Cell

Pathogenic *SYNGAP1* Mutations Impair Cognitive Development by Disrupting Maturation of Dendritic Spine Synapses

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http://dx.doi.org/10.1016/j.cell.2012.08.045

SUMMARY

Mutations that cause intellectual disability (ID) and autism spectrum disorder (ASD) are commonly found in genes that encode for synaptic proteins. However, it remains unclear how mutations that disrupt synapse function impact intellectual ability. In the *SYNGAP1* mouse model of ID/ASD, we found that dendritic spine synapses develop prematurely during the early postnatal period. Premature spine maturation dramatically enhanced excitability in the developing hippocampus, which corresponded with the emergence of behavioral abnormalities. Inducing *SYNGAP1* mutations after critical developmental windows closed had minimal impact on spine synapse function, whereas repairing these pathogenic mutations in adulthood did not improve behavior and cognition. These data demonstrate that SynGAP protein acts as a critical developmental repressor of neural excitability that promotes the development of life-long cognitive abilities. We propose that the pace of dendritic spine synapse maturation in early life is a critical determinant of normal intellectual development.

INTRODUCTION

Disruptions to the molecular mechanisms controlling glutamatergic synapse structure and function are believed to underlie certain neurodevelopmental disorders of cognition, such as intellectual disability (ID) and autism spectrum disorder (ASD), which are two disorders that are often codiagnosed in afflicted children (Bear et al., 2004; Penzes et al., 2011; Ramocki and Zoghbi, 2008; Südhof, 2008). Deleterious mutations in synaptic proteins are linked to these disorders and many animal models display deficits related to synapse structure and/or function (Gauthier et al., 2011; Gilman et al., 2011; Guilmatre et al., 2009; Hamdan et al., 2011a; Hamdan et al., 2011b; Hamdan et al., 2009; Südhof, 2008). However, it remains largely unknown how synaptic dysfunction resulting from pathogenic mutations during development impacts circuit function and behavior. This is a particularly important consideration in ID and ASD because these brain disorders are often first diagnosed in very young children. Disruption of excitatory/inhibitory (E/I) balance is emerging as a common neurophysiological phenotype common to many brain disorders, including ID and ASD (Rubenstein and Merzenich, 2003). Recently, it was demonstrated that increasing neural excitation is sufficient to disrupt cognition and sociability (Yizhar et al., 2011). Therefore, genetic mutations that selectively increase glutamatergic synaptic strength in pyramidal neurons would be expected to significantly impact E/I balance, information processing, and behavior, particularly during early postnatal development when GABAergic interneuron systems are still maturing (Danglot et al., 2006).

Recently, autosomal-dominant de novo mutations in *SYNGAP1* that lead to truncation of the full-length protein were reported as a cause of sporadic ID in ~4% of screened cases (Hamdan et al., 2011a; Hamdan et al., 2009; Krepischi et al., 2010). All identified patients with *SYNGAP1* haploinsufficiency have moderate to severe forms of ID, and several of these patients also have an ASD (Hamdan et al., 2011a; Pinto et al., 2010). Interestingly, these patients present with nonsyndromic ID, as there are no physical abnormalities other than those observed in the cognitive/behavioral domain. Thus, de novo mutations that disrupt *SYNGAP1* are highly pathogenic and selectively impact brain function. Early prevalence data indicate that these mutations are unexpectedly common (predicted to be >1 million afflicted individuals world-wide and more prevalent than fragile X syndrome), underscoring the impact that *SYNGAP1* has on cognitive development (Hamdan et al., 2011a; Hamdan et al., 2011b; Hamdan et al., 2009).

SYNGAP1 encodes a synaptic RasGAP (SynGAP) that is largely localized to dendritic spines in neocortical pyramidal neurons (Chen et al., 1998; Kim et al., 1998; Zhang et al., 1999), where it suppresses signaling pathways linked to NMDA receptor (NMDAR)-mediated synaptic plasticity and AMPA receptor (AMPAR) membrane insertion (Kim et al., 2005; Krapivinsky et al., 2004; Rumbaugh et al., 2006). This is a complicated gene with alternative transcriptional start sites and several alternatively spliced C-terminal exons that result in many possible isoforms of SynGAP (Chen et al., 1998; Kim et al., 1998). Not surprisingly, the impact of SynGAP protein expression in neurons is unclear. Both the N and C termini expression can influence SynGAP protein function, and depending on the variant expressed, SynGAP can either stimulate (Rumbaugh et al., 2006) or suppress dendritic spine synapse function (McMahon et al., 2012). In addition, disrupting SynGAP expression in dissociated hippocampal neurons can enhance dendritic spine function (Kim et al., 2005; Rumbaugh et al., 2006) or suppress it (Krapivinsky et al., 2004). Based on these data, it is difficult to predict how inactivating mutations of *SYNGAP1* would impact the development of brain circuits and the cognitive modalities subserved by them. Regardless, considering that this protein is restricted to dendritic spines and copy number variation directly impacts cognition, mice that harbor *SYNGAP1* truncating mutations provide an excellent model to study how a genetic mutation influences synaptic maturation and cognitive development. Interestingly, adult SynGAP Heterozygous knockout mice (Hets), which model human *SYNGAP1* haploinsufficiency and offer construct validity, are reported to have normal synaptic transmission and only modest defects in synaptic plasticity (Kim et al., 2003; Komiyama et al., 2002). Despite the lack of pervasive functional synaptic defects in adulthood, these animals have profound cognitive abnormalities (Guo et al., 2009; Komiyama et al., 2002; Muhia et al., 2010). These data suggest that SynGAP's role in regulating synapse development may be particularly important to cognitive and behavioral maturation. However, the role of this critical gene in brain development remains largely unexplored. Therefore, we hypothesized that *SYNGAP1* haploinsufficiency is particularly disruptive to neonatal dendritic spine synapse development, which, as a consequence, contributes to deficits in cognition and behavior.

In this study, we found that a mouse model of human *SYNGAP1* haploinsufficiency had glutamatergic synapses that matured at an accelerated rate during the first few weeks of neonatal development. Loss of this essential glutamatergic synapse repressor dramatically disrupted E/I balance in neural networks that support cognition and behavior and these effects were linked to life-long intellectual disability. These studies provide a neurophysiological mechanism linking abnormal glutamatergic synapse maturation during development to enduring abnormalities in behaviors indicative of neurodevelopmental disorders.

RESULTS

SYNGAP1 Haploinsufficiency Accelerates the Maturation of Hippocampal Synaptic Function

SynGAP is expressed throughout the forebrain, with particularly high levels in the hippocampus (Porter et al., 2005). The hippocampus is a central mediator of cognition and memory because it receives and integrates information from sensory cortices that is then relayed to associational regions. Indeed, development of the hippocampus is disrupted in ID and ASD patients (Saitoh et al., 2001). SynGAP expression in the hippocampus of WT mice peaks around postnatal day (PND) 14 (Figure 1A), suggesting that this period of brain development may be vulnerable to the reduced levels of full-length SynGAP protein expressed in Het mice (Figure 1B). We began to probe for possible hippocampal circuit dysfunction in Het mice by measuring synaptic transmission in the medial perforant path (MPP) of the dentate gyrus (DG), the major input pathway into the hippocampus. Synaptic function was normal at very young ages (~PND9), but transmission increased dramatically in Het mice by PND14 (Figures 1C and 1D). Interestingly, synaptic function was again equivalent between genotypes by PND21 and later (Figures 1E and 1F), indicating that SynGAP controls the trajectory of synapse maturation during a particularly critical period (PND10–20) of hippocampal development.

We next sought to better understand the neurophysiological mechanism responsible for enhanced synaptic function during development. NMDAR-evoked synaptic transmission was unchanged in PND14 Het mice (Figure 2A), suggesting that the elevated synaptic transmission at PND14 was mediated by a postsynaptic increase in the sensitivity of AMPARs to evoked glutamate release. Consistent with this idea, we observed a selective increase in the ratio of AMPA/NMDA currents during whole-cell recordings of DG granule neurons (DGNs) only in PND14 Het mice (Figure 2B). We found no differences in the rise or decay of these evoked currents at any age tested (Figure S1 available online). These data also demonstrate that pathogenic *SYNGAP1* mutations cause a premature acquisition of adult levels of functional AMPARs. We also observed a selective increase in mEPSC amplitude and frequency in SYNGAP1 Het mice at PND14, which also normalized by PND21 (Figures 2C and 2D). Synaptic disruptions first seen at PND 14 were largely specific to postsynaptic function because we did not detect changes in MPP release probability (Figure 2E) or intrinsic spiking of DGNs at this age (Figure 2F). We observed no changes in resting membrane potential or input resistance at any age tested (Figure S1). Interestingly, we observed a significant increase in mIPSC frequency and amplitude in DGNs (Figure 2G). Considering that SYNGAP is not expressed at inhibitory synapses (Chen et al., 1998; Kim et al., 1998), these data suggest the intriguing possibility that changes to the inhibitory system represent a compensatory response to increased excitation caused by elevated postsynaptic dendritic spine synapse AMPAR function in these neurons (Lau and Murthy, 2012).

SYNGAP1 Haploinsufficiency Disrupts Dendritic Spine Dynamics during the Second Postnatal Week

In pyramidal neurons, spine structure is tightly correlated with synapse function (Matsuzaki et al., 2001; Noguchi et al., 2011). Thus, we next sought to determine whether *SYNGAP1* haploinsufficiency disrupted the development of DGN dendritic spine structure over the same time-course as that observed for synaptic function. At PND9, SynGAP mutants had normal dendritic spine structure (Figure 3A), but spines became larger

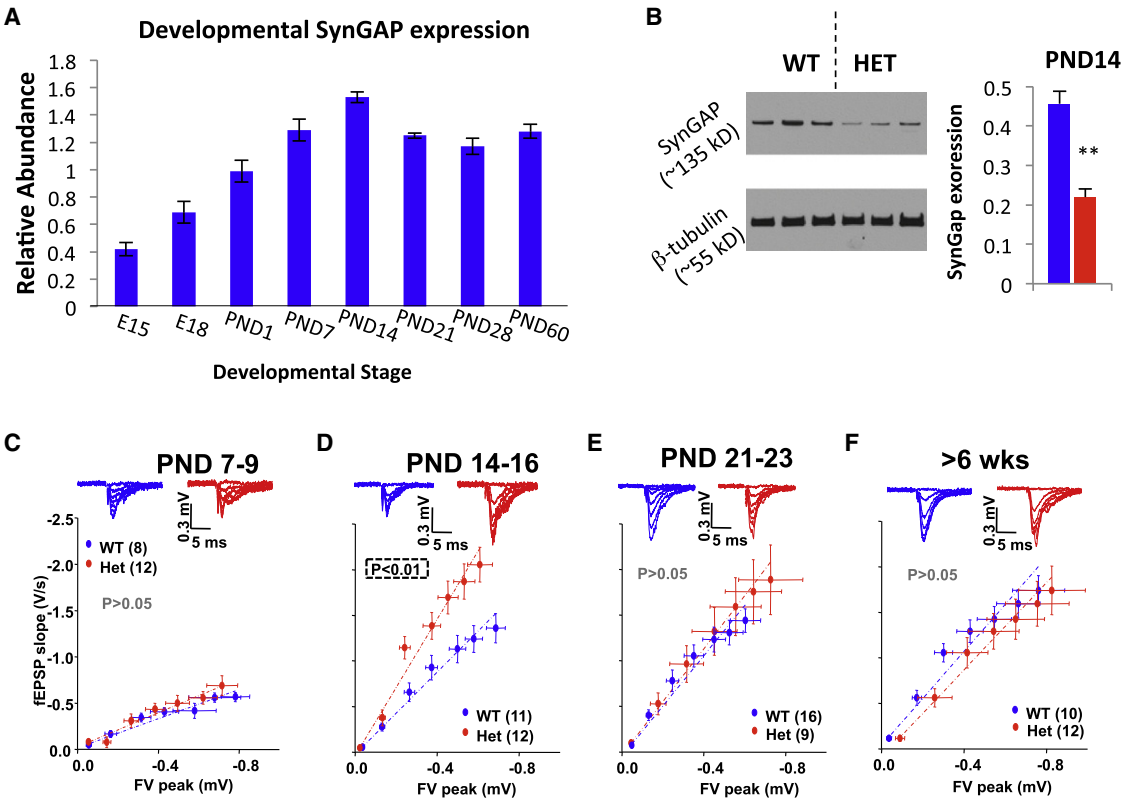

Figure 1. A Restricted Period of Elevated Excitatory Synaptic Transmission in Developing SynGAP Mutants

(A) SYNGAP1 transcript levels were measured in WT C57/Bl6J mice by qPCR throughout development. Relative abundance was calculated by normalizing SynGAP transcript levels to GAPDH levels, which was previously determined to not change during development.

(B) Hippocampi from WT (n = 3) and Het (n = 3) PND14 mice were extracted and probed for SynGAP and β-tubulin expression; (ANOVA, $F_{(1,5)} = 28.2$, $p = 0.006$).

(C–F) Representative traces of field EPSPs and summary graphs of input-output relationships from the MPP input into the DGNs measured during different developmental epochs. Significance was determined with an ANOVA to compare slopes after linear regression. Number in parenthesis represents number of slices. At least three animals per genotype per age were used.

Error bars represent SEM.

relative to WT by PND14 (Figure 3B). These abnormalities persisted into adulthood (Figure 3C), which is consistent with the "spine dysfunction" theory of cognitive disorders (Penzes et al., 2011). The disruption to spine head size altered the distribution of spine classes in Het mice, resulting in more mushroom-type spines and fewer stubby spines beginning in the second postnatal week (Figure 3D). We did not observe differences in spine density (Figure 3E) at any stage of development in the DG, indicating that synapse density in the hippocampus is not affected by *SYNGAP1* haploinsufficiency at the ages tested. Importantly, we confirmed that *SYNGAP1* haploinsufficiency alters dendritic spine size in early development by characterizing these structures from internally perfused and fixed Thy1-GFP SYNGAP1 Hets (Figure S2). Additional structural abnormalities were observed at PND14. Although dendritic arborization was unchanged in SYNGAP1 Hets (not shown), we did observe a decrease in the spatial volume occupied by individual DGN dendritic trees (Figures 3F–3G).

Gradual acquisition of synaptic AMPARs and subsequent functional unsilencing of glutamatergic inputs is a hallmark of early postnatal development (Kerchner and Nicoll, 2008). Therefore, premature acquisition of functional AMPARs into synapses, like that observed in SynGAP Hets, is suggestive of an aberrant acceleration of normal neurodevelopmental milestones. Therefore, we next investigated the idea that dendritic spine dynamics in SynGAP Het mice also displayed characteristics of early maturation. We observed that spines from PND14 DGNs in Het mice were significantly less motile than WT spines (Figures 4A and 4B). Because spine motility rates decrease during development (Dunaevsky et al., 1999; Majewska and Sur, 2003), this measure is a mark of synapse maturation. Indeed, spines from young SynGAP Het mice appeared to have dynamics normally seen in adult animals. Spine motility rates dropped in WT animals between PND14 and adult (Figure 4B) as expected (Dunaevsky et al., 1999; Majewska and Sur, 2003). However, motility rates did not drop in Het mice because they already displayed adult-like motility rates by the end of the second postnatal week (Figure 4B). Defects in spine motility and synaptic function in neonatal Het mice were linked to spine signaling abnormalities. Cofilin signaling regulates spine structure and AMPAR trafficking by controlling actin dynamics (Gu et al., 2010). Indeed, we observed that Cofilin was hyperphosphorylated in young

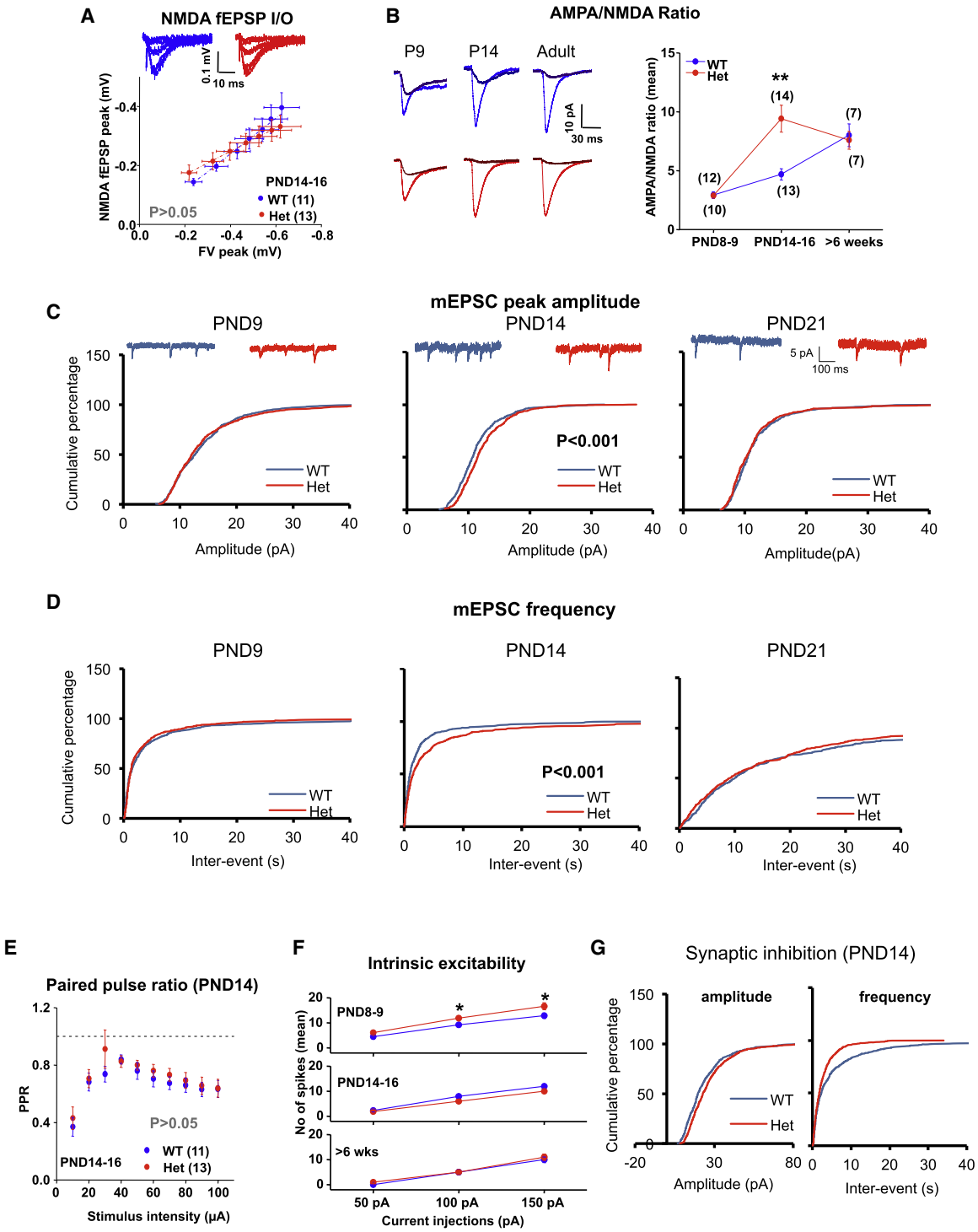

Figure 2. Developmental Disruptions to Het Synaptic Transmission Are Caused by Enhanced Sensitivity of AMPARs to Released Glutamate

(A) Isolated NMDAR-mediated fEPSPs at PND14–16. Number in parenthesis represents number of slices. At least three animals per genotype per age were used.

(B) Representative traces (left) and summary data of AMPA/NMDA ratios evoked from mild stimulation of MPP in patch-clamped DGGCs (ANOVA; PND7–9: $F_{1,20} = 0.031$, $p > 0.05$; PND14–16: $F_{1,25} = 13.76$, $p < 0.01$; adult: $F_{1,12} = 0.118$, $p > 0.05$).

(C) Cumulative percentage of mEPSC amplitude from PND9 (n = 800), PND14 (n = 1300), PND21 (n = 500). Note an increase in mEPSC amplitude only at PND14 ($p < 0.05$; two-sample K-S test).

(D) Summary of cumulative percentage of mEPSC inter-event interval, which is reduced at PND14 ($p < 0.05$; two-sample K-S test).

SynGAP mutants (Figure S3). These results are consistent with signaling deficits previously reported in adult SynGAP mice (Carlisle et al., 2008; Komiyama et al., 2002).

During spine motility experiments, we made the surprising observation that, regardless of genotype, a subset of PND14 DGN spines displayed a novel form of structural plasticity defined by a spontaneous increase in head volume (Figure 4C and Movie S1). As a group, spines that displayed this previously unreported form of structural plasticity maintained the elevated volume for at least 1 hr. Although the dynamics of spontaneous spine head enlargement were similar between genotypes (Figure 4C), the frequency of spontaneous plasticity events was significantly lower in Het brain slices at PND14 (Figure 4D). The frequency also dramatically decreased with age in WT, suggesting that this phenomenon might be related to developmental maturation of glutamatergic synapses in the DG. The frequency of plasticity events in Hets did not change between PND14 and adult time points (Figure 4D), suggesting that spines in PND14 Het mice may have already undergone structural plasticity. In support of this idea, we observed that Het spines that did not display this plasticity were significantly larger than Het spines that did eventually enlarge (Figure 4E). In addition, the population of PND14 Het spines that failed to enlarge was also significantly larger than both populations of WT spines (Figure 4E). These data suggest that abnormal spine dynamics in early development may account for the persistent disruption to spine morphology observed in adult SynGAP Hets (Figures 3A and 3B).

SYNGAP1 Haploinsufficiency Alters Hippocampal Information Processing, E/I Balance, and Memory

We next sought to determine whether accelerated maturation of dendritic spine synapses in SynGAP Hets leads to altered excitability at the circuit level in the developing hippocampus. To directly test this idea, we performed laser photolysis of caged glutamate paired with fast voltage-sensitive dye imaging to monitor signal propagation throughout the entire hippocampus. Indeed, we found that the dynamics of signal propagation through the neonatal hippocampus were significantly different between genotypes (Figures 5A and 5B). Photostimulation-evoked signals originating in the WT DG were progressively attenuated as they traveled through the trisynaptic circuit (Figures 5A and 5C and Movie S2). In Het mice, however, signals originating in the DG were dramatically amplified as they spread through the hippocampus (Figures 5B and 5C and Movie S2), demonstrating that *SYNGAP1* haploinsufficiency disrupts information processing in the hippocampus. These data also provided direct evidence that *SYNGAP1* inactivating mutations shift the balance of hippocampal networks toward excitation in early development. Hyperactivity across the Het hippocampus suggested that synapses in addition to the MPP-DGN pathway would be abnormally strong. Indeed, we found that synaptic transmission in the Schaeffer Collateral pathway in area CA1 was also abnormally strong in early development but not in adult Hets (Figure 5D and Figure S4), indicating that enhanced synaptic function during early neural development is a common outcome of *SYNGAP1* haploinsufficiency.

Hippocampal hyperactivation indicated that SynGAP Het mice may be prone to seizures, a condition highly comorbid in patients with *SYNGAP1* haploinsufficiency (Hamdan et al., 2011a; Hamdan et al., 2009). Indeed, young SynGAP Het mice had a reduced fluorothyl-induced seizure threshold (Figure 5E) and were prone to audiogenic seizures (Table S1 and Movie S3), a phenotype shared with other mouse models of neurodevelopmental disorders (Musumeci et al., 2000). We next wished to determine whether behavior was altered in neonatal SynGAP mutants. Although behavioral analysis is challenging in preweanling mice, we did observe that activity in the open field arena was much higher in PND14 Hets compared to WT littermates (Figure 5F). Exploratory behavior in this paradigm is guided by several factors, including spatial cognition (Dvorkin et al., 2008), and hyperactivity is associated with developmental hippocampal dysfunction (Daenen et al., 2002). Together, these data link early maturation of dendritic spine synapses and E/I imbalance to the onset of behavioral abnormalities in neonatal SynGAP Het mice.

Developmental disruptions in the maturation of hippocampal circuits would be expected to cause persistent, ongoing deficits in brain function, including memory encoding (Squire, 1992). Proper DG circuit function is necessary to resolve two closely overlapping neural representations (Kesner, 2007). Experimentally, contextual discrimination is highly dependent on DG function (Sahay et al., 2011). Thus, context discrimination is an ideal paradigm to link synapse dysfunction in the DG to abnormal adult cognition. To probe for potential context discrimination deficits in *SYNGAP1* haploinsufficiency, we first trained animals in a fear conditioning paradigm over several days in a unique context (A+). The animals were then exposed to the original training context (A+), followed by exposure to a slightly different contextual environment (B−) (Figure 6A). As we reported previously (Guo et al., 2009), SynGAP Het mice exhibited normal contextual fear memory when compared to WT littermates (Figure 6B). WT mice were also able to discriminate between the similar contexts A and B, as they learned to freeze less in context B (Figure 6C). Consistent with the idea that SynGAP Hets have functional deficits in the dentate gyrus, Het mice were unable to discriminate between the two contexts over the same period of time (Figure 6D).

Developmental SYNGAP1 Mutations Lead to Persistent Behavioral Abnormalities

The results thus far demonstrate that *SYNGAP1* mutations responsible for persistent, life-long intellectual disability disrupt rodent synapse development in circuits that support cognition.

(E) Paired-pulse ratio in WT and SynGAP Hets at PND 14–16 (RMANOVA; $F_{1,23} = 0.898$; $p > 0.05$). Number in parenthesis represents number of slices. At least three animals per genotype per age were used.

(F) Intrinsic excitability of DGGNs observed by variable current injections into patch-clamped cells at PND14–16 (RMANOVA; PND8–9; $F_{1,24} = 5.139$, $p < 0.05$; PND14–16: $F_{1,40} = 2.280$, $p > 0.05$; > 6 weeks: $F_{1,11} = 0.353$, $p > 0.05$; asterisks denote significance after Bonferroni Post Hoc test, $p < 0.05$).

(G) Cumulative probability of mIPSC amplitude ($p < 0.05$, two-sample K-S test) and frequency ($p < 0.05$, two-sample K-S test), respectively, from PND14; n = 700. Error bars represent SEM. See also Figure S1.

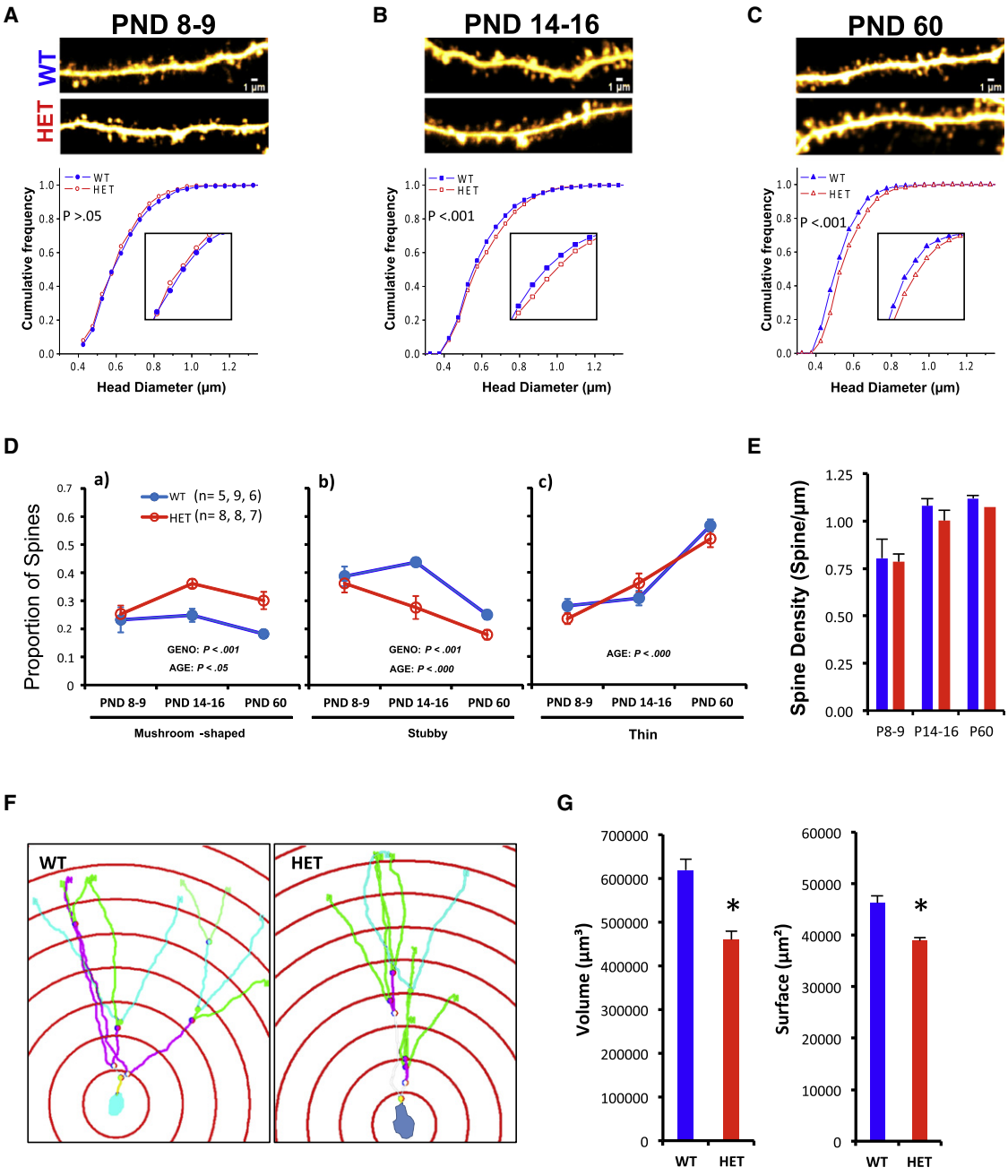

Figure 3. Emergence of Abnormal Spine Size and Shape in *SYNGAP1* Mutants during the Second Postnatal Week

(A–C) Representative multiphoton excitation images of dendrites (scale bar = 1 µm) obtained in live acute brain slices in both *WT* and SYNGAP1 Hets. Cumulative frequency curves of spine head diameter in both groups across three stages of development (PND8–9, WT [n = 687 spines] versus Het [n = 687]; PND14–16, WT [n = 1650] versus Het [n = 1650]; PND60, WT [n = 963] versus Het [n = 963]). K-S test was performed because a population defined by spine head diameter results in a clear nonnormal distribution. Inset shows spine diameters from 0.6–0.9 µm.

(D) Graphs depicting the proportion of Mushroom (left), Stubby (middle), and Thin (right) spines in WT and Het mice at three different developmental stages; Mushroom: genotype ($F_{(1, 37)}$ = 15.243, p = 0.00039); age ($F_{(2, 37)}$ = 4.1677, p = 0.02332), Stubby: genotype ($F_{(1, 37)}$ = 13.167, p = 0.00086); age ($F_{(2, 37)}$ = 17.451, p = 0.00000), Thin: age ($F_{(2, 37)}$ = 52.569, p = 0.00000). Number in parenthesis represents number of slices. At least three animals per genotype per age were used.

(E) Density of WT (blue) and Het (red) spines at three different developmental time points were calculated (ANOVA; genotype [$F_{(1, 38)}$ = 0.98887, p = 0.32631]; age [$F_{(2, 38)}$ = 14.048, p = 0.00003]; genotype × age [$F_{(2, 38)}$ = 0.13787, p = 0.87164]).

(F) Representative examples of 3D reconstruction and Sholl ring analysis in dentate gyrus granular neurons (PND 16).

(G) Histograms showing volumetric and surface extension field of dentate gyrus neurons in both WT (n = 40 traced neurons from four animals) and Het (n = 40 traced neurons from four animals) mice; Student t test, *p < 0.05.

Values represent means ± SEM. See also Figure S2.

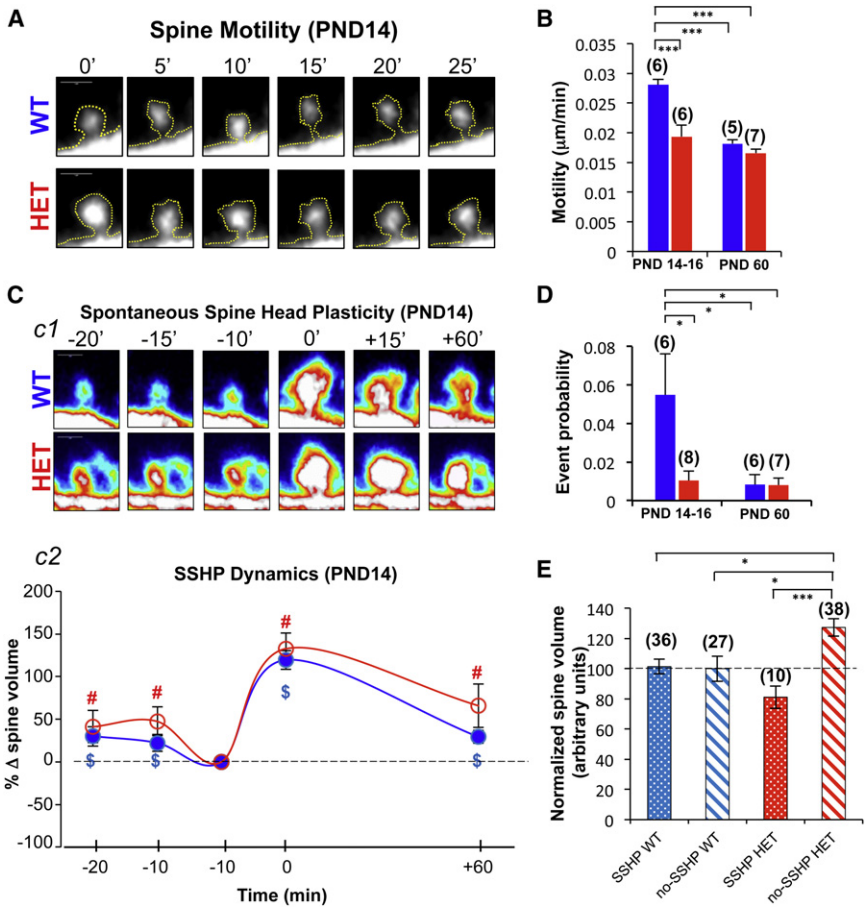

Figure 4. *SYNGAP1* Haploinsufficiency Disrupts Developmental Spine Dynamics

(A) Multiphoton excitation images of an individual spine over time taken from acute slices in WT and Het mice at PND14.

(B) Motility of Het (red) and WT (blue) spines from slices taken at PND14–16 and PND60 (ANOVA two-way; genotype [$F_{(1,20)}$ = 19.439, p = 0.00027]; age [$F_{(1,20)}$ = 29.338, p = 0.00003]; interaction [$F_{(1,20)}$ = 9.3201, p = 0.00628]); number in parenthesis represents number of slices. At least three animals per genotype per age were used.

(C) *c1,* frames of individual spines in acute slices showing spontaneous spine head plasticity (SSHP) at PND14–16. *c2,* Spontaneous spine head plasticity kinetic curves of WT (n = 36) and Het (n = 10) spine populations demonstrating this phenomenon at PND14–16 (one-sample t test [population mean = 100], $ = WT significance,# = Het significance, p < 0.05).

(D) Graph depicting SSHP event probability (probability that any observed spine in the brain slice would change volume > 50% between 2 successive frames) in WT (blue) and Het (red) slices at PDN14–16 and PND60; (ANOVA two-way; genotype [$F_{(1,23)}$ = 4.6586, p = 0.04157]; age [$F_{(1,23)}$ = 5.6275, p = 0.02643]; interaction [$F_{(1,23)}$ = 4.6034, p = 0.04270]); number in parenthesis represents number of slices. At least three animals per genotype per age were used.

(E) Spine head volume measurements were made in spines that underwent plasticity (10 min before observation of >50% spine volume change) and spines that did not display this behavior (no-SSHP; spine chosen at random and volume measurement chosen at a randomly selected time point); thus dividing spines into two populations—plastic and not plastic; ANOVA one-way, ($F_{(3,107)}$ = 6.720, p = 0.0003). Number in parenthesis represents number of spines analyzed. At least three animals per genotype per age were used. A Bonferroni post hoc test was applied where appropriate, *p < 0.05, ***p < 0.001. Error bars depict SEM. See also Figure S3 and Movie S1.

However, it remains unclear whether developmental synapse disruptions contribute to enduring cognitive and behavioral abnormalities. Therefore, we next performed a series of studies to link developmental synapse abnormalities to life-long cognitive disruptions.

In the first set of studies, we sought to test the hypothesis that developing dendritic spine synapses are particularly sensitive to *SYNGAP1* mutations. To test this idea, we engineered a conditional mouse line that allows for efficient temporal induction of SYNAGP1 haploinsufficiency. This newly engineered mouse line contained loxP sites flanking the same exons targeted in our conventional Het mouse. We confirmed that the inserted LoxP sites were correctly targeted in this mouse line and that Cre recombinase expression significantly reduced SynGAP protein levels in SYNGAP1$^{+/fl}$ neurons (Figure S5). We confirmed (Figure S5) a previous report (Pilpel et al., 2009) that demonstrated AAV8 pseudotyped vector particles drive high levels of transgene expression within days of injection into the newborn mouse brain, indicating that it was technically possible to induce haploinsufficiency in the first week of life. For temporal induction of haploinsufficiency, AAV8 Cre virus particles were unilaterally injected into the hippocampus of either neonatal or adult SYNGAP1$^{+/fl}$ mice (Figure 7A). To assess the effect of age-restricted haploinsufficiency, we recorded DGN AMPA/NMDA ratios from both infected (SYNGAP1 Haploinsufficiency) or uninfected (SynGAP WT) hemispheres 14 days after injections. To facilitate the identification of DGNs with haploinsufficiency, we crossed *SYNGAP1* conditional KO mice with Ai9 Cre reporter mice, which express tdTomato in response to Cre activity. Indeed, 2 weeks after Cre virus injections, we observed a mosaic expression of tdTomato in neurons throughout the DG (Figure 7B), confirming that the virus drives active Cre recombinase within 14 days of injection. As we had found in the conventional Het mice, PND1 virus injections into *SYNGAP1*$^{+/fl}$ conditional mice resulted in a robust increase in DGN AMPA/NMDA ratios (Figure 7C). This effect was most likely caused by altered SynGAP expression because there was no effect of Cre virus injection in SYNGAP1$^{+/+}$ mice (Figure 7C). We did not observe any other effects on synaptic or neuronal function in this neonatal haploinsufficiency experiment (data not shown), indicating that a critical role of SynGAP during development is to control the maturation rate of dendritic spine synapses. Importantly, these data also demonstrate that pathogenic SYNGAP1 mutations affect synaptic maturation through cell-autonomous

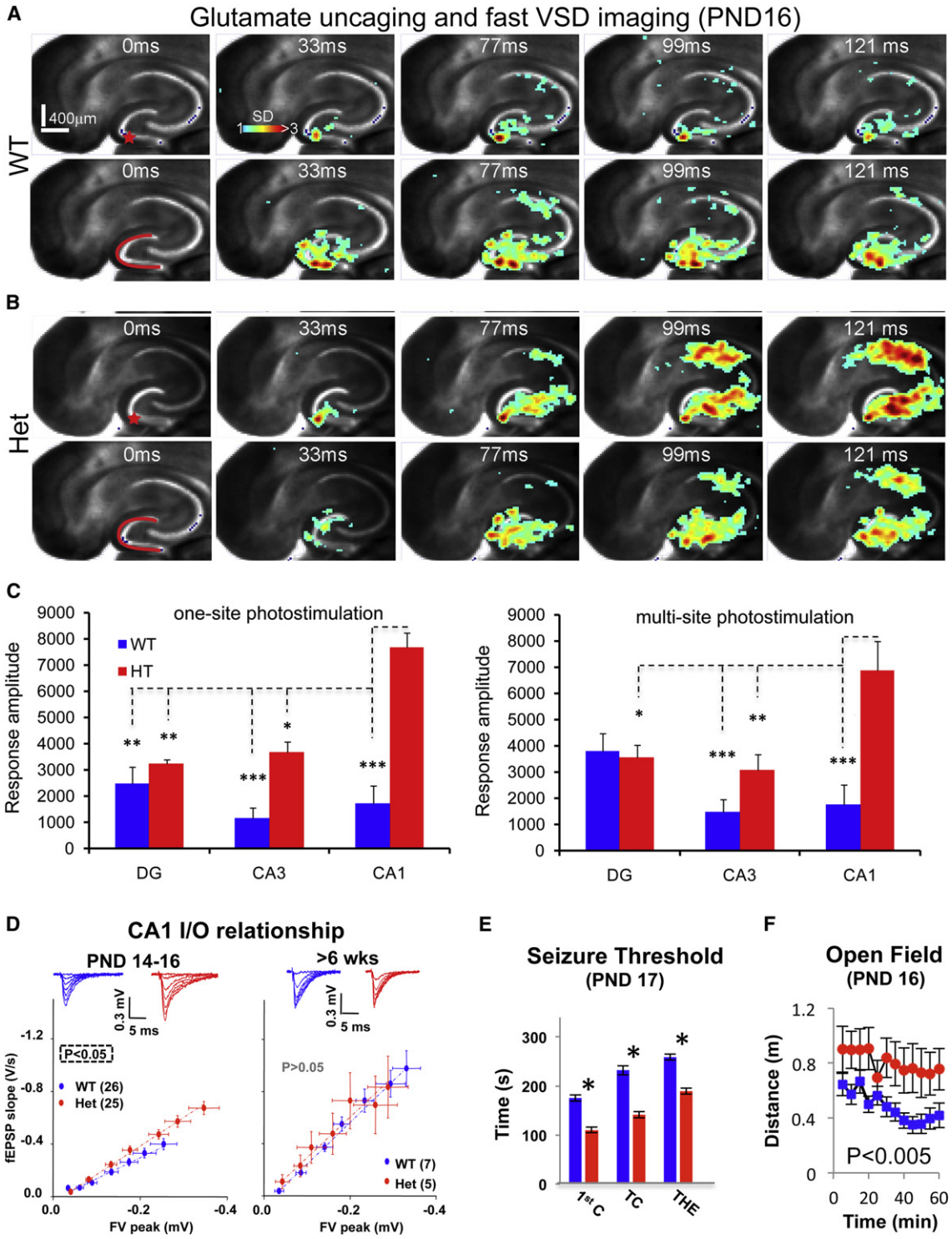

Figure 5. *SYNGAP1* Haploinsufficiency in Young Mice Causes Abnormal Hippocampal Signal Processing, E/I Imbalance, and Seizures

(A and B) Time series data of voltage-sensitive dye (VSD) imaging of responses to single-site photostimulation (indicated by the red star) in DG and near-simultaneous, multisite photostimulation across DG (indicated by the red curve), respectively, from the same PND16 WT (A) or Het (B) slice. VSD frames were acquired at 2.2 ms/frame, but are displayed at specific time points. Time progresses from left to right in the row. Color code is used to indicate VSD signal amplitudes expressed as SD multiples above the mean baseline. The warmer the color, the stronger the response.

(C) Average response amplitude in SD units for DG, CA3, and CA1 to single-site (left) and multisite (right) DG photostimulation from WT (n = 6) and Het (n = 7) slices (four animals from each genotype); ANOVA two-way: Single Site [Genotype ($F_{(1,33)}$ = 20.8, p = 0.00007); Brain Region ($F_{(2,33)}$ = 4.27, p = 0.022); Interaction

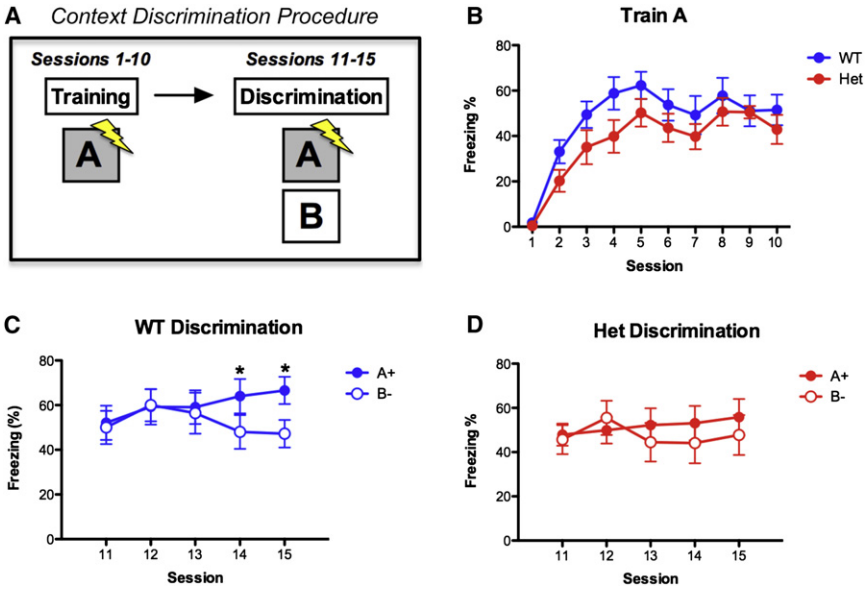

Figure 6. Adult *SYNGAP1* Hets Display Learning Deficits in a Task Selective for the Dentate Gyrus

(A) Schematic depicting context discrimination paradigm.

(B) WT (n = 10) and Het (n = 12) mice in the multiday training paradigm. Both groups showed a significant and equivalent increase in freezing in context A across sessions (main effect of session $F_{(9, 180)} = 31.7$, $p < 0.05$, no effect of genotype $F_{(1, 20)} = 2.08$, $p > 0.05$, no genotype × session interaction $F < 1$).

(C) WT mice learned to discriminate the shock context (A+) from the safe environment (B−) across five sessions (main effect of context $F_{(1,9)} = 7.05$, $p < 0.05$, context × session interaction $F_{(4, 36)} = 3.35$, $p < 0.05$).

(D) Hets did not learn to discriminate between the shock context and safe environment across five sessions (no effect of context $F_{(1, 11)} = 1.72$, $p > 0.05$, no context × session interaction $F_{(4, 44)} = 2.13$. $p > 0.05$). *$p < 0.05$.

Error bars depict SEM.

mechanisms because we did not observe TdTomato-positive neurons outside the hippocampus. In the next experiment, virus was injected unilaterally into adult animals (Figure 7A) to determine the effect of *SYNGAP1* haploinsufficiency in the mature hippocampus. In contrast to the PND1 injections, there was no effect of Cre expression on AMPA/NMDA ratio in either the *SYNGAP1*$^{+/fl}$ or *SYNGAP1*$^{+/+}$ mouse lines when recordings were performed 2 weeks after virus injection (Figure 7D). However, we did find that adult induction of *SYNGAP1* haploinsufficiency increased the intrinsic excitability of DGNs (Figure S5). This effect was not observed in either PND1 virus-injected animals (Figure S5) or in conventional adult Hets (Figure 2F), indicating that this is a cell-autonomous effect specific to adult-induced haploinsufficiency. Together, these data demonstrate that a critical period of SynGAP protein function exists in the first 2 weeks of hippocampal development, when this protein plays a key role in determining the rate of dendritic spine synapse development.

In the second series of experiments, we sought to determine whether neonatal *SYNGAP1* haploinsufficiency causes enduring cognitive and behavioral disability. The rationale behind this experiment was that if *SYNGAP1* haploinsufficiency disrupted the organization of brain circuits during development, then rescue of SynGAP protein in adult mutants would have minimal positive impact on behavior and cognition. To test this idea, we constructed a novel mouse line that enabled conditional reversal of *SYNGAP1* haploinsufficiency in adult mice. This mouse line contained a LoxP-STOP-LoxP cassette downstream of exon 5 of the mouse *SYNGAP1* gene (Figure S6). This cassette would be expected to cause a truncation of full-length protein, which is known to inactivate SynGAP (Rumbaugh et al., 2006). Indeed, all known cases of *SYNGAP1* haploinsufficiency result from a truncation of the full-length protein (Hamdan et al., 2009; Hamdan et al., 2011a, Hamdan et al., 2011b). Southern blot analysis confirmed that the STOP cassette was appropriately targeted and that this targeted insertion disrupted expression of full-length SynGAP (Figure S6). Importantly, behavioral endophenotyping demonstrated that LoxP-stop Het mice had robust behavioral abnormalities similar to what we have previously published (Guo et al., 2009) in our conventional Hets (data not shown). We next confirmed in cell culture experiments that the expression of Cre recombinase significantly increased SynGAP protein levels (data not shown), indicating that removal of the STOP cassette could rescue SynGAP expression in a comprehensive behavioral study of adult mice. To test the effect of genetic rescue on behavioral and cognitive performance, we crossed heterozygous *SYNGAP1* LoxP-Stop animals with a hemizygous inducible and ubiquitously-expressing, CreERt2 driver line (Hayashi and McMahon, 2002) previously shown to effectively rescue gene expression in adult mice (Guy et al., 2007) after tamoxifen (TMX) administration. The offspring resulting from this cross fell into four genotypes (Figure 8A), which

($F_{(2,33)} = 5.12$, $p = 0.011$)], Multisite [Genotype ($F_{(1,33)} = 13.5$, $p = 0.00083$); Brain Region ($F_{(2,33)} = 4.23$, $p = 0.023$); Interaction ($F_{(2,33)} = 7.20$, $p = 0.0025$)]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$ after a Bonferroni post hoc test.

(D) Representative traces and pooled data of input-output relationship from the Schaffer-collateral pathway in CA1 in developing and mature SynGAP mice. Significance was determined by comparison of slopes after linear regression.

(E) Time taken to reach three benchmarks in fluorothyl-induced seizures in PND17 WT (n = 25) and Het (n = 21) mice [ANOVA; First Clonus (1st C): ($F_{(1,42)} = 41.8$, $p < 0.001$), Tonic-Clonic (TC): [$F_{(1,42)} = 46.5$, $p < 0.001$], Tonic Hindlimb Extension (THE): ($F_{(1,42)} = 34.0$, $p < 0.001$]). *$p < 0.001$.

(F) Activity levels of PND16 WT (n = 27) and Het (n = 10) mice during an exposure to a novel open field arena; RMANOVA ($F_{(1,33)} = 11.2$, $p = 0.002$).

Error bars depict SEM. See also Movies S2 and S3, Figure S4 and Table S1.

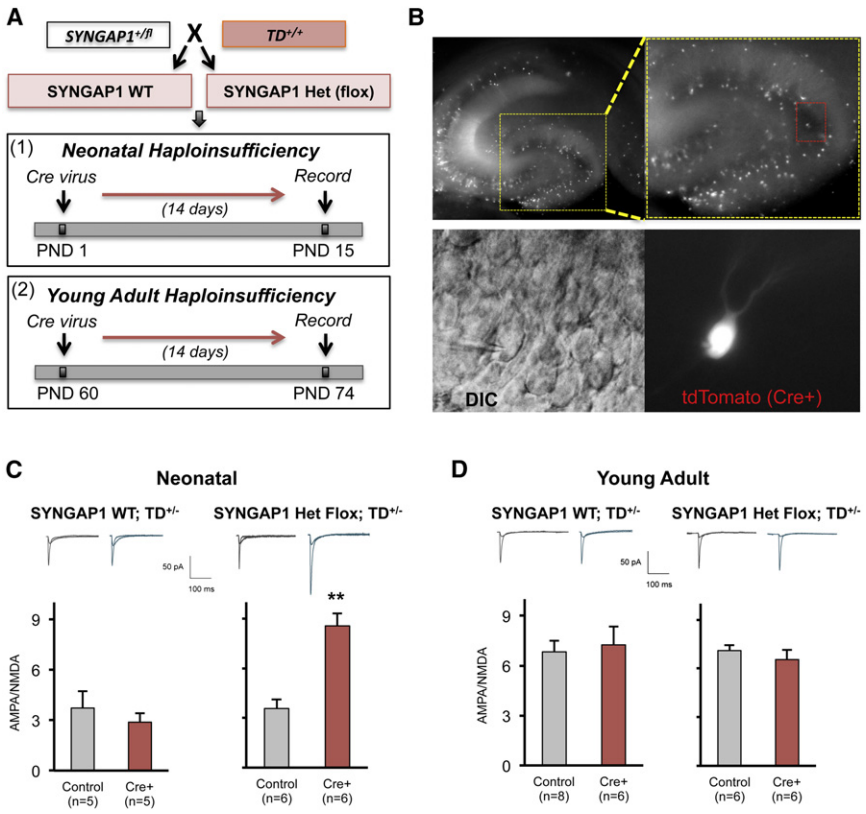

Figure 7. Developing, but Not Adult, Dendritic Spine Synapses Are Cell Autonomously Suppressed by SynGAP Protein

(A) Schematic depicting the strategy for temporal dissection of SYNAGAP1 haploinsufficiency on cell-autonomous neuronal properties. Briefly, *SYNGAP1* mice heterozygous for flanking LoxP *(SYNGAP1$^{+/fl}$)* sites were crossed to homozygous Ai9 Cre reporter mice *(TD$^{+/+}$)* and resulting offspring were injected with Cre virus at either PND1 or PND60. Whole-cell patch clamp recordings were carried out 14 days later.

(B) Photos were taken of an acute brain slice at PND14 with a mosaic expression of TdTomato+ neurons. Red box depicts a neuron that was successfully patched clamped.

(C and D) Mean AMPA/NMDA ratios from either control (tdTomato-negative) or Cre+ (tdTomato-expressing) neurons patch-clamped in the two genotypes at the two developmental stages (neonatal = $p < 0.01$; adults = $p > 0.05$; Student's t test).

See also Figure S5. Error bars depict SEM.

enabled us to test the effect of the LoxP-Stop allele while also controlling for the Cre-ERt2 background. At 8 weeks of age, all animals were then endophenotyped in a minibehavioral battery that tested for the presence of cognitive and noncognitive behavioral abnormalities. Importantly, these behaviors were selected due to the ability to test the same animals before and after genetic rescue. The presence of the LoxP-stop cassette again resulted in behavioral abnormalities similar to that seen in our conventional *SYNGAP1* Hets and the presence of CRE-ERt2 had minimal impact on the behaviors tested (Figures 8B–8D). Specifically, both Cre+ and Cre− *SYNGAP1* LoxP-Stop Hets exhibited increased time in the open arm of the elevated plus maze, increased open field activity levels and the absence of spontaneous alternation in a T-maze. This latter behavior is striking, as it represents a failure of basic working memory that promotes survival (Lisman, 1999). Next, animals were injected with TMX to induce Cre-ERt2 activity, which was expected to reverse *SYNGAP1* haploinsufficiency and restore SynGAP protein levels in these adult animals. One month after reversal, the same animals were again tested in the behavioral battery. Interestingly, there was no apparent effect of adult SynGAP rescue in any behaviors tested. In open field and elevated plus maze, Cre-positive LoxP-Stop Hets continued to show significant behavioral deficits relative to their WT controls (Figures 8B and 8C). In addition, we compared the performance of each individual animal before and after rescue in these two tests by calculating difference scores. There was no significant difference between Cre-positive WT and LoxP-Stop Het behavior when comparing these difference scores (Figure S6), further supporting the idea that rescue did not impact behavior in Cre-positive LoxP-Stop mice. Finally, Cre-positive LoxP-Stop Hets continued to fail the spontaneous alternation tests in post-TMX trials (Figure 8D), indicating that basic working memory in these mice did not improve after adult reversal of haploinsufficiency. Importantly, we confirmed by Southern blot that TMX injections reversed *SYNGAP1* haploinsufficiency in adult Cre-positive LoxP-Stop mice (Figure 8E) and that SynGAP protein expression was restored in these animals (Figure 8F).

DISCUSSION

SYNGAP1 Haploinsufficiency Accelerates the Maturation of Dendritic Spine Synapses during Neonatal Development

In this study, we report that dendritic spine synapses are profoundly impacted by *SYNGAP1* haploinsufficiency during early postnatal development. This critical period of robust synaptogenesis and functional synapse maturation is marked by a steep rise in neural excitability. A major mechanism occurring during these first few weeks of rodent brain development is synapse unsilencing (Kerchner and Nicoll, 2008). Although many synapse types are morphologically intact and capable of releasing glutamate, the majority of glutamaterigic postsynapses on excitatory neurons are not yet functional (Ashby and Isaac, 2011; Isaac et al., 1997; Petralia et al., 1999). Gradually, these synapses acquire functional AMPARs, driving an increase in neural circuit excitation. Our data demonstrate that an essential function of SynGAP during early brain development is to control the gain of excitatory synapses by restricting AMPAR accumulation apposed to presynaptic release sites, particularly in the

phase of development where glutamatergic synapses gradually acquire AMPARs (e.g., the first 21 days of rodent development). Mechanistically, SynGAP shapes developmental synaptic function through its synaptic GAP activity, where it suppresses many biochemical signaling cascades within dendritic spines that promote growth of synapses and insertion of AMPARs (Kim et al., 2005; Krapivinsky et al., 2004; Rumbaugh et al., 2006). This broad action of SynGAP on dendritic spine signaling arises from its central location in the NMDAR complex (Kennedy et al., 2005) and its endowment with a promiscuous GAP domain that can regulate a variety of small G-proteins (Krapivinsky et al., 2004; Pena et al., 2008). However, the role that SynGAP plays in neurons to regulate dendritic spine synapse function has been controversial because some studies report that SynGAP is a repressor of these synapses (Kim et al., 2005; Rumbaugh et al., 2006; Vazquez et al., 2004), whereas others report that it stimulates them (Krapivinsky et al., 2004; McMahon et al., 2012). Adding to the confusion, a recent report has demonstrated that SynGAP can enhance or inhibit dendritic spine synapse function depending on the particular isoform expressed (McMahon et al., 2012). Although the roles of SynGAP may be complex at the cellular level, an important advance arising from our current study is the clear demonstration that *SYNAGAP1* inactivating mutations present during early development have the net effect of derepressing the maturation of dendritic spine synapse in the hippocampus. Thus, we propose that a core neurophysiological outcome of human *SYNGAP1* haploinsufficiency is developmental hyperexcitability triggered by early dendritic spine synapse maturation.

The profound increase in glutamatergic synaptic strength caused by *SYNGAP1* haploinsufficiency was only observed in an early period of hippocampal development. In addition, we observed this transient change in postsynaptic function at multiple synapses in the hippocampus, indicating that this phenomenon is widespread across neocortical dendritic spine synapses and is a primary outcome of pathogenic *SYNGAP1* mutations. This effect in Het mice could arise from abnormally strong synapses that overshoot a predetermined level of function. Alternatively, because we show that SynGAP is a developmental repressor, it could also be caused by accelerated synapse development. Our findings support the latter possibility. Indeed, Het levels of postsynaptic function during the second postnatal week closely resembled that of adult WT animals, indicating that Het synapses achieve adult levels of strength earlier than their WT counterparts. This idea was further supported by the minimal impact of adult-induced SYNGAP1 haploinsufficiency on dendritic spine synapse function. Dendritic spines were less dynamic in the young Het brain, and these spines obtained adult levels of motility and plasticity earlier than WT animals. These findings are consistent with the role of SynGAP as an essential repressive factor that contributes to the developmental trajectory of glutamatergic synapse maturation (Kim et al., 2003; Rumbaugh et al., 2006; Vazquez et al., 2004). Our proposed "accelerated maturation" hypothesis also explains why others have failed to detect changes in hippocampal synaptic strength in adult SynGAP Het mice (Kim et al., 2003; Komiyama et al., 2002).

The Impact of Aberrant Dendritic Spine Synapse Maturation on Circuit Function and Behavior

A major outstanding question in the field of neurodevelopmental disorders is how developmental disruptions to synapse maturation are translated into abnormal systems level alterations that impact cognition and behavior. E/I imbalance is a defining neurophysiological feature of neurodevelopmental disorders (Rubenstein and Merzenich, 2003) and elevated excitation is sufficient to disrupt cognition and sociability (Yizhar et al., 2011). We have found that the effects of *SYNGAP1* haploinsufficiency on synapse maturation are translated into striking changes to E/I balance and hippocampal information processing in the early neonatal period. These data support a mechanism where premature maturation of glutamatergic synapses directly shifts the balance of networks toward aberrant excitation. These circuit-level excitability changes in neonates were accompanied by a reduced seizure threshold and elevated activity in the open field arena, suggesting that abnormal synapse maturation is directly altering behavioral performance and cognition.

Our data suggest that the large increase in neonatal hippocampal excitability occurs through selective loss of the normal repressive action of SynGAP on glutamatergic synapses in glutamatergic neurons. A selective effect on excitatory synapses during early development would be expected to produce a particularly profound hyperexcitation in the hippocampus, a brain region that is already sensitive to overstimulation due to the high level of recurrent excitation within the trisynaptic network (Lisman, 1999). Recurrent excitation is balanced by feed forward inhibition. However, the local GABAergic networks that counteract excitation develop slowly between PND10–21 (Danglot et al., 2006), contributing to the susceptibility of the neonatal mammalian brain to seizure (Bender et al., 2004). Thus, the mammalian brain is ill-equipped to compensate for the enhanced developmental excitability caused by *SYNGAP1* haploinsufficiency. Interestingly, GABAergic synaptic currents were elevated in DGNs in early development. However, because SynGAP is selectively localized to excitatory synapses (Chen et al., 1998; Kim et al., 1998), this effect is likely explained by a homeostatic upregulation of the GABAergic system caused by elevated network excitation triggered by prematurely developing spine synapses. Thus, we propose that rapid dendritic spine synapse maturation in *SYNAGAP1* mutants triggers a chain-reaction of compensatory cellular and systems-level events that may ultimately support organismal survival at the expense of intellectual development. This hypothesis presents a framework for understanding how reduced SynGAP expression in the neonate can have such a profound impact on cognitive development that persists throughout life.

In conclusion, we propose that altered E/I balance in early development, which is a major outcome of *SYNAGP1* haploinsufficiency, directly contributes to cognitive and behavioral abnormalities observed in this disorder. E/I balance influences the duration and efficacy of critical period plasticity windows (Hensch, 2004), which permit refinement of connections that ultimately give rise to cognitive and behavioral modalities. Due to the pervasive disruption of synapse development and E/I balance in the neonatal SynGAP Het brain, we believe that

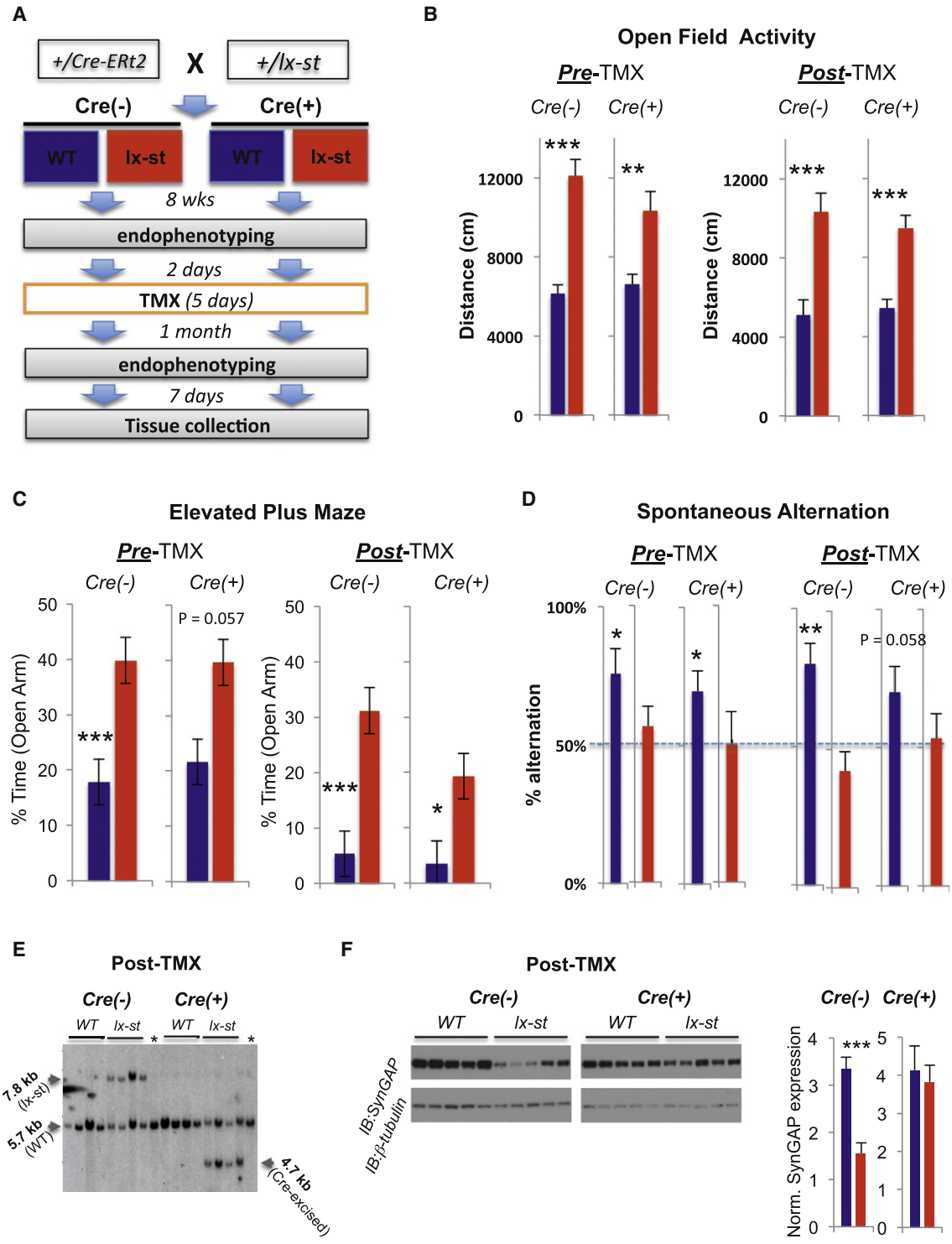

Figure 8. Rescuing SynGAP Protein Expression in Adult Mice Has Minimal Impact on Cognition and Behavior

(A) Experimental scheme—hemizygous male Cre mice were mated with female heterozygous *SYNGAP1* lox stop (rescue) mice to generate four different genotypes: Cre-/WT, Cre-/lx-st, Cre+/WT, Cre+/lx-st, which were run through a behavioral battery at 8 weeks, administered TMX for 5 consecutive days, retested in the behavioral battery 1 month later, and brains extracted and prepared for Southern and western blots.

(B) The mice were run for 30 min sessions in a standard open field test before and after TMX administrations and analyzed for distances traveled. Students t test: *pre-TMX*, *Cre*−,WT (n = 11) versus Het (n = 16); *Cre*+, WT (n = 11) versus Het (n = 10); *post-TMX*: *Cre*−,WT (n = 10) versus Het (n = 15); *Cre*+, WT (n = 10) versus Het (n = 10); *p < 0.05, **p < 0.01, ***p < 0.005.

critical windows of neural plasticity prematurely close or perhaps never open in the first place. In support of this hypothesis, the timing of synapse disruptions seen in Het mice precedes many of the known critical periods of neocortical development (Hensch, 2004). In addition, we show that dendritic spines become larger and functionally stronger earlier in development compared to WT animals. These features are characteristic of stable synapses that are less likely to be eliminated in vivo (Holtmaat et al., 2005). Most strikingly, however, SynGAP mutations cause dendritic spines to become less motile in early development. In fact, spine motility rates in young SynGAP Hets are indistinguishable from adult WT animals. The precise function of spine motility is still unknown, but there is strong evidence that it serves to promote initial wiring or rewiring of neuronal circuits during development (Konur and Yuste, 2004; Majewska and Sur, 2003; Yuste, 2011). Thus, a mutation that causes excessive developmental neural excitation, larger and stronger synaptic connections, and reduced spine motility is highly suggestive of neural networks that are initially miswired and thus resistant to later phases of experience-dependent refinement. A network with these irregular features would be expected to have altered windows of cortical development, resulting in cognitive dysfunction. In support of this idea, rescue of pathogenic SYNGAP1 mutations after critical periods close (e.g., adulthood) did not improve basic behavioral and cognitive abnormalities seen in the mouse model of the disease, suggesting that early spine synapse defects contribute to the disorganization of developing neural circuits that guide these behaviors in adulthood. Future studies will be necessary to understand precisely how developmental synapse disruptions influence the organization of neural circuits that govern intellectual and cognitive development.

EXPERIMENTAL PROCEDURES

For all studies, the experimenter was blind to genotypes. The heterozygous SynGAP KO mouse line has been described previously (Kim et al., 2003), and all studies utilized both males and females. The *SYNGAP1* conditional KO line and the *SYNGAP1* rescue line are described in the supplemental materials. For behavioral tests, adult animals were at least 12 weeks of age unless otherwise noted. For electrophysiological studies, acute brain slices were prepared from PND7–PND9, PND14–16, PND21–23 and 6- to 9-week-old mice. For input-output studies, slices were prepared from one WT and one Het pair each day. Whole-cell current/voltage clamp experiments were made from visually identified DGNs in the molecular layer with glass microelectrodes with an open-tip resistance of 5–8 MΩ. Two-photon imaging of DGN dendrites was performed with a multiphoton laser-scanning microscope (Olympus FV1000MPE-TWIN), equipped with a water immersion objective lens (ULTRA 25x, numerical aperture 1.05, Olympus) and FluoView software. Spines were imaged in acute slices from both Het and WT brain at PND8–9, PND14–16 and PND > 60. Six to nine dendritic segments of ~20–30 μm were collected and considered for analysis. Segments were traced and each individual spine was marked and measured (spine width, length, head diameter). Spontaneous spine head plasticity was evaluated in terms of relative change in spine volume and occurrence of probability of events in both Het and WT PND 14–16 and PND > 60 brain slices. We quantified relative changes in spine-head volume by measuring intensity of the spine head relative to the nonsaturated parent dendrite (background signal was nominal). For voltage-sensitive dye imaging studies, optical recording of voltage sensitive dye (VSD) signals was performed by the MiCAM02 system with a sampling rate of 2.2 ms per frame (frame resolution 88 (w) × 60 (h) pixels). Stimulation was achieved by UV uncaging of MNI-glutamate. Under the 2× objective, the imaging field covered the area of 2.56 × 2.14 mm^2 with a spatial resolution of 29.2 × 35.8 μm/pixel. Complete methods for each type of experiment, including all behavioral paradigms and mouse targeting strategies, are documented in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, one table, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.08.045.

ACKNOWLEDGMENTS

We thank Drs. Ronald Davis, Damon Page, and Fadi Hamdan, as well as members of the Rumbaugh Laboratory, for their helpful comments on this manuscript. We also thank Zachary Collier, Samuel Bacharach, Bryce Grier, and Gopi Patel for assistance in context discrimination studies. This work was supported by grants to G.R. from the National Institute for Neurological Disorders and Stroke (R01NS064079), The Eunice Kennedy Shriver National Institute for Child Health and Human Development (R03HD060672), and the National Alliance for Research on Schizophrenia and Depression (NARSAD). The laser photostimulation and voltage-sensitive dye imaging part of this work was performed in X.X.'s laboratory, supported by a grant from the National Institute for Drug Abuse (DA023700-04S1). J.P.C. and E.D.O. performed electrophysiological recordings; M.A. performed multiphoton imaging studies and spine analysis; T.K.C., N.J.R., A.G.A., and B.J.W. performed behavioral studies; Y.S. and X.X. performed laser photo-stimulation and imaging studies; G.R., C.A.M., T.K.C., J.P.C., M.A., B.J.W., X.X. designed experiments; B.M. and T.K.C. performed protein/mRNA studies; G.R. conceived the study and wrote the manuscript; G.R., C.A.M., J.P.C., and T.K.C. edited the manuscript.

(C) The mice were run in a standard 5 min elevated plus maze test before and after TMX administrations and analyzed for percent time spent in the open arms of the maze. Student's t test: *pre-TMX*, *Cre*−,WT (n = 11) versus Het (n = 16); *Cre*+,WT (n = 11) versus Het (n = 10); *post-TMX*: *Cre*−, WT (n = 10) versus Het (n = 15); *Cre*+, WT (n = 10) versus Het (n = 10); *p < 0.05, ***p < 0.01.

(D) The mice were run in a standard automated discrete-trials spontaneous alternation test three times before and after TMX administrations and analyzed for average percent alternation. Cre−/WTs and Cre+/WTs alternated significantly above chance (50%) level before and after TMX administrations, whereas the corresponding het groups did not. Dashed line represents chance levels of alternation (50%). Student's one-sample t test against population mean of 50%: *pre-TMX*: *Cre*−, WT (n = 11), Het (n = 16); *Cre*+, WT (n = 11), Het (n = 10); *post-TMX*: *Cre*−, WT (n = 10) versus Het (n = 15); *Cre*+, WT (n = 10) versus Het (n = 10); *p < 0.05, **p < 0.005.

(E) Frontal cortical brain tissue from all four groups of mice was dissected and processed for Southern blot analysis, which confirmed that TMX excised the LoxP-Stop cassette in adult Cre-ERt2-positive animals. Four subjects from each group were randomly chosen for genetic analysis. Lanes with asterisks were loaded with C57/Bl6-positive control genomic DNA.

(F) Hippocampal tissues from the mice were dissected and processed for western blot analysis of SynGAP protein levels normalized to β-tubulin after TMX administrations and behavioral testing (n = 7 per group; t test, p < 0.001).

See also Figure S6. Error bars depict SEM.

Received: January 5, 2012
Revised: July 21, 2012
Accepted: August 20, 2012
Published: November 8, 2012

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