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***Syngap1* haploinsufficiency damages a postnatal critical period of pyramidal cell structural maturation linked to cortical circuit assembly**

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Abstract

Background—Genetic haploinsufficiency of *Syngap1* commonly occurs in developmental brain disorders, such as intellectual disability (ID), epilepsy, schizophrenia (SCZ), and autism spectrum (ASD) disorder. Thus, studying mouse models of *Syngap1* haploinsufficiency may uncover pathological developmental processes common among distinct brain disorders.

Methods—A *Syngap1* haploinsufficiency model was used to explore the relationship between critical period dendritic spine damage, cortical circuit assembly and the window for genetic rescue in order to understand how damaging mutations disrupt key substrates of mouse brain development.

Results—*Syngap1* mutations broadly disrupted a developmentally sensitive period that corresponded to the period of heightened postnatal cortical synaptogenesis. Pathogenic *Syngap1* mutations caused a coordinated acceleration of dendrite elongation and spine morphogenesis, and pruning of these structures in neonatal cortical pyramidal neurons. These mutations also prevented a form of developmental structural plasticity associated with experience-dependent reorganization of brain circuits. Consistent with these findings, *Syngap1* mutant mice displayed an altered pattern of long-distance synaptic inputs into a cortical area important for cognition. Interestingly, the ability to genetically improve the behavioral endophenotype of *Syngap1* mice decreased slowly over postnatal development and mapped onto the developmental period of coordinated dendritic insults.

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Conclusions—Pathogenic *Syngap1* mutations have a profound impact on the dynamics and structural integrity of pyramidal cell postsynaptic structures known to guide the *de novo* wiring of nascent cortical circuits. These findings support the idea that disrupted critical periods of dendritic growth and spine plasticity may be a common pathological process in developmental brain disorders.

Keywords

Intellectual disability; Autism spectrum disorder; Epilepsy; *Syngap1*; development; synapse; Mouse Model

Introduction

Hundreds of genetic loci are believed to contribute to commonly diagnosed neurodevelopmental disorders (NDDs), such as ID, ASD, epilepsy and SCZ. Recent genomic sequencing studies in enriched patient populations have shown that damaging mutations commonly occur in patients with these developmental brain disorders (1–11). Collectively, these studies have demonstrated that certain commonly mutated genes, while rare in overall occurrence, have an unusually large effect on brain function during development. Based on these findings, mouse models of these mutations may provide unique insight into the pathobiology of NDDs. Indeed, because similar mutations in the same gene can lead to distinct psychiatric disorders (12, 13), identification of pathobiological processes in these mouse models may also provide insight into the shared neurobiology of related, but distinct, developmental brain disorders.

Damaging mutations in *SYNGAP1/Syngap1* are causally-linked to a spectrum of developmental brain disorders, including ID, ASD, epileptic encephalopathy (EE), and SCZ (1, 3, 4, 11, 14–20). Indeed, *Syngap1* has been highlighted as a high-impact neurodevelopmental disease gene based on the convergence of several developmental brain disorders seen in patients with these damaging mutations (12, 13). *De novo* nonsense mutations in *Syngap1* that lead to genetic haploinsufficiency are one of the most common causes of sporadic and non-syndromic ID. Current estimates indicate that pathogenic *Syngap1* mutations account for 2–8% of sporadic ID cases (2, 3, 14, 17, 19) and up to 1% of EE cases (1), suggesting that there are tens-of-thousands of undocumented individuals carrying these mutations. By reducing expression of SynGAP protein, haploinsufficiency causes a severe comorbid syndrome with commonly overlapping diagnoses of ID, ASD and epilepsy (19). However, less severe *Syngap1* mutations, such as splicing mutations, are linked to milder forms of cognitive impairment, such as those seen in SCZ (10). Furthermore, missense mutations that moderately impact SynGAP protein function lead to relatively less severe forms of ID (19). Together, the genomic data compiled from enriched patient populations point to a relationship between the extent of *Syngap1* genetic damage and severity of mental dysfunction.

There is a convergence of findings indicating that abnormal function of dendritic spine synapses contribute importantly to intellectual disability and related brain developmental disorders (21). Many high-risk loci for NDDs encode proteins that directly regulate dendritic

spine synapse function (22). *Syngap1* encodes a neuron-specific RasGAP (SynGAP) that is predominantly expressed in glutamatergic neurons of the mammalian forebrain (23, 24). Within these neurons, the protein is trafficked exclusively to dendritic spine synapses (24) where it negatively regulates excitatory synaptic function and dendritic spine size through suppression of the Ras/ERK pathway (25–27). While it is known that two intact copies of *Syngap1* are essential for proper mouse brain development and cognitive maturation (25, 26), it remains unclear how haploinsufficiency of this gene impacts key neurodevelopmental substrates known to influence cognitive maturation, such as the structural development of dendrites and spines and their relationship to cortical circuit assembly.

Methods and Materials (Summary)

All procedures were performed in accordance with the Scripps Research Institutional Animal Care and Use Committee. Complete methods, experimental procedures, and statistical analyses are reported in the Supplemental Materials.

Results

To investigate the potential impact of *Syngap1* mutations on structural alterations of developing neurons, we first tracked apical dendritic arborization in young and adult somatosensory cortical pyramidal cells in *wt* and *Syngap1* heterozygous knockout (*het*) (28, 29) mice (Fig. 1A–E). Arbors traced from PND21 *het* neurons exhibited increased total neurite length (Fig. 1B–C), higher complexity (Fig. 1D) and occupied a larger volume (Fig. 1E) compared to *wt* neurons from mice of the same age. Strikingly, *het* arbors in these young mice already had several features of older neurons, such as higher-order branching and adult-like dendritic length (Fig. 1B–E), indicating that the neuronal phenotype of PND21 *het* mice resulted from early maturation of arbors rather than abnormal overgrowth of dendrites. We observed that soma size predicted dendritic length in *wt* neurons at both ages, but not in *hets* (Fig. 1F), consistent with disruptions to growth-related processes in *Syngap1* mutants. To probe for possible signaling abnormalities underlying accelerated neuronal growth in *hets*, we examined levels of phosphorylated S6 (p-S6), a marker of mTORC1-mediated cellular growth and protein synthesis (30, 31). We found that PND4 *het* mice had elevated p-S6 immunoreactivity in L5 neurons compared to *wt*s (Fig. 1G). Our results in L5 neurons are consistent with a recent report demonstrating that SynGAP suppresses protein synthesis in cultured forebrain neurons (32). Together, these findings indicate that *Syngap1* mutations cause accelerated growth and/or early differentiation of developing deep cortical pyramidal cells *in vivo*.

We next investigated the relationship between altered dendrite maturation and spine morphogenesis in *het* L5 pyramidal neurons. We observed a striking developmental shift in both spine formation and spine pruning in *het* neurons relative to *wt*s (Fig. 2A–B), indicative of an overall acceleration in the normal development of neuronal structure. Peak supernumerary spine density and the final density after pruning were indistinguishable between genotypes (Fig. 2B). However, the timing of each of these key developmental milestones was clearly shifted in *hets*, with each happening at least one week earlier than in the cortex of *wt* mice, and appeared to map onto the time-course of accelerated dendritic

elongation (Fig. 1B–F). Filopodia and spine steady-state density exhibit an inverse relationship in developing neurons because filopodia can be precursors of spines (33). Thus, their levels fall as dendritic spine density increases. Interestingly, we also observed a premature age-dependent reduction in filopodia density in *het* mice that was inversely related to dendritic spine density (Fig. 2C), consistent with a dramatic early structural maturation of deep cortical pyramidal neurons. Finally, we also observed significantly longer dendrites in very young *het* neurons without spines (Fig. S1), indicating that coordinated increases in spine density and dendritic elongation are influenced by a common upstream factor, such as the rate of postmitotic differentiation.

We next investigated if early structural maturation of neurons in *het* mice was associated with lower levels of dendritic plasticity. To test this idea, we first determined if whisker-related activity contributed to the disparities between *wt* and *het* spine density at PND 21. Whisker deprivation during the period of peak spinogenesis in *hets* (PND7–21) did not normalize spine density, though this experimental manipulation did reduce peak spine density equally in each genotype (Fig. 3A–C). These data indicated that intrinsic processes in the *het* brain, rather than environmental influences, drive developmental increases in steady-state spine density in superficial dendrites of deep cortical pyramidal cells. As expected, whisker deprivation caused a 2.5-fold increase in *wt* filopodia density at PND21 (Fig. 3D), a cellular response associated with the functional remapping of sensory networks in response to altered experience (34). Remarkably, this response was entirely absent in *het* mice (Fig. 3D), consistent with a reduction in the capacity to reorganize cortical circuits.

It is unclear how transient alterations to steady-state spine density and altered dendritic plasticity in developing *Syngap1* mutants could impact cognitive ability. One possibility is that these steady state measures reflect altered spine and filopodia dynamics, which could impact organization and target specificity of developing neural circuits. Indeed, the dynamic formation and elimination of dendritic spines reflects functional reorganization of neural circuits (33, 34). Therefore, we next performed transcranial *in vivo* serial dendritic spine imaging to determine how *Syngap1* haploinsufficiency impacts dendritic spine dynamics during the developmental period of heightened cortical synaptogenesis. First, we imaged spines over a four-hour period in ~PND17 mice in order to measure spine formation rates in the developing brain (Fig. 4A–E). We observed that *Syngap1* haploinsufficiency increased the conversion rate of filopodia into spines, which may explain the enhanced density of dendritic spines observed in young mutants (Fig. 2A–B). Next, we performed serial spine imaging over a two-day period starting at PND21 (Fig. 4F) and found that spine formation outpaced elimination in *wt* mice (Fig. 4G,H), which is consistent with neurons that are rapidly increasing the steady-state supply of dendritic spines (Fig. 2B). However, spine elimination outpaced spine formation in *het* neurons at this age (Fig. 4G,H), which is consistent with developmentally older neurons (35) undergoing steady-state spine pruning (Fig. 2B). To directly test the idea that spine dynamics in PND21 *hets* reflected processes normally occurring in developmentally older neurons, we repeated this experiment in slightly more mature animals (Fig. 4F). Similar to neurons from *hets* at PND21, spine elimination in PND30 *wt* neurons now outpaced formation over a two-day period (Fig. 4J–K). This is consistent with past studies demonstrating that spine dynamics of this nature

reflect activity-dependent spine pruning (35). Neurons from PND30 *het* mice, however, displayed reduced spine elimination rates that did not differ from the formation rate (Fig. 4J–K), a feature seen in adult *wt* Layer 5 neurons (33) that have already undergone significant spine pruning (Fig. 2B). Importantly, *wt* spine formation and elimination rates in PND30 animals appeared similar to PND21 *het* dynamics (Fig. 4H,K), confirming the notion that *Syngap1* haploinsufficiency accelerates the appearance of spine dynamics indicative of more mature neurons. Next, we calculated the total spine turnover rate in both genotypes because this measure reflects the capacity for network plasticity and this rate is known to decrease as neurons mature (33, 34). Interestingly, the spine turnover rate in *hets* was reduced relative to *wts* at both stages of development (Fig. 4I,L), which is a disruption most likely caused by the early maturation of these neurons. Together, these data demonstrate that pathogenic *Syngap1* mutations accelerate the growth and maturation of cortical neuron structures associated with *de novo* neural circuit formation and refinement.

Is *Syngap1*'s influence on dendritic spine properties related to a critical period of development? To investigate this idea, we performed a series of experiments to determine how the presence or absence of *Syngap1* mutations during the early postnatal period affected dendritic spine properties. We focused on dendritic spine size for these experiments because it is the only dendritic spine abnormality in *het* mice known to emerge during development and persist into adulthood (25). First, we probed dendritic spine size in two different types of cortical neurons in the cortex, L5 and L2/3 pyramidal neurons. Interestingly, we observed a substantial shift in dendritic spine size at PND14 in both L2/3 and L5 neurons in *het* mice (Fig. 5A). We also observed an enlarged PSD area from spines contacted by presynaptic terminals in the young mutants (Fig. 5B), which indicates that enlarged spines are incorporated into nascent neural circuits. In addition, we found that spines were enlarged in adult *het* L2/3 and L5 neurons (Fig. 5C, E), confirming that this is a persistent cortical structural abnormality in *Syngap1* mutants. *In vivo* serial spine imaging experiments performed in developing (e.g. PND21–PND23) *het* mice demonstrated that newly formed spines from L5 neurons were larger compared to spines born in *wt* neurons (Fig. 5D). These results suggested that the level of SynGAP expression in nascent dendritic spines is a critical factor that determines the size of these structures. However, an alternative possibility is that SynGAP expression levels dynamically regulate spine size regardless of age. To distinguish between these two possibilities, we measured dendritic spine size in developmentally normal animals with haploinsufficiency induced in adulthood (26). Strikingly, inducing haploinsufficiency in animals with mature and stabilized dendritic spines did not significantly alter the size of these structures (Fig. 5E), consistent with the emerging view that *Syngap1* is a gene with a developmentally sensitive period with respect to dendritic spine synapse regulation (25, 26, 36). This finding also supports the intriguing possibility that the *Syngap1* expression level in a newborn spine is an intrinsic factor that determines its size.

To test the idea that developmental disruptions to the dynamics and morphology of dendritic spines may influence the organization of long-range cortical circuits, we used a modified rabies virus, RV-GFP (37), to map long-distance monosynaptic inputs into the mPFC of *Syngap1 wt* and *het* mice. We first determined the sensitivity and accuracy of RV-GFP-

generated cortical input maps (Fig. S2A). We found that injections of RV-GFP particles into the mPFC resulted in a pattern of neuronal labeling distinct from injections into primary visual cortex (V1) that was consistent with reports from prior anatomical studies (Fig. S2B–C). In the course of analyzing these input maps, we observed a surprisingly low coefficient of variance in the total number of labeled neurons in various cortical areas (Fig. S2D–E), indicating that this method is a highly sensitive way to detect quantitative changes in relative synaptic connectivity with an experimental manipulation. Therefore, we next injected RV-GFP into the mPFC of *wt* or *het* mice (Fig. 6A–B) and generated brain-wide input maps to understand how *Syngap1* mutations alter long-range neural connectivity into this associational brain region implicated in higher cognition. The relative proportion of long-range synaptic inputs into the mPFC were surprisingly similar between adult *wt* and mutant mice (Fig. 6C–F). Importantly, *Syngap1* mutations did not alter the basic properties of RV-GFP neuronal entry or eGFP reporter expression (Fig. S3A). Brain weight and cortical layer thickness were similar between genotypes (Fig. S3B–G). Thus, the basic structural and anatomical aspects of the cortex appear unaffected in *het* mice, a finding consistent with the human *Syngap1* haploinsufficiency syndrome (19, 38), which lends additional face validity to this disease model. These data motivated us to probe connectivity at the regional level. We did not observe genotype differences between relative ipsilateral and contralateral projections to the mPFC (Fig. 6D), or between areas subdivided by developmental origin (Fig. 6E). However, we began to see changes in relative connectivity between genotypes when cortical regions were parsed into primary and secondary areas (Fig. 6F), suggesting that individual brain areas might have altered relative mPFC connectivity in *het* mice.

Focusing on individual brain areas, we next generated a brain-wide map based on the percent labeling of neurons located in each region displaying afferent connectivity to the mPFC. Selection of the most appropriate statistical test for large data sets represents a challenge. Therefore, to determine if there were changes in connectivity between genotypes for each brain region, we performed the statistical analyses employed in a recent study that used RV-GFP tracing tools to probe point-to-point circuit organization in mice (39). In both cases, the data sets conform to the independence of observations requirement of unpaired t-tests. Specifically, because RV-GFP is taken up by synaptic terminals and retrogradely transported, a distantly-labeled neuron can only contribute to one connection in the analysis. Therefore, each GFP+ neuron represents a unique projection to the mPFC (e.g. circuit), such that the retrograde labeling of each neuron is independent of other labeled neurons. Each neuron bears an additional level of independence because it is only counted once in the analysis (e.g. it cannot be part of two brain regions). This regional analysis revealed specific cortical areas with altered mPFC connectivity (Fig. 7A–B,E), including both hemispheres of the frontal association cortex (i/cFrA) and the ipsilateral primary somatosensory cortex (iS1), which were hypoconnected in *Syngap1* mutants. We next considered the potential for false positives as a result of multiple t-tests. A p-value of 0.05 would predict one false positive for every 20 t-tests performed. Twenty-eight comparisons were performed for the cortical analyses depicted in Figure 4A–B. Therefore, the expectation would be 1.4 false positives. Here, three regions achieved significance. Furthermore, two of the significant comparisons were from the same brain region (FrA), located in opposite hemispheres. Given that false positives occur at random, it is unlikely that both iFrA ($P = 0.003$) and cFrA ($P =$

0.046) would achieve significance in error. A center-of-gravity analysis (39) of GFP-labeled iS1 neurons revealed that the location of these cells was not altered in *hets* (Fig. 7F). Furthermore, we observed trends toward hyperconnectivity of the amygdala, secondary auditory cortex and perirhinal cortex inputs to the mPFC (Fig. 7C–E). Qualitatively, it appeared that posterior cortical afferents into the mPFC were more likely to trend towards hyperconnectivity, with the converse for more anterior regions. Therefore, we plotted the relationship between the connectivity change in *het* mice for each mPFC afferent relative to regional distance from bregma (Fig. 7G). This analysis determined that there is, indeed, a correlation between the cortical anterior/posterior axis and the valence of altered connectivity in mutant mice. The correlation remained significant even in the absence of the two data points that fell furthest from the regression line in the posterior part of the brain ($P < 0.05$), indicating that the correlation cannot be attributed solely to a handful of brain regions. This finding suggests a subtle, but widespread, disruption to the organization of prefrontal cortical circuits in mice with pathogenic *Syngap1* mutations.

The time course of alterations to dendritic spine structure and synaptic dynamics by *Syngap1* mutations suggests that these disruptions may contribute to the behavioral endophenotype of the mice. We reasoned that if alterations to the maturation of neuronal dendrites and spines contribute to the mouse endophenotype, then the ability to genetically rescue disrupted behaviors should map onto a similar developmental time course. Therefore, we explored the idea that reversing mutations at PND1, which marks a time in development that precedes the critical period of dendritic growth and heightened spine dynamics, would protect animals born with pathogenic *Syngap1* mutations from developing behavioral abnormalities. To do this, we mated *Syngap1* conditional reversal mice with a line that ubiquitously expresses a form of Cre that is induced by tamoxifen (25, 26). This strategy enabled Cre activation during the first postnatal week. Validation experiments confirmed that this tamoxifen dosing scheme in young mice effectively induced Cre activity in nearly all brain cells by PND7 (Fig. S4A). All offspring from this cross were treated with tamoxifen and we confirmed these injections enhanced both hippocampal (Fig. 8A) and cortical (Fig. S4B) SynGAP protein levels in Cre-positive *het* animals. At 12 weeks of age, they were tested for core behavioral abnormalities indicative of mouse *Syngap1* haploinsufficiency (25, 28, 40), such as increased risk-taking, novelty-induced hyper-locomotion and long-term memory. As expected, *het* mice lacking the inducible form of Cre displayed behavioral abnormalities consistent with mice harboring pathogenic *Syngap1* mutations (Fig. 8B–D). Remarkably, *het* mice carrying the inducible Cre allele were behaviorally indistinguishable from their Cre-positive *wt* counterparts in all three behavioral domains (Fig. 8B–D), indicating that tamoxifen injections in the first postnatal week completely protected these mice from developing common cognitive and behavioral abnormalities associated with *Syngap1* haploinsufficiency. In a separate set of experiments, we tested the hypothesis that the efficacy of genetic rescue would be reduced once peak precocious spine density (e.g. wiring) was reached in *het* mice (e.g. ~PND21). To test this, we repaired a pathogenic *Syngap1* mutation starting at PND21 and then observed how this treatment impacted behavior and cognition in adulthood. Overall, this strategy was less efficacious compared to reversal experiments initiated at PND1 (Fig. 8E–H). There was no effect of PND21 genetic reversal on *het* performance in the elevated plus maze, a measure of anxiety and risk-taking, which

indicated that irreversible brain abnormalities had already occurred by this stage of development. Performance in the open field was slightly improved by the PND21 genetic rescue strategy relative to Cre(–) *het* animals, but was still significantly elevated relative to Cre(+) *wt*s, indicating a partial rescue of brain dysfunction. In contrast, we observed that contextual memory was completely rescued when reversal was initiated at PND21, indicating that dysfunction that affects some forms of long-term memory had not yet occurred. These findings demonstrate that there is no single *Syngap1* critical period for behavioral modalities. Rather, different behavioral modalities are modulated by *Syngap1* at different times in development. These findings suggest that distinct neurobiological processes contribute to the shaping of different behavioral modalities, though the best overall behavioral outcomes in this mouse model are achieved when mutations are repaired before the rapid phase of dendritic growth and spine morphogenesis.

Discussion

The key finding in this study is that *Syngap1* mutations associated with developmental brain disorders disrupt a critical period of neuronal growth, maturation and dynamics of dendritic structures known to influence *de novo* assembly of developing cortical circuits. We found that the ability to prevent the onset of behavioral abnormalities in mice with *Syngap1* haploinsufficiency mapped onto a disrupted critical period of cortical pyramidal neuron development, which encompassed a period between the first and third postnatal weeks. Damage to this critical period was evident from the premature growth and/or differentiation of dendritic arbors, the early accumulation and subsequent pruning of dendritic spines, the emergence of abnormally large dendritic spines, as well as the premature reduction in dendritic spine dynamics and plasticity. A genetic rescue strategy that was completely successful immediately after birth lost efficacy by the end of the third postnatal week, suggesting that the damage to the critical period of dendritic structural maturation influences behavior in these mice. Based on the findings, we conclude that one route to altered cognition in *Syngap1* mutant mice is through an alteration in the organization of cortical circuits, particularly in brain areas that support higher cognitive function (e.g. the mPFC). However, considering that *Syngap1* mutations lead to a spectrum of developmental brain disorders (1, 3, 4, 11, 14–20), it is possible that damage to the critical period of dendritic structural maturation may be a common pathological substrate across mouse models of these disorders. Indeed, altered developmental spine dynamics have been described in a mouse model of Fragile X syndrome (41, 42).

This conclusion is supported by several key observations. L5 cortical pyramidal neurons in *het* mice developed spine synapses at an accelerated rate relative to *wt* littermates. These data indicate that certain neuronal connections form too quickly in the mutant brain. Adult spine density in *hets* was no different from controls, supporting the idea that *Syngap1* strongly influences neurodevelopmental processes that promote cortical wiring. Interestingly, spine pruning was also initiated earlier in the *het* brain. Spine pruning is believed to be an essential developmental process that reflects the refinement of cortical circuits and improved mental ability (21, 43, 44). Consistent with this idea, monosynaptic retrograde circuit tracing indicated that some brain areas are misconnected in *Syngap1* mutants. The current findings in developing cortical neurons are quite distinct from past

observations made in the developing hippocampus of *Syngap1* mutant mice. In dentate gyrus granule neurons (DGNs), spine density is not changed during development, despite spine volume being significantly enhanced during the second postnatal week (25). In addition, DGN dendritic arborization and maturation was also normal in young *Syngap1* mutants. Furthermore, spine density in mature CA1 neurons is normal in *Syngap1* mice (45). Thus, to our knowledge, this is the first report of a coordinated disruption to the rate of dendritic spine morphogenesis and corresponding elongation of dendrites in developing *Syngap1* mutants. Because this type of developmental insult was not observed in the hippocampus, these data raise the possibility that there is a distinct form of developmental damage in the cortex relative to the hippocampus.

We also report here that the ability to protect animals from developing *Syngap1* mutant behavioral endophenotypes decreased by PND21. Prior studies demonstrated that complete protection is possible when *Syngap1* mutations are reversed in cortical progenitor cells in the mid-to-late embryonic period (26). However, no improvement is achieved when CNS-wide reversal is initiated in adulthood (25). In this context, our finding that the efficacy for postnatal rescue had already decreased by PND21 supports the idea that damage during the critical period of dendrite structural maturation contributes to the endophenotypes in *Syngap1* mutants. Therefore, translational mouse studies aimed at discovery of therapeutics to treat this genetic form of ID and epilepsy may benefit from a focus on protecting the structural critical period. Considering our findings here that early neonatal genetic reversal and re-expression of SynGAP protein can protect animals from developing severe behavioral problems (Fig. 8), identifying novel chemical or biological agents that effectively boost *Syngap1* expression during the developmental critical period of synaptogenesis may prove to be a particularly powerful translational strategy. A therapeutic with this property in humans would likely have efficacy limited to patients with *Syngap1* loss-of-function (LOF) mutations, though the translational approach may be generally useful for developing tailor-made therapies for other sporadic developmental brain disorders caused by autosomal dominant LOF mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Carvill GL, Heavin SB, Yendle SC, McMahon JM, O'Roak BJ, Cook J, et al. Targeted resequencing in epileptic encephalopathies identifies de novo mutations in CHD2 and SYNGAP1. *Nature genetics*. 2013; 45:825–830. [PubMed: 23708187]
2. de Ligt J, Willemssen MH, van Bon BW, Kleefstra T, Yntema HG, Kroes T, et al. Diagnostic exome sequencing in persons with severe intellectual disability. *The New England journal of medicine*. 2012; 367:1921–1929. [PubMed: 23033978]
3. Rauch A, Wieczorek D, Graf E, Wieland T, Ende S, Schwarzmayr T, et al. Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. *Lancet*. 2012; 380:1674–1682. [PubMed: 23020937]
4. Stefansson H, Meyer-Lindenberg A, Steinberg S, Magnusdottir B, Morgen K, Arnarsdottir S, et al. CNVs conferring risk of autism or schizophrenia affect cognition in controls. *Nature*. 2014; 505:361–366. [PubMed: 24352232]
5. Iossifov I, Ronemus M, Levy D, Wang Z, Hakker I, Rosenbaum J, et al. De novo gene disruptions in children on the autistic spectrum. *Neuron*. 2012; 74:285–299. [PubMed: 22542183]
6. Jiang YH, Yuen RK, Jin X, Wang M, Chen N, Wu X, et al. Detection of clinically relevant genetic variants in autism spectrum disorder by whole-genome sequencing. *American journal of human genetics*. 2013; 93:249–263. [PubMed: 23849776]
7. Neale BM, Kou Y, Liu L, Ma'ayan A, Samocha KE, Sabo A, et al. Patterns and rates of exonic de novo mutations in autism spectrum disorders. *Nature*. 2012; 485:242–245. [PubMed: 22495311]
8. O'Roak BJ, Vives L, Girirajan S, Karakoc E, Krumm N, Coe BP, et al. Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. *Nature*. 2012; 485:246–250. [PubMed: 22495309]
9. Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ, et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. *Nature*. 2012; 485:237–241. [PubMed: 22495306]
10. Fromer M, Pocklington AJ, Kavanagh DH, Williams HJ, Dwyer S, Gormley P, et al. De novo mutations in schizophrenia implicate synaptic networks. *Nature*. 2014; 506:179–184. [PubMed: 24463507]
11. Purcell SM, Moran JL, Fromer M, Ruderfer D, Solovieff N, Roussos P, et al. A polygenic burden of rare disruptive mutations in schizophrenia. *Nature*. 2014; 506:185–190. [PubMed: 24463508]
12. Zhu X, Need AC, Petrovski S, Goldstein DB. One gene, many neuropsychiatric disorders: lessons from Mendelian diseases. *Nat Neurosci*. 2014; 17:773–781. [PubMed: 24866043]
13. Hoischen A, Krumm N, Eichler EE. Prioritization of neurodevelopmental disease genes by discovery of new mutations. *Nat Neurosci*. 2014; 17:764–772. [PubMed: 24866042]
14. Hamdan FF, Gauthier J, Spiegelman D, Noreau A, Yang Y, Pellerin S, et al. Mutations in SYNGAP1 in autosomal nonsyndromic mental retardation. *The New England journal of medicine*. 2009; 360:599–605. [PubMed: 19196676]
15. Krepischi AC, Rosenberg C, Costa SS, Crolla JA, Huang S, Vianna-Morgante AM. A novel de novo microdeletion spanning the SYNGAP1 gene on the short arm of chromosome 6 associated with mental retardation. *American journal of medical genetics Part A*. 2010; 152A:2376–2378. [PubMed: 20683986]
16. Pinto D, Pagnamenta AT, Klei L, Anney R, Merico D, Regan R, et al. Functional impact of global rare copy number variation in autism spectrum disorders. *Nature*. 2010; 466:368–372. [PubMed: 20531469]
17. Hamdan FF, Daoud H, Piton A, Gauthier J, Dobrzyniecka S, Krebs MO, et al. De novo SYNGAP1 mutations in nonsyndromic intellectual disability and autism. *Biological psychiatry*. 2011; 69:898–901. [PubMed: 21237447]
18. Hamdan FF, Gauthier J, Araki Y, Lin DT, Yoshizawa Y, Higashi K, et al. Excess of de novo deleterious mutations in genes associated with glutamatergic systems in nonsyndromic intellectual disability. *American journal of human genetics*. 2011; 88:306–316. [PubMed: 21376300]

19. Berryer MH, Hamdan FF, Klitten LL, Moller RS, Carmant L, Schwartzentruber J, et al. Mutations in SYNGAP1 Cause Intellectual Disability, Autism, and a Specific Form of Epilepsy by Inducing Haploinsufficiency. *Human mutation*. 2012
20. Writzl K, Kneigt AC. 6p21.3 microdeletion involving the SYNGAP1 gene in a patient with intellectual disability, seizures, and severe speech impairment. *American journal of medical genetics Part A*. 2013; 161:1682–1685. [PubMed: 23687080]
21. Penzes P, Cahill ME, Jones KA, VanLeeuwen JE, Woolfrey KM. Dendritic spine pathology in neuropsychiatric disorders. *Nature Neuroscience*. 2011; 14:285–293.
22. Verpelli C, Sala C. Molecular and synaptic defects in intellectual disability syndromes. *Current opinion in neurobiology*. 2012; 22:530–536. [PubMed: 22000839]
23. Chen HJ, Rojas-Soto M, Oguni A, Kennedy MB. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron*. 1998; 20:895–904. [PubMed: 9620694]
24. Kim JH, Liao D, Lau LF, Huganir RL. SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. *Neuron*. 1998; 20:683–691. [PubMed: 9581761]
25. Clement JP, Aceti M, Creson TK, Ozkan ED, Shi Y, Reish NJ, et al. Pathogenic SYNGAP1 mutations impair cognitive development by disrupting maturation of dendritic spine synapses. *Cell*. 2012; 151:709–723. [PubMed: 23141534]
26. Ozkan EDC, Kramar TK, Seese EA, Babyan RR, Rojas AH, Shi CS, Lucero Y, Xu R, Noebels X, Miller JL, Lynch CA, Rumbaugh GSG. Reduced cognition in Syngap1 mutants is caused by isolated damage within developing forebrain excitatory neurons. *Neuron*. 2014; 82:1317–1333. [PubMed: 24945774]
27. Rumbaugh G, Adams JP, Kim JH, Huganir RL. SynGAP regulates synaptic strength and mitogen-activated protein kinases in cultured neurons. *Proc Natl Acad Sci U S A*. 2006; 103:4344–4351. [PubMed: 16537406]
28. Guo X, Hamilton PJ, Reish NJ, Sweatt JD, Miller CA, Rumbaugh G. Reduced expression of the NMDA receptor-interacting protein SynGAP causes behavioral abnormalities that model symptoms of Schizophrenia. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*. 2009; 34:1659–1672. [PubMed: 19145222]
29. Kim JH, Lee HK, Takamiya K, Huganir RL. The role of synaptic GTPase-activating protein in neuronal development and synaptic plasticity. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2003; 23:1119–1124. [PubMed: 12598599]
30. Cloetta D, Thomanetz V, Baranek C, Lustenberger RM, Lin S, Oliveri F, et al. Inactivation of mTORC1 in the developing brain causes microcephaly and affects gliogenesis. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2013; 33:7799–7810. [PubMed: 23637172]
31. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012; 149:274–293. [PubMed: 22500797]
32. Wang CC, Held RG, Hall BJ. SynGAP Regulates Protein Synthesis and Homeostatic Synaptic Plasticity in Developing Cortical Networks. *PloS one*. 2013; 8:e83941. [PubMed: 24391850]
33. Bhatt DH, Zhang S, Gan WB. Dendritic spine dynamics. *Annual review of physiology*. 2009; 71:261–282.
34. Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, et al. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature*. 2002; 420:788–794. [PubMed: 12490942]
35. Zuo Y, Yang G, Kwon E, Gan WB. Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. *Nature*. 2005; 436:261–265. [PubMed: 16015331]
36. Clement JP, Ozkan ED, Aceti M, Miller CA, Rumbaugh G. SYNGAP1 Links the Maturation Rate of Excitatory Synapses to the Duration of Critical-Period Synaptic Plasticity. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2013; 33:10447–10452. [PubMed: 23785156]
37. Wickersham IR, Lyon DC, Barnard RJ, Mori T, Finke S, Conzelmann KK, et al. Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron*. 2007; 53:639–647. [PubMed: 17329205]

38. Hamdan FF, Gauthier J, Spiegelman D, Noreau A, Yang Y, Pellerin S, et al. Mutations in SYNGAP1 in autosomal nonsyndromic mental retardation. *The New England journal of medicine*. 2009; 360:599–605. [PubMed: 19196676]
39. Wall NR, De La Parra M, Callaway EM, Kreitzer AC. Differential innervation of direct- and indirect-pathway striatal projection neurons. *Neuron*. 2013; 79:347–360. [PubMed: 23810541]
40. Muhia M, Yee BK, Feldon J, Markopoulos F, Knuesel I. Disruption of hippocampus-regulated behavioural and cognitive processes by heterozygous constitutive deletion of SynGAP. *The European journal of neuroscience*. 2010; 31:529–543. [PubMed: 20105235]
41. Cruz-Martin A, Crespo M, Portera-Cailliau C. Delayed stabilization of dendritic spines in fragile X mice. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2010; 30:7793–7803. [PubMed: 20534828]
42. Pan F, Aldridge GM, Greenough WT, Gan WB. Dendritic spine instability and insensitivity to modulation by sensory experience in a mouse model of fragile X syndrome. *Proc Natl Acad Sci U S A*. 2010; 107:17768–17773. [PubMed: 20861447]
43. Petanjek Z, Judas M, Simic G, Rasin MR, Uylings HB, Rakic P, et al. Extraordinary neoteny of synaptic spines in the human prefrontal cortex. *Proc Natl Acad Sci U S A*. 2011; 108:13281–13286. [PubMed: 21788513]
44. Rakic P, Bourgeois JP, Eckenhoff MF, Zecevic N, Goldman-Rakic PS. Concurrent overproduction of synapses in diverse regions of the primate cerebral cortex. *Science*. 1986; 232:232–235. [PubMed: 3952506]
45. Carlisle HJ, Manzerra P, Marcora E, Kennedy MB. SynGAP regulates steady-state and activity-dependent phosphorylation of cofilin. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2008; 28:13673–13683. [PubMed: 19074040]

Figure 1. Pathogenic *Syngap1* mutations accelerate the rate of normal neocortical pyramidal cell growth and maturation

(A) 3D reconstructed confocal imaging sections of somatosensory cortex obtained in post-fixed brain slices in *Syngap1*^{+/-} and *wt* (PND 21 and PND 60). (B) Representative example of 3D reconstruction layer V pyramidal neurons (PND 21 and 60), scale bar = 5 μ m. (C–E) Graphs depicting the total length, intersections and volume of traced neurons * $p < .05$ within genotype group comparison, # $p < .05$ within age group comparison. (F) Scatter plot graphs showing the Correlation Index between cell body volume and the total length of the neuronal dendrites measured by Sholl analysis quantification. (G) Immunostaining for p-S6 (red) and NeuN (green) in L5 neurons in prefrontal cortex of PND 4 *Syngap1*^{+/-} ($n=5$ animals, 150 cells) mice and wild type ($n=3$ animals, 90 cells) littermates. * $p < 0.05$, Student t-Test. Values represent means; error bars indicate SEMs. Complete statistical reporting can be found in Table S1.

Figure 2. Accelerated spine formation and premature spine pruning in developing neocortical neurons in *Syngap1* hets

(A) Representative dendritic branches at different time points. (B–C) Line graphs show spine density and filopodia density trajectories over time; PND14 (*wt*-n=5, *Syngap1*^{+/-}-n=4), PND21 (*wt*-n=6, *Syngap1*^{+/-}-n=6), PND30 (*wt*-n=5, *Syngap1*^{+/-}-n=5), PND60 (*wt*-n=5, *Syngap1*^{+/-}-n=4). Values represent means; error bars indicate SEMs. * *p*<.05 within genotype group comparison, # *p*<.05 within age group comparison. Complete statistical reporting can be found in Table S2.

Figure 3. The impact of whisker deprivation on dendritic protrusion dynamics in developing *Syngap1* hets

(A) Whisker trimming experimental design. (B) Representative dendritic samples of layer 5 pyramidal cell tuft for *Syngap1*^{+/-} and *wt* and conditions (un-trimmed and trimmed) in PND 21 mice, (scale bar=5 μ m). (C) Spine density for trimmed (*wt*, n=4; *Syngap1*^{+/-}, n=4) and un-trimmed (*wt*, n=3; *Syngap1*^{+/-}, n=3) animals. Values represent means, error bars indicate SEMs. Multiple comparisons were evaluated within each independent factor. * p<.05. Complete statistical reporting can be found in Table S3.

Figure 4. Premature cellular maturation causes altered spine dynamics in *Syngap1* hets

(A) Schematic demonstrating the sequential time-lapse recording experiment. (B) Representative dendritic segments. In vivo time-lapse imaging of the same dendritic segments over 2 and 4 h in the primary somatosensory cortex (scale bar = 5 μ m). (C–E) Graphs depicting filopodia density (C), proportion of stable spines over 2 and 4 h recording (D), or proportion of filopodia that converted into spines in *Syngap1*^{+/–} (n=5) and *wt* (n=5); # *p* < .05. (F–I) Diagram showing time lapse recording experimental design at PND21–23 and PND30–32. (G–M) 2PLMS imaging session of the same dendritic segments over 2 days (scale bar = 5 μ m). (H–I) PND 21–23 dynamic properties of dendritic spines. Graphs showing the proportion of new formed spines and spine eliminated detected over 2 days each time point (H) and Turnover index (I), Student t-Test [*t*(5) = 3.223, *p* = .023] *Syngap1*^{+/–} (n=4) and *wt* (n=3) animals. (J–L) Spine formation and elimination from PND30 to PND32. Graphs showing the dynamic plasticity of spine imaged over 2 days (K) and Turnover index (L), Student t-Test [*t*(6) = 2.46, *p* = .0492] *Syngap1*^{+/–} (n=4) and *wt* (n=3) animals. Arrowheads: green = new formed; yellow = lost; red = stable spines; white = filopodia; Purple filopodia converted into spines. Values represent mean, error bars indicate SEM. LSD post-hoc test was used for multiple comparisons. Complete statistical reporting can be found in Table S4.

Figure 5. Widespread spine enlargement in *Syngap1* mutants is caused by a disrupted developmental critical period

(A) Representative dendrites of PND 14 layer 2 neurons. Cumulative frequency curves depict spine head diameter in Layer 2 and Layer 5 apical branches (located in Layer 1) in PND14 *Syngap1*^{+/-} and *wt*. (B) Representative electron microscopy (EM) images of pre- (yellow) and post- (green) synaptic area at PND14 in which post-synaptic density (PSD) area is outlined. Cumulative frequency curves depict PSD area observed in Layer 1. (C) Cumulative frequency curves depicting spine head diameter observed in Layer 5 at PND60. (D) Cumulative frequency curves showing spine head diameter of newborn (NB) spines detected during a second in vivo transcranial imaging (i.e. spines present at PND23 but not at PND21). (E) Representative dendrites of PND > 90 layer 2 neurons in *Syngap1*^{+/-} and *Syngap1*^{+/-} adult-induced haploinsufficiency model. Cumulative frequency curves show that wider spine heads persist throughout adulthood in conventional *Het* mutants, while this effect does not occur in animals with *Syngap1* haploinsufficiency induced in adulthood. Complete statistical reporting can be found in Table S5.

Figure 6. Gross anatomical long-distance synaptic connectivity appears normal in *Syngap1* mutants

(A) Experimental design. (B) Representative coronal sections in the anterior to posterior axis of long-range connectivity to the mPFC. Scale bar, 1 mm. (C) Quantitative characterization of the injection sites and total number of direct-labeled inputs to the mPFC. A.U., arbitrary unit. (D) Relative hemispheric representations of the contributions of inputs from ipsilateral and contralateral sides. (E) Relative representation of inputs clustered by cortical, subcortical and limbic brain areas. (F) Relative representation of primary and secondary cortical inputs including areas from ipsilateral motor, auditory, visual and soma- to sensory cortex and contralateral motor and somatosensory cortices. Values represent means, error bars indicate SEMs. P values indicate two-tailed unpaired t-test comparisons of *wt* versus *Syngap1*^{+/-} mice. Data are presented as a proportion of the total number of direct inputs to the mPFC between *wt* and *Syngap1*^{+/-} mice.

Figure 7. Evidence of macroscale disruptions in long-distance intracortical synaptic connectivity in *Syngap1* mutants

(A–D) Comparison of the relative strength of monosynaptic inputs to the mPFC in the ipsilateral cortical (A), contralateral cortical (B), subcortical (C) and limbic (D) areas. Values represent means, error bars indicate SEMs. P values indicate two-tailed unpaired t-test comparisons between *wt* (n=9) and *Syngap1*^{+/-} (n=9) animals. (E) Monosynaptic inputs to the mPFC labeled with RV-GFP in *wt* and *Syngap1*^{+/-} for brain areas located in cortical (Layer 5 pyramidal neurons in the somatosensory cortex), subcortical (thalamus), and limbic (amygdala) regions. Pia is indicated with a dotted line. Scale bar shown in all figures, 200 μ m. (F) Cumulative frequency curves of numbers of primary somatosensory neurons in both groups; $p<0.0001$; two-sample Kolmogorov-Smirnov test. (G) Relative

strength in connectivity represented as a measure of the location of specific brain regions. Location of the brain area biased the strength of connectivity of cortical inputs to the prefrontal cortex ($n = 19$ brain areas, $p=0.006$ for linear regression of connectivity ratio and anatomical location). The connectivity ratio was established as the differential inputs between *wt* and *Syngap1*^{+/-} relative to the number of inputs in *wt*. Grey indicates 95% confidence interval for the regression line.

Figure 8. Early, but not late, postnatal repair of a pathogenic *Syngap1* mutation prevents cognitive and behavioral abnormalities

Four groups of mice (*Syngap1*^{+/+}; CAG-CreER^{-/-}, *Syngap1*^{+/lox-stop}; CAG-CreER^{-/-}, *Syngap1*^{+/+}; CAG-CreER^{+/-}, *Syngap1*^{+/lox-stop}; CAG-CreER^{+/-}) were administered either a single injection (SQ) of tamoxifen (TM) (20 mg/kg) on PND1 or five daily injections (IP) of TM starting on PND21. Behavioral batteries consisting of elevated plus maze (EPM), open field (OF) and remote (30d) contextual fear conditioning (FC) measured by activity suppression ratio (1st 2 min of the test)/[(1st 2 min of the test)+(1st 2 min of the training)] tests, respectively, were conducted starting at 12 wks. Hippocampi were dissected and processed for immunoblot of Syngap protein levels in the four genotype groups after conclusions of the behavioral batteries. (A) Genotypic reversal of *Syngap1* is induced in *Syngap1*^{+/lox-stop}; CAG-CreER^{+/-} mice administered TM at PND1. Densitometric values were normalized to β -tubulin levels and subsequently transformed. (B–D) Performances of *Syngap1*^{+/lox-stop}; CAG-CreER^{+/-} mice are rescued relative to that of *Syngap1*^{+/+}; CAG-CreER^{+/-} mice in EPM, OF, and FC tests. (E) Genotypic reversal of *Syngap1* is induced in *Syngap1*^{+/lox-stop}; CAG-CreER^{+/-} mice administered TM at PND21. Densitometric values were normalized to β -tubulin levels and subsequently transformed. (F–H) Performances of *Syngap1*^{+/lox-stop}; CAG-CreER^{+/-} mice are not rescued relative to that of *Syngap1*^{+/+}; CAG-CreER^{+/-} mice in EPM, OF and FC tests. Multiple comparisons within each independent factor were calculated. **p*<.05, ***p*<.01, ****p*<.001, difference within Cre, #*p*<.05, ##*p*<.01, ###*p*<.001, difference within genotype. Number of animals are indicated within bar graphs. Complete statistical reporting can be found in Table S6.