synthesizing enzyme, GAD65, in the balance of GABAergic and glutamatergic synaptic transmission, and of associative and non-associative forms of long-term synaptic plasticity in the LA. GAD65 deficiency and stressful fear experience both result in a loss of this balance, which at the behavioral level appears to be associated with reduced stimulus discrimination for conditioned fear responses.

Structural and Functional Consequences of GAD65 Deficiency at GABAergic Synapses in the Amygdala

GAD is thought to be expressed in the majority, if not all subsets, of GABA neurons in the amygdala (Ehrlich *et al*, 2009; Pape and Pare, 2010) and is thus likely to serve as a general mechanism contributing to inhibitory control. In fact, GAD expression is regulated upon both stress (Bowers *et al*, 1998) and fear conditioning (Bergado-Acosta *et al*, 2008; Heldt and Ressler, 2007). While GAD67 is the cytosolic isoform controlling metabolic GABA synthesis (Kaufman *et al* 1991), it has been suggested on the basis of subcellular localization, membrane, and cofactor association studies that GAD65 is the synaptically localized isoform generating GABA in an activity-dependent manner required for rapid synaptic release (Kaufman *et al*, 1991; Patel *et al*, 2006). The expression of GAD65 is reduced 24 h after fear conditioning in the BLA (Bergado-Acosta *et al*, 2008), concomitant with a decrease in GABAergic LTP in LA PNs (Szinyei *et al*, 2007). Our data show that Gad65 deficiency

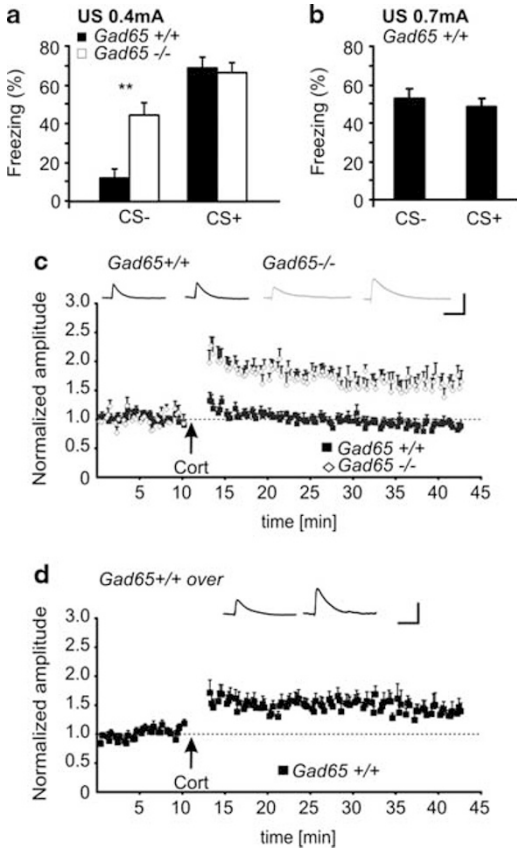

Figure 5 Generalized fear and non-associative LTP in GAD65-deficient mice upon standard fear training and in wild-type mice upon fear training with high shock intensities. (a, b) Freezing during fear retrieval in conditioned mice. (a) Freezing of $Gad65^{+/+}$ ($n=3$) and $Gad65^{-/-}$ ($n=3$) mice in response to CS− and CS+ presentation during fear retrieval after standard training with low shock intensity. Freezing time was calculated as percentage during the 10 s CS presentation. (b) Freezing of $Gad65^{+/+}$ ($n=6$) during CS− and CS+ presentation after training with high shock intensity. US stimulation strengths as indicated. Note that $Gad65^{+/+}$ differentiate between the CS+ and CS−, while null mutants after training with low shock intensities and wild types after training with high shock intensities display similar levels of freezing during CS+ and CS−, indicating fear generalization. (c, d) LTP at cortical inputs recorded in LA PNs *ex vivo*, 24 h after fear retrieval. (c) Time course of synaptic changes after STS at cortical afferents (marked by arrow) in $Gad65^{-/-}$ ($n=11/3$; open squares) and $Gad65^{+/+}$ ($n=12/3$; filled squares) after standard low shock intensity fear training. Example traces of eEPSPs before and 25 min after STS in $Gad65^{+/+}$ (black) and $Gad65^{-/-}$ (gray) mice. (d) Same as in (c), but recordings were obtained from LA PNs in $Gad65^{+/+}$ after training with high shock intensities ($n=14/3$). Note that null mutants after standard training and wild types after training with high shock intensities display homosynaptic cortico-LA LTP, whereas $Gad65^{+/+}$ after training with low shock intensity lack this form of LTP. Scale bars: 6 mV and 100 ms; $^{**}p<0.01$.

impairs the efficacy of evoked GABAergic synaptic transmission. These functional alterations are not associated with gross neuroanatomical changes, as indicated by an unaltered number and density of interneurons and GABAergic terminals in the LA. The reduction in frequency of sIPSCs suggests that GAD65 is required for efficient action potential-driven GABA release. In keeping with this, the vast majority of GAD65 is present as an inactive apoenzyme, and phosphorylation by PKC isoforms is required for activation of GAD65 (Battaglioli *et al*, 2003; Wei and Wu, 2008). The modest increase in synaptic length observed in GAD65-deficient mice might be reflected by the increase in frequency of miniature IPSCs, indicating facilitated, action potential-independent presynaptic release (Pierce and Lewin, 1994; Murthy *et al*, 2001). The unchanged amplitudes of miniature IPSCs indicate that postsynaptic sites remain unaffected. Previous studies in $Gad65^{-/-}$ have indeed concluded that there is no postsynaptic change in $GABA_A$ receptor density (Kash *et al*, 1999). The most parsimonious explanation for the reduced efficacy of GABAergic synaptic transmission in the LA upon GAD65 deficiency therefore is a reduction in GABA availability and impairment of synaptic release. This deficiency will be of particular relevance during periods of high demand, as GAD65 is the only isoform that processes reuptaken glutamate into GABA during times of high activity (Hartmann *et al*, 2008; Jin *et al*, 2003).

Link Between Synaptic and Behavioral Phenotypes in GAD65 Deficiency

One critical consequence of the reduced efficacy of GABAergic synaptic transmision is an impaired heterosynaptic activation of presynaptic $GABA_B$ receptors, which would otherwise dampen subsequent transmitter release (Szinyei *et al*, 2000). Although presynaptic $GABA_B$ receptors exist on glutamatergic afferents to both GABAergic interneurons and PNs in the LA, they mediate a target-cell-specific control of glutamate release (Pan *et al*, 2009). Impaired GABA availability and reduced GABA spillover may well explain the impairment of $GABA_B$ receptor-dependent short-term plasticity in the LA of GAD65-deficient mice. Along the same line, LTP is suppressed via activation of presynaptic $GABA_B$ receptors (Pan *et al*, 2009). Consistent with such a regulatory function, a genetic deficiency of $GABA_{B(1a)}$ receptors resulted in a shift from the associative, NMDA receptor-dependent form of LTP towards a non-associative, NMDA receptor-independent form at cortico-amygdala afferents (Shaban *et al*, 2006). A similar shift in LTP specificity occurred upon ablation of GAD65, and this phenotype was mimicked by blockade of $GABA_B$ receptors. Furthermore, a GABA reuptake inhibitor used to elevate extracellular GABA levels prevented this shift in LTP in $Gad65^{-/-}$. Spillover of GABA to glutamatergic terminals thus appears to control the associative nature of plasticity at cortical inputs to LA PNs.

Interfering with this synaptic mechanism at both downstream (presynaptic $GABA_B$ receptors) and upstream (GAD65 activity) sites is associated with conditioned fear generalization (present study; Shaban *et al*, 2006). Fear training with high shock intensities in wild-type mice mimicks both the synaptic and the behavioral phenotype. The question arises of how GAD65 may be involved in maintaining stimulus-specific fear memory. Available data suggest the following scenario. Under baseline conditions, the GABAergic synaptic influence is high, allowing inhibitory control of signal flow in the amygdala. Upon fear conditioning, GAD65 expression and extracellular GABA levels decrease (Stork *et al*, 2002; Bergado-Acosta *et al*, 2008), resulting in a relieve of glutamatergic inputs from presynaptic $GABA_B$ blockade and facilitation of LTP.

Behaviorally, conditioned fear responses can occur with high specificity for the conditioned stimulus. If the availability of GABA or stimulation of $GABA_B$ receptors is further reduced (as, for instance, in GAD65 or $GABA_B$ null mutants), LTP at cortical inputs to the LA shift from associative to non-associative forms, whereas LTP at thalamic inputs is unaltered or enhanced. Fear responses occur with reduced stimulus discrimination, ie, in a generalized manner. Through these mechanisms, GABAergic regulation of synaptic plasticity may help control both the induction of conditioned fear and the stimulus specificity of the conditioned responses.

The loss of GAD65 activity in the null mutants may thus be seen as an equivalent of an exaggerated endogenous GAD65 downregulation by stress or fear conditioning. However, these conditions are known to regulate also GAD67 expression (Heldt and Ressler, 2007), which may functionally compensate for GAD65 (Asada *et al*, 1996; Kash *et al*, 1997). This appears unlikely because fear training with high shock intensities mimicked both the synaptic and behavioral phenotypes in *Gad65*$^{-/-}$, whereas the gross synaptic network structure and function displayed no severe abnormalities, and glutamatergic transmission was not affected. Moreover, *Gad65* genotypes do not differ in behavioral responses to increasing foot-shock currents or in fear-related behavior before and immediately after fear conditioning, and they display impaired cued but not contextual components of fear memory and extinction (Bergado-Acosta *et al*, 2008; Sangha *et al*, 2009), which is difficult to reconcile with GAD65-related changes in pain perception (Kubo *et al*, 2009; Zhang *et al*, 2011).

Fear Generalization and GAD Variations Relating to Anxiety Disorders

Generalization of fear to stimuli resembling the conditioned cue is one robust marker of clinical anxiety (Lissek, 2012). The impact of GAD and GABA availability are indicated by two major findings. First, association studies suggested a contribution of *GAD1* variations to individual susceptibility across a range of anxiety disorders (Hettema *et al*, 2006). Hypomethylation of *GAD1* was reported in patients with panic disorder (Domschke *et al*, 2013). In addition, an association between *GAD2* and behavioral inhibition, an anxiety-related trait, was inferred from a family-based study in children (Smoller *et al*, 2001), and polymorphisms in the *GAD2* gene-region are associated with risk factors for anxiety disorders (Unschuld *et al*, 2009). Second, subjects with mood and anxiety disorders display abnormally low GABA levels in plasma (Petty and Sherman, 1984; Petty *et al*, 1999) and cerebral spinal fluid (Kasa *et al*, 1982; Kendell *et al*, 2005).

CONCLUSIONS

GAD65 is required for efficient GABAergic synaptic transmission and plasticity in the LA, and for maintaining extracellular GABA at a level needed for associative plasticity at cortical inputs to PN, which, if disturbed, results in an impairment of cue specificity of conditioned fear responses typifying anxiety disorders.

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