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Species-Conserved *SYNGAP1* Phenotypes Associated with Neurodevelopmental Disorders

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Abstract

SYNGAP1 loss-of-function variants are causally associated with intellectual disability, severe epilepsy, autism spectrum disorder and schizophrenia. While there are hundreds of genetic risk factors for neurodevelopmental disorders (NDDs), this gene is somewhat unique because of the frequency and penetrance of loss-of-function variants found in patients combined with the range of brain disorders associated with *SYNGAP1* pathogenicity. These clinical findings indicate that *SYNGAP1* regulates fundamental neurodevelopmental processes that are necessary for brain development. Here, we describe four phenotypic domains that are controlled by *Syngap1* expression across vertebrate species. Two domains, the maturation of cognitive functions and maintenance of excitatory-inhibitory balance, are defined exclusively through a review of the current literature. Two additional domains are defined by integrating the current literature with new data indicating that *SYNGAP1*/*Syngap1* regulates innate survival behaviors and brain structure. These four phenotypic domains are commonly disrupted in NDDs, suggesting that a deeper understanding of developmental *Syngap1* functions will be generalizable to other NDDs of known or unknown etiology. Therefore, we discuss the known molecular and cellular functions of *Syngap1* and consider how these functions may contribute to the emergence of disease-relevant phenotypes. Finally, we identify major unexplored areas of *Syngap1* neurobiology and discuss how a deeper understanding of this gene may uncover general principles of NDD pathobiology.

Keywords

Syngap1; SynGAP; Intellectual Disability; Autism Spectrum Disorder; Neurodevelopment; Circuits; Synapse; Microcephaly; Cognitive impairment; Epilepsy

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SYNGAP1 gene function is important in health and disease

The advent of genomic sequencing in previously undefined patients with NDDs has demonstrated that there is a subset of autosomal genes that confer near 100% risk for developing ID, ASD and/or epilepsy (Deciphering Developmental Disorders, 2015, 2017; Epi et al., 2013). There is considerable interest in understanding the molecular and cellular mechanisms regulated by this subset of high-impact genetic risk factors (Hoischen et al., 2014; Zhu et al., 2014). In-depth study of animal models harboring pathogenic variants common to patient populations is a powerful approach for determining linkages between molecular and cellular functions of distinct disease risk factors, and perhaps more importantly, how possible convergent molecular mechanisms contribute to disease-relevant phenotypes. Through in-depth biological investigations of highly-penetrant risk genes in model systems, it may be possible to identify common molecular mechanisms that converge to influence disease-relevant phenotypes. With such knowledge, therapeutic approaches developed through modeling of one gene may be successfully applied to other NDDs of known or unknown etiology.

The NDD risk-factor, *SYNGAP1,* is a major cause of genetically-defined childhood brain disorders and an attractive candidate for in-depth investigations that span multiple model systems. The *SYNGAP1* gene has emerged as a high-risk locus for neuropsychiatric disorders that cross diagnostic barriers (Hoischen et al., 2014; Zhu et al., 2014). Indeed, causal rare variants are found in enriched populations with ID (Deciphering Developmental Disorders, 2015, 2017; Hamdan et al., 2009; Rauch et al., 2012), ASD (Hamdan et al., 2011; O'Roak et al., 2014), severe epilepsy (Carvill et al., 2013; von Stulpnagel et al., 2015) and schizophrenia (Purcell et al., 2014). Severe *de novo* variants in *SYNGAP1* resulting in haploinsufficiency lead to a defined phenotype characterized by ID with epilepsy [termed Mental Retardation- Type 5(MRD5); OMIM#603384] that may explain up to 1% of ID cases (Berryer et al., 2013; Deciphering Developmental Disorders, 2015, 2017). A recent study has found that there are no severe or obvious pathogenic *SYNGAP1* variants in more than 60,000 subjects lacking any known neuropsychiatric conditions, solidifying the notion that pathogenic *SYNGAP1* loss-of-function variants are both highly penetrant and sufficient to cause NDDs (Kosmicki et al., 2017).

SynGAP proteins, the products encoded by the *SYNGAP1*/*Syngap1* gene (Chen et al., 1998; Kim et al., 1998), lie at a critical intersection of protein signaling networks strongly linked to a spectrum of NDDs. Mechanisms that drive brain dysfunction associated with neuropsychiatric disorders intersect at excitatory synapse regulation in glutamatergic neurons. For example, the NMDA receptor signaling complex within dendritic spine synapses is enriched with proteins encoded by a high-proportion of genes with pathogenic variants linked to a range of neuropsychiatric disorders marked by cognitive impairment (Bayes et al., 2014; Volk et al., 2015). SynGAP is both a core postsynaptic density (PSD) protein and a major constituent of the NMDA receptor signaling complex (Bayes et al., 2012; Chen et al., 1998; Kim et al., 1998). SynGAP protein-protein interactions are believed to promote organization of macromolecular complexes within dendritic spines (Walkup et al., 2016; Zeng et al., 2017). Moreover, SynGAP also regulates mRNA translation

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machinery (Barnes et al., 2015; Wang et al., 2013) that regulates excitatory synapse plasticity, which is a cellular process believed to contribute to ASD pathogenesis (Huber et al., 2015; Richter et al., 2015).

Species-Aligned Phenotypic Domains Regulated by *Syngap1* Function

A relatively homogenous human phenotype emerges in patients harboring pathogenic variants causing *SYNGAP1* haploinsufficiency (Berryer et al., 2013; Mignot et al., 2016; Parker et al., 2015). Indeed, ~85% of known patients with pathogenic *SYNGAP1* variants have rare, loss-of-function variants predicted to cause reduced protein expression or function (Mignot et al., 2016). The high proportion of loss-of-function variants, combined with a relatively homogenous core phenotype in humans, indicates that *SYNGAP1* has essential natural functions during brain development.

Here, we discus known human phenotypes (Berryer et al., 2013; Mignot et al., 2016; Parker et al., 2015) in the context of conservation across several vertebrate species to highlight the fundamental importance of *SYNGAP1/Syngap1* in sculpting brain function. Studies performed across species demonstrate that this gene has retained functions throughout vertebrate evolution to promote cognitive functions, excitatory-inhibitory (E-I) balance, brain structure, and innate behavioral adaptations *(see following sub-sections)*. The alignment of *SYNGAP1/Syngap1* phenotypes across vertebrate species suggests that it controls fundamental cellular processes that promote the assembly and function of neural circuits that underlie behavior and cognition. Thus, in-depth study of *Syngap1* phenotypes in animal models may provide molecular insight into the shared pathobiology of NDDs.

Phenotype 1: Cognitive function

Reduced cognitive function is a common feature of many NDDs, including intellectual disability, ASD, epilepsy and schizophrenia. In humans, proper *SYNGAP1* expression is essential for the development of cognitive abilities. *SYNGAP1* haploinsufficiency leads to an intellectual disability disorder characterized by severe cognitive impairment (Berryer et al., 2013; Mignot et al., 2016; Parker et al., 2015). Most patients have reduced capacity for language and are non-verbal. IQ is usually <50 and patients have impaired executive functions. Animal models with disrupted *Syngap1* expression also display behaviors and neurophysiological abnormalities consistent with cognitive impairment (Guo et al., 2009; Komiyama et al., 2002; Muhia et al., 2010; Ozkan et al., 2014). For instance, *Syngap1* heterozygous KO mice (Hets) express disruptions in various forms of learning and memory, including impaired spatial learning, altered spatial working memory, weakened social memory, and deficits in remote contextual memory consolidation. These learning and memory phenotypes are highly reproducible in mice with *Syngap1* haploinsufficiency, having been observed across several laboratories using independently generated *Syngap1* knockout lines (Guo et al., 2009; Komiyama et al., 2002; Muhia et al., 2010; Ozkan et al., 2014).

Syngap1 also regulates synaptic plasticity in the same brain regions that support memory and cognition. Disruptions to this cellular process may contribute to the cognitive impairments displayed by *Syngap1* haploinsufficient animals. Indeed, reduced germline

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Syngap1 expression causes deficits in various forms of hippocampal synaptic plasticity. Long-term potentiation (LTP) is a cellular correlate of learning and memory and the synaptic strengthening that accompanies LTP is believed to be a mechanism for storing newly acquired information within neural circuits (Lynch et al., 2007; Nicoll, 2017). Alterations in this cellular process is, therefore, an attractive substrate for cognitive impairments commonly observed in NDDs. Consistent with this, many animal models of NDD risk gene pathogenicity display impaired synaptic plasticity (Araujo et al., 2017; Lauterborn et al., 2007; Lee et al., 2014; Li et al., 2016), including LTP impairments in *Syngap1* Het mice (Clement et al., 2013; Kim et al., 2003; Komiyama et al., 2002; Ozkan et al., 2014). Similar to learning and memory impairments, several laboratories using independently generated *Syngap1* Het mouse lines and distinct induction protocols have routinely observed severely impaired LTP.

LTP deficits in *Syngap1* mice are associated with alterations in NMDAR-activated Ras signaling dynamics in dendritic spines (Ozkan et al., 2014). SynGAP is a GTPase activating protein (GAP) (Chen et al., 1998; Kim et al., 1998), which inactivates several small GTPases from the Ras superfamily, including Ras, Rap1/2 and Rab5 (Krapivinsky et al., 2004; Pena et al., 2008; Tomoda et al., 2004). SynGAP content within spines is dynamically reduced in response to neuronal activation (Araki et al., 2015). This process promotes a transient elevation in Ras activation, which is known to drive AMPA receptor membrane insertion required for LTP expression (Zhu et al., 2002). Whole brain extracts (Clement et al., 2013; Kim et al., 2003; Komiyama et al., 2002) and even hippocampal dendritic spines (Ozkan et al., 2014) within *Syngap1* Het mice display basally elevated Ras signaling, which occludes further Ras activation in response to synaptic stimulation. Restoring normal SynGAP protein levels in adult *Syngap1* Het mice rescues both LTP expression and Ras-related signaling impairments (Ozkan et al., 2014). Together, these data support the view that a function of SynGAP protein within dendritic spines is to maintain low basal level Ras-ERK signaling in an unstimulated state, which may be a mechanism to maximize the signal/noise ratio of this pathway upon synaptic stimulation to promote LTP during learning.

Studies in long-term depression (LTD) are consistent with the role of SynGAP to balance Ras-ERK signaling at synapses. LTD is a unique form of synaptic plasticity that weakens neural connections in response to activity and may act to enhance computational flexibility within neural networks (Pinar et al., 2017). In contrast to the clear impact of *Syngap1* on LTP, this gene has a more complex role in LTD. For example, LTD in response to a standard input-specific and synaptically-driven induction paradigm in CA1 is normal in *Syngap1* Hets (Kim et al., 2003). Additionally, no change was observed in CA1 tissue slices from *Syngap1* Hets after LTD induced by bath application of NMDA (Carlisle et al., 2008). In contrast, mGlur5-dependent CA1 LTD was enhanced and resistant to protein synthesis inhibitors (Barnes et al., 2015). This mGlur5-dependent LTD phenotype is similar to what was reported in *Fmr1* KO mice (Osterweil et al., 2010), which is an animal model of Fragile X syndrome. In both *Syngap1* Het and *Fmr1* KO mice, pharmacological targeting of elevated Ras signaling rescued LTD phenotypes (Barnes et al., 2015). This finding demonstrates a form of molecular convergence at the synapse caused by two distinct NDD risk factors and suggests that targeting aberrant Ras signaling may improve behaviors associated with genetically distinct NDDs. Indeed, Ras-targeted therapies improve seizure phenotypes in

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Fmr1 mice (Osterweil et al., 2013) and memory in models of Neurofibromatosis (Li et al., 2005) and Noonan syndrome (Lee et al., 2014). However, the efficacy of Ras-regulating pharmacological therapies in similar animal-level phenotypes in *Syngap1* mice remain unknown. Thus, it remains an open question if elevated Ras-ERK signaling is a causal factor driving impaired cognitive function in animals with *Syngap1* heterozygosity.

Phenotype 2: Excitatory balance/Seizure

In humans, *SYNGAP1* haploinsufficiency leads to impaired excitability, as nearly all patients exhibit some form of epilepsy (Berryer et al., 2013; Mignot et al., 2016; Parker et al., 2015). Early clinical reports indicated that prevalence in *SYNGAP1*-related disorders was 70-80%. However, when only MRD5 cases are considered (i.e. cases with clear loss-of-function variants), prevalence is believed to approach 100% (Weldon et al., 2018). Seizure type and age of onset varies among patients. Some cases involve early onset and intractable neonatal seizures (Carvill et al., 2013; Okazaki et al., 2017), including drop attacks and eyelid myoclonus, while other reports involve seizure episodes occurring later in development that are responsive to standard pharmacotherapies (Berryer et al., 2013). EEG abnormalities are common in MRD5 patients. Frequent inter-ictal cortical discharges and high-frequency oscillations are common in these patients (Berryer et al., 2013; Carvill et al., 2013).

In animal models, *Syngap1* heterozygosity leads to altered neural circuit excitability, spontaneous seizure, and altered EEG waveforms. Spontaneous seizure-like behaviors are observed in a Zebrafish model of reduced *syngap1* expression (Kozol et al., 2015). Similar to what was found in MRD5 patients, generalized, bilateral spike discharges were frequently observed during EEG recordings in *Syngap1* Het mice (Ozkan et al., 2014). Consistent with altered neural excitability, the threshold for pharmacological seizure induction was lower in *Syngap1* mice (Clement et al., 2012; Guo et al., 2009). Interestingly, restricting *Syngap1* haploinsufficiency to cortical glutamatergic neurons and glia was sufficient to lower seizure threshold, while selectively repairing *Syngap1* haploinsufficiency within these brain cells protected animals from developing seizure threshold phenotypes (Ozkan et al., 2014). It is important to mention that *Syngap1* is also expressed in GABAergic neurons (Moon et al., 2008; Zhang et al., 1999). Restricting *Syngap1* haploinsufficiency to GABAergic neurons disrupts oscillatory activity within cortical networks (Berryer et al., 2016), but does not change seizure threshold (Ozkan et al., 2014).

Maintaining a balance between excitation and inhibition (E-I balance) within neural circuits is essential for normal brain function (Deneve and Machens, 2016; Haider et al., 2006). Disrupting this balance is sufficient to impair cognitive functions and behavior (Marlin et al., 2015; Yizhar et al., 2011). As such, impaired E-I balance is a substrate of NDDs (Rubenstein and Merzenich, 2003; Tatti et al., 2017). *Syngap1* controls E-I balance in the developing brain. *Syngap1* is primarily a negative regulator of excitatory synaptic structure and function in developing neurons, which may explain, in part, how this gene shapes E-I balance in the brain. Downregulation of total SynGAP protein levels within glutamatergic neurons leads to an enhancement of excitatory synaptic function (Kim et al., 2003; Rumbaugh et al., 2006; Vazquez et al., 2004), while overexpression of SynGAP leads to excitatory synapse

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depression (Rumbaugh et al., 2006), but see (McMahon et al., 2012). Consistent with a predominantly repressive role in excitatory synapses, a larger population of GluR1 positive excitatory synapses are present in hippocampal cultures prepared from Syngap1 knockout animals (Kim et al., 2003; Vazquez et al., 2004). Indeed, *Syngap1* represses excitatory synapse function though inhibition of surface AMPAR content and baseline synaptic activity (Araki et al., 2015; Rumbaugh et al., 2006; Vazquez et al., 2004; Zeng et al., 2016). *In vivo*, the repressive effect of *Syngap1* on excitatory synapse function in mice is largely limited to a critical period of postnatal development that spans the first three postnatal weeks (Clement et al., 2012; Clement et al., 2013). Reducing SynGAP protein levels after this critical period has limited effect on excitatory synaptic function in hippocampal neurons. Seizure phenotypes and other behavioral abnormalities are present during these early developmental time periods in *Syngap1* Het mice (Clement et al., 2012). Thus, haploinsufficiency of *Syngap1* may trigger circuit hyperexcitability, at least in part, through elevated excitatory synapse function, tipping the balance of circuits toward excitation. The intrinsic functions of *Syngap1* in interneurons is also consistent with circuit hyperexcitability. However, the role of *Syngap1* in these neurons appears to promote the formation of inhibitory synapses onto glutamatergic neurons (Berryer et al., 2016). Thus, loss of SynGAP protein through haploinsufficiency further disrupts E-I balance by reducing a form of synaptic inhibition. It remains unclear how E-I balance and cognitive function are related in *Syngap1* mice. There are many possible forms of E-I balance and all of them have yet to be explored experimentally. However, measures of seizure susceptibility can be dissociated from cognitive impairment in mice when *Syngap1* haploinsufficiency is induced in adulthood (Ozkan et al., 2014). This indicates at least some cellular processes mediated by SynGAP that suppress seizure are distinct from cellular processes that contribute to cognitive functions.

Syngap1 also represses spine formation and maturation. Precocious dendritic spine maturation from distinct neuronal subtypes in *Syngap1* Het mice has been reported by several groups. Dendritic spine formation occurs earlier in cultured neurons from *Syngap1* knockout mice (Vazquez et al., 2004). *In vivo* studies have also shown accelerated spine formation in cortical pyramidal neurons of *Syngap1* Het mice and spines that form in cortical neurons are larger than those found in wild type (WT) neurons (Aceti et al., 2015). *Syngap1* modulates multiple growth-related pathways regulating protein synthesis, receptor content and cytoskeletal arrangement of dendritic spines, which may underlie alterations in dendritic spine properties in *Syngap1* mutants. Reduced SynGAP expression in neurons results in increased growth promoting signaling, such as elevated Ras, mTOR and PAK activity, (Barnes et al., 2015; Carlisle et al., 2008; Komiyama et al., 2002; Rumbaugh et al., 2006; Wang et al., 2013) while decreasing activity of growth limiting pathways such as Rap1/2 and p38 MAPK (Krapivinsky et al., 2004; Rumbaugh et al., 2006).

Phenotype 3: Innate (unlearned) behavior

Recent reports suggest that MRD5 patients express impairments in innate behaviors. Specifically, parents note extreme risk-taking behaviors by their children (Weldon et al., 2018). Children do not typically require prior experience falling from especially high places to avoid such situations, as this is an innate instinct that promotes survival. However,

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climbing and jumping from high places is reported in MRD5 and is a major challenge for some caregivers. It is unclear what neural domains underlie these abnormal behaviors. ADHD, an impulse control disorder, is a common comorbid diagnosis with *SYNGAP1*-related disorders (Berryer et al., 2013; Hamdan et al., 2011). ADHD is associated with both impulsivity and risky behaviors (Dekkers et al., 2016), suggesting that behaviors observed in *SYNGAP1* patients may be related to impulsivity. However, risk-taking may be due to impairments in their ability to appropriately process visual fields, leading to alterations in the perception of height or other innately dangerous situations.

Syngap1 Het mice also display impaired innate behaviors and increased risk-taking. The elevated plus maze (EPM) can quantify risk assessment in mice, as it challenges animals to balance the drive to explore a novel space with an innate fear of open spaces (Carobrez and Bertoglio, 2005). It is thought that mice generally avoid open arms of the maze because they prefer the safety of vertical walls. Anxiogenic compounds decrease open arm time (OAT) and anxiolytic compounds increase it, indicating that OAT reflects anxiety-like levels elicited from innate fear of open spaces (Walf and Frye, 2007). *Syngap1* Het mice have increased OAT in EPM (Berryer et al., 2016; Guo et al., 2009). This phenotype is observed in different labs using distinct *Syngap1* lines, indicating that it is highly reproducible. It remains unclear if increased OAT reflects reduced anxiety or increased exploratory drive, or both. *Syngap1* mice exhibit increased locomotion in the open field test, but not in a Pavlovian fear conditioning box (Guo et al., 2009). Some groups have found no changes in horizontal locomotion in EPM (Guo et al., 2009), while others have found the opposite (Berryer et al., 2016). *Syngap1* mouse activity in the homecage remains an open question.

Changes in OAT in EPM may reflect impaired learning (Bertoglio et al., 2006), suggesting that *Syngap1* phenotypes in this test are unrelated to innate fear of open spaces. To confirm that increased OAT occurs through impaired innate processes rather than learning, OAT should be calculated for each one-minute interval of the test (Jurgenson et al., 2010). We reasoned that if deficits in OAT in *Syngap1* mice are observed in the first minute, then altered innate processes are likely disrupted. To do this, we performed a re-analysis of EPM data previously published from our lab (Ozkan et al., 2014). In this re-analysis, we calculated open arm entries (OAE) and OAT at each minute of the test in EMX1-*Syngap1* Hets, which have heterozygosity of the *Syngap1* gene restricted to forebrain excitatory neurons and some glia. We chose this experiment because we observed an overall cumulative increase in OAT and this study had very high statistical power (~n=30 per genotype). Here, we found that *Syngap1* mutant mice had significant overall differences in open arm entries during the duration of the test, but not during the first one-minute bin (Fig. 1A). In contrast, OAT was significantly different over all binned periods (Fig. 1B), including the initial minute of testing, indicating that these *Syngap1* mice were innately less fearful of the open arm. This idea was strengthened by observing the aggregate location of animals within the maze during the first minute of the test (Fig. 1C). WT mice entered the open arm, but then quickly moved back to the center. In contrast, *Syngap1* Hets were equally likely to enter the open arm, but were more likely to thoroughly explore it before returning to the center. This behavior appeared to be the major factor driving increased OAT between the genotypes in the first minute.

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The EPM findings in *Syngap1* Het mice suggest that they exhibit a form of reduced innate fear leading to an increase in risk-taking. To further investigate this idea, we utilized the cliff avoidance task (Matsuoka et al., 2005; Yokota et al., 2013) to determine if risky behaviors associated with heights observed in MRD5 patients was evolutionarily conserved in mice with *Syngap1* haploinsufficiency. We reasoned that if *Syngap1* Het mice were less afraid of heights, then they would be more likely to venture off the elevated platform. We performed two versions of the test. In the first, we reproduced the experimental conditions of Matsuoka and colleagues, and then quantified height-related risk-taking in adult (>PND60) conventional *Syngap1* Het mice. We found that *Syngap1* Het mice were much more likely to leave the elevated platform (Fig. 2A), consistent with the idea that these mice were less fearful of the apparatus. In the second study, we were interested in assessing innate fear of heights in developing *Syngap1* Het mice. Assessing behaviors in young mice (PND21) required modifications of the task (Fig. 2B). This modified apparatus allowed us to measure two distinct behaviors, "edge departures" and "full departures" (Fig. 2C). We found that *Syngap1* Het mice ventured over the sides of the platform more often than controls, as measured by increases in edge departures (Fig. 2C-E). *Syngap1* Het mice were also more likely to completely leave the platform compared to *Syngap1* WT mice (Fig. 2E). It is unlikely that severely impaired vision is related to this *Syngap1* Het phenotype. *Syngap1* Het mice are able to find the visible platform during Morris Water Maze training (Komiyama et al., 2002) and they have normal object recognition memory (Muhia et al., 2010). Along with increased OAT in EPM, these data support the idea that *Syngap1* Het mice are more likely to engage in ethologically-relevant risk-taking behaviors. Prior experience was not necessary to elicit these behaviors in *Syngap1* Het mice, indicating they reflect impaired innate, rather than learned, neural functions.

While the data on impaired innate survival behaviors in MRD5 patients remain suggestive (Weldon et al., 2018), the data obtained in animal models support the idea that the *Syngap1* gene regulates the function of circuits that control innate survival behaviors. More clinical research in MRD5 is therefore warranted to determine the extent of risk-taking behaviors in these patients. Studies in animal models will be necessary to understand the neural basis of impaired risk-taking. It would be useful if clinical studies assessed depth perception and other forms of visual processing to understand if these behaviors are cognitive or affective in nature. Moreover, studies in *Syngap1* animal models will enable in-depth neurobiological studies, such as visualization and possible manipulation of circuits that are activated during execution of behaviors that elicit innate fear of open spaces or height.

Phenotype 4: Gross/Fine brain morphology

Some MRD5 patients have mild microcephaly (Parker et al., 2015; Prchalova et al., 2017), or a reduction in head/brain size. *Syngap1* heterozygous mice express a phenotype consistent with this. Small animal MRI measurements revealed a reduction in total absolute brain volume of Het mice compared to WT at PND90 (Fig. 3A-B). The effect on brain size was largely driven by males within this large cohort (Fig. 3C; Supplemental Tables 1-3). We did not measure body weight in these animals. Indeed, it is possible that a change in body weight is a confounding variable in these studies. However, brain size and body weight can vary independently from each other in mice (Ellegood et al., 2013). Moreover, at a regional

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level, only ~51% of the 182 anatomically distinct regions were significantly different between male *Syngap1* WT and Hets (Supplemental Table 2). This indicates that decreased total volume is not driven by global downscaling of the brain. Thus, if body size is a confound, it is unlikely to be the only factor driving a change in brain size. More studies are needed to determine mechanisms driving the reduced brain size. It is notable that many cortical areas did not reach significance, including the somatosensory cortex (Supplemental Tables 1-2). This result agrees with past literature that reported no changes in somatosensory cortex lamination in developing *Syngap1* Hets (Barnett et al., 2006). However, the volume of many cortical areas related to the visual system were changed in *Syngap1* Hets. This finding is interesting in the context of risk-taking phenotypes related to innate fear of open spaces or height (Figs. 1-2). It will be important to probe circuit function in visual processing areas of *Syngap1* animal models and to understand how these areas interact with circuits that guide impulse control, decision making, and/or emotional valance.

In addition to changes in brain volume, prior studies in *Syngap1* Het mice demonstrated that there are more fine-grained alterations in brain structures. *Syngap1* Het mice demonstrated an impairment in cellular barrel segregation in the somatosensory cortex (Barnett et al., 2006). *Syngap1* heterozygosity results in alterations in dendritic morphogenesis. L5 pyramidal neuron dendritic morphogenesis is accelerated during postnatal development in *Syngap1* Hets (Aceti et al., 2015). This same dendritic phenotype was not observed in adult animals, indicating that *Syngap1* represses the developmental maturation rate of dendritic morphogenesis in this neuronal subtype. A similar phenotype was also observed in deep neurons of the developing prefrontal cortex. Because dendrites are a substrate for synapse formation, altered rates of dendritic arborization are suggestive of impaired assembly of circuits during developmental critical periods. Interestingly, cell type-independent monosynaptic tracing of presynaptic inputs into the prefrontal cortex of *Syngap1* Het mice revealed very few changes in anatomical long-range connectivity (Aceti et al., 2015). These findings suggest that altered patterns of circuit assembly in *Syngap1* Het mice may be restricted to specific cell types or circuits. More sophisticated anatomical and functional tracing studies are required to determine how changes in dendritic morphogenesis translate into altered circuit assembly. Future studies will also be necessary to understand possible links between reduced brain volume and altered dendritic morphogenesis in *Syngap1* Het mice.

Major Unexplored Areas of *Syngap1* Neurobiology

A major unexplored area of *Syngap1* biology relates to the molecular and/or cellular mechanisms that connect gene function to species-conserved phenotypes. Do diverse phenotypes driven by *Syngap1* gene function arise through a single function of SynGAP protein? Alternatively, the *Syngap1* gene may produce a range of protein functions, and these distinct functions may contribute to the range of disease-relevant phenotypes. In this latter example, termed the “single-gene multiple-hit” model, *Syngap1* controls multiple and distinct molecular and/or cellular processes that converge in either simple or complex ways to produce the wide range of phenotypes observed in animal models. In its most simplistic form, this hypothesis predicts that individual animal-level phenotypes can be assigned to a precise molecular function of SynGAP. A more complex manifestation of this hypothesis is

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that several distinct molecular functions of SynGAP converge to produce individual phenotypes. These diverse SynGAP functions may interact in a spatial and/or temporal manner to regulate phenotypes. This hypothesis is useful because it provides a framework for drawing links between defined molecular functions driven by this gene (and resultant proteins) to emergent animal-level phenotypes. Research that establishes these links would be expected to further our basic understanding of this critical neurodevelopmental gene. Moreover, identifying these linkages would be therapeutically relevant because many phenotypes are commonly associated with NDDs.

SynGAP is best known as a modulator of glutamatergic synapses through regulation of GTPase signaling associated with the PSD (Araki et al., 2015; Chen et al., 1998; Kim et al., 1998; Ozkan et al., 2014; Rumbaugh et al., 2006; Vazquez et al., 2004). While this function of SynGAP is well accepted, it remains unclear how this, or any, function of SynGAP contributes to species-conserved phenotypes observed in response to *Syngap1* loss-of-function. This uncertainty exists because animal models targeting the *Syngap1* gene have, thus far, been limited to null mutations. The incorporation of more subtle variants into *Syngap1* that disrupt known biological functions of the protein have yet to be carried out. This approach would be useful because it would enable a better understanding of how specific molecular functions of *Syngap1* contribute to various species-aligned and disease-relevant phenotypes. The dissociation of key phenotypes from each other in animals expressing more subtle variants would promote a better understanding of how diverse molecular and/or cellular functions of SynGAP proteins contribute to brain function.

Syngap1 gene structure is well understood and the protein has been extensively studied at the biochemical level (Fig. 4A-B). Thus, there are clear strategies to disrupt specific features of the protein. A principle cellular function of SynGAP is regulation of Ras-like GTPases (Chen et al., 1998; Kim et al., 1998). This function arises through activity of the GAP domain, which stimulates the conversion of GTP-to-GDP in small GTPases. GAP function in SynGAP is unique, as it has been shown to also directly regulate Rap1/2 (Krapivinsky et al., 2004) and Rab GTPases (Tomoda et al., 2004). RapGAP activity requires an interaction between the GAP and C2 domains (Pena et al., 2008). The ability to regulate multiple GTPases enables the protein to have a powerful control over different signaling pathways within brain cells (Kennedy et al., 2005). SynGAP is heavily phosphorylated and this process regulates the activity of the GAP domain. CAMKII phosphorylation drives Ras-dominant regulation, while CDK5 phosphorylation biases activity toward inactivation of RAP1 (Walkup et al., 2015). This type of regulation is likely biologically meaningful because Ras and Rap are known to drive opposing cellular process that promote circuit assembly and synaptic plasticity (Zhu et al., 2002). SynGAP also regulates axon growth through regulation of Rab5 (Tomoda et al., 2004). Although it has only been shown in cerebellar granule neurons, SynGAP regulation of Rab5 constitutes a strong candidate for contribution to MRD5 disease mechanisms, due to its influence on early endosome formation. Disruptions in early endosome formation is associated with various brain disorders (Ouyang et al., 2013). Furthermore, this property of SynGAP is unrelated to dendritic spine biology, supporting the “multiple-hit” model of *Syngap1* gene function. Animal models with engineered variants that selectively target activity at individual

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GTPases would help to identify signaling pathways that drive disease-relevant *Syngap1* phenotypes.

It has been suggested that SynGAP performs a structural role in the PSD that is independent of GTPase activity (Dosemeci et al., 2016; Walkup et al., 2016). Certain splice variants of SynGAP contain a C-terminal motif that enables binding to PDZ-containing proteins (Chen et al., 1998; Kim et al., 1998), such as PSD95 and SAP102, which are scaffolding proteins that organize macromolecular structures within glutamatergic synapses that enable plasticity supporting cognitive functions (Feng and Zhang, 2009). Due to its abundance, SynGAP is a dominant binding partner of major PDZ domain-containing proteins in the synapse and may therefore act as a PDZ-blocking molecule within the PSD (Dosemeci et al., 2016; Walkup et al., 2016; Zeng et al., 2016). In the context of this work, this idea is relevant because it implies that there are structural/scaffolding functions of SynGAP protein that are independent from GAP domain activity, which is consistent with our proposed "multiple-hit" hypothesis of *Syngap1* gene function. The so-called "slot" hypothesis (Walkup et al., 2016) posits that SynGAP occupies PDZ protein-containing slots in the PSD, and SynGAP occupation of these slots is essential for structural and functional organization of this macromolecular complex. According to this hypothesis, reduced SynGAP within the synapse, which occurs during genetic haploinsufficiency or during LTP-like stimuli (Araki et al., 2015), may enhance synaptic function in a GAP-independent manner. It is argued that when SynGAP is at low abundance in the PSD, more PDZ binding "slots" become available, which become occupied by other proteins with PDZ binding domains. Some of these proteins may stabilize and/or recruit AMPARs at the synapse, leading to an increase in synaptic strength. This hypothesis is supported by data demonstrating that AMPAR stabilizing proteins, such as TARPs, are increased in the PSD of *Syngap1* Het mice (Walkup et al., 2016). The general hypothesis that SynGAP acts as a PDZ-blocking molecule is also supported by the recent finding that SynGAP prevents Tau accumulation in the PSD (Bi et al., 2017). This finding broadens the relative importance of the slot hypothesis because SynGAP-mediated prevention of synaptic Tau accumulation is neuroprotective. Multilevel analysis (i.e. biochemical, cell-biological, electrophysiological, and behavioral approaches) in an animal model engineered to have impaired GTPase activity, combined with similar studies in a separate model engineered to selectively disrupt SynGAP-PDZ binding, could in theory test critical aspects of slot hypothesis, while also testing the relative importance of GAP activity on key *Syngap1* phenotypes.

Genetic features of *Syngap1* also support the "multiple-hit" model. *Syngap1* mRNAs are heavily spliced (Fig. 4A-B), leading to at least twelve distinct SynGAP protein isoforms (McMahon et al., 2012). Alternative splicing of the same locus is a common mechanism for generating protein isoforms with completely unique molecular functions (Yang et al., 2016). Thus, the genetic complexity of *Syngap1* may yield protein isoforms with distinct functions. C-terminal splicing is of particular importance because it is known to regulate molecular and cellular functions of SynGAP. There are four possible SynGAP C-termini, Alpha1, Alpha2, Beta and Gamma (Li et al., 2001; McMahon et al., 2012), which arise from the alternative splicing of the final three exons of the *Syngap1* gene. The Alpha1 spliced event, the only C-terminus studied in any detail, gives rise to a protein with a C-terminal PDZ ligand that regulates binding to PSD proteins (Kim et al., 1998), such as PSD95 and SAP102. This

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binding is believed to promote PSD organization and may regulate the threshold for activity-dependent AMPA receptor insertion required for LTP (Walkup et al., 2016; Zeng et al., 2017; Zeng et al., 2016).

The most current framework for understanding how *Syngap1* functions in the brain is heavily dominated by PDZ-dependent molecular functions of SynGAP proteins (Walkup et al., 2016; Zeng et al., 2017). However, there is evidence that the other C-terminal sequences also regulate SynGAP protein function. For instance, alternative splicing of Exon 21 encodes the Alpha2 C-terminus (Fig. 4A), which reverses the function of SynGAP at glutamatergic synapses. Inclusion of this C-tail in SynGAP promotes synaptic function (McMahon et al., 2012), rather than represses it, as in the case when the Alpha1 C-terminus is present (Rumbaugh et al., 2006). This function of Alpha2 appears to refine SynGAP function at the synapse rather than to drive key synaptic phenotypes. Heterozygosity of *Syngap1* leads to enhanced synaptic function in hippocampal neurons on a global scale in vitro (Rumbaugh et al., 2006), as well as during developmental epochs *in vivo* (Clement et al., 2012; Clement et al., 2013), indicating that non-Alpha2 mechanisms are dominant with respect to synaptic function. However, Alpha2 inclusion within SynGAP molecules may act to balance functions at selected synapses. While this possible role of Alpha2 remains untested, it could arise through synapse-specific regulation of the Alpha1/Alpha2 ratio. Alpha2 transcripts appear to contain unique regulatory elements in the 3′ untranslated region that lead to selective regulation of its expression levels (Yokoi et al., 2017).

The signaling mechanisms that underlie the Alpha isoform-specific differential function of SynGAP at synapses remain unknown. Indeed, it is unclear if the protein sequence of Alpha2 leads to differential signaling within the synapse. This is presumably the case because GTPase function of SynGAP is known to regulate synapse function (Rumbaugh et al., 2006) and Alpha2 leads to a distinct effect on synapse regulation, relative to Alpha1 (McMahon et al., 2012). Moreover, it remains unknown how each of the Alpha isoforms contribute to species-conserved *Syngap1* phenotypes. Creating animal models that selectively target Alpha1 versus Alpha2 function or expression at the organismal level, combined with targeted cell biological studies that probe the molecular functions of each isoform, may help to clarify the roles of these two important SynGAP variants as they related to key disease-relevant phenotypes. It would be of interest to understand how loss of PDZ binding of SynGAP, which is encoded through Alpha1 splicing, impacts species-conserved *Syngap1* phenotypes. It is assumed that this interaction is crucial for maintaining E/I balance *in vivo* and for promoting cognitive functions (Walkup et al., 2016; Zeng et al., 2017), though these assumptions have not been tested directly. In addition, it would be of interest to understand how the other major *Syngap1* phenotypes, such as brain structure and innate behavioral regulation, are impacted by PDZ-dependent functions of SynGAP.

The clear impact that Alpha1/2 spliced motifs have on SynGAP regulation at the synapse illustrates the importance of splicing on protein function. This would suggest that the other two remaining C-terminal variants may also act to shape SynGAP function. Beta and Gamma isoforms have received the least attention, and there is very little known about how each isoform regulates cellular signaling, neuronal function or relevant animal-level phenotypes. SynGAP Gamma-containing mRNAs, which arise through selective inclusion

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of Exon 20 (Fig. 4A), can be found in publically available databases containing rodent and human cDNA sequences. However, unlike the other three C-terminal sequences (Kim et al., 1998; McMahon et al., 2012; Moon et al., 2008), there is no specific antibody for these isoforms. Thus, there is essentially nothing known about Gamma isoforms other than that they are likely expressed in mammals. SynGAP-Beta, which arises from alternative splicing of Exon19 (Fig. 4A), has been studied in neurons (Li et al., 2001; Moon et al., 2008). It was found to have a unique subcellular expression pattern in neurons compared to other isoforms, suggesting that it may have unique functions compared to the Alpha isoforms. As with SynGAP-Gamma, there is no information on how Beta isoforms regulate neuronal signaling and synapse function. It will be of considerable interest to determine how their expression contributes to disease-relevant phenotypes. Importantly, these isoforms can be selectively disrupted using standard genetic engineering approaches, which would provide an opportunity to explore potential SynGAP diversity through isoform-specific functions.

Methods

Animals

Syngap1+/- (*Syngap1* Het mice) constitutive knockout mice (Kim et al., 2003) were generated and maintained as previously described (Clement et al., 2012). Males and females were used for all studies. Animals were >PND60 for risk-taking studies and >PND90 for MRI studies.

Platform Departures Test of Risk-taking

The risk-taking/impulsivity test in adult *Syngap1* mice was carried out using an apparatus and protocol that was similar to published reports (Yokota et al., 2013, Matsuoka et al., 2005). For young (~PND21) mice, several modifications were made to the published apparatus and protocol. C57BL/6 wild type and mutant mice were weaned the day before the test was conducted. Each mouse, two at a time, was placed on an overturned stainless-steel wire Galaxy pencil/utility cup (7.8cm bottom diameter (“platform”), 10.8cm high, Yang, Silverman, Crawley, 2011) situated on a black granite bench top in a vivarium procedure room within demarcated areas (30×30×27 cm) using box cardboard dividers to separate the two mice from each other and two other sides on the bench with a front open side where a notebook web camera videotaped each 10min session. White duct tape was affixed to the black foam bottom of both cups for easier cleaning between trials and better contrasts with black mice. Each mouse's activities were hand scored thereafter. The number of “edge departures” were the principle measure. This behavior was defined by the mouse forepaws grasping the platform edge or wire mesh that made up the vertical walls exhibiting a forward stance over the edge. The latency to first full departure was also scored. Platform departures occurred when all four paws were the top platform and on the vertical bars. Pilot tests using other cups or beakers of different heights (unpublished observations from our lab) did not alter scoring parameter measures. However, we preferred the wire sided cups because we could measure intended edge departures as well as climbing tendencies not possible with a glass beaker eliminating most unintended loss-of-balance movements from the platform. Experimenters and scorers were blind to group identities with several individual scorers attaining similar parameter measurements.

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Small Animal MRI

A multi-channel 7.0 Tesla MRI scanner (Varian Inc., Palo Alto, CA) was used to image the brains within their skulls. Sixteen custom-built solenoid coils were used to image the brains in parallel (Bock et al., 2005).

Anatomical Scan—To detect volumetric changes, we used the following parameters for the MRI scan: T2- weighted, 3-D fast spin-echo sequence, with a cylindrical acquisition of k-space, a TR of 350 ms, and TEs of 12 ms per echo for 6 echoes, field-of-view equaled to 20 × 20 × 25 mm3 and matrix size equaled to 504 × 504 × 630. Our parameters output an image with 0.040 mm isotropic voxels (Spencer Noakes et al., 2017). The total imaging time was 14 hours.

MRI Registration and Analysis—To visualize and compare any changes in the mouse brains the images are linearly (6 followed by 12 parameter) and non-linearly registered together. Registrations were performed with a combination of mni_autoreg tools and ANTS (advanced normalization tools). All scans are then resampled with the appropriate transform and averaged to create a population atlas representing the average anatomy of the study sample. The result of the registration is to have all images deformed into alignment with each other in an unbiased fashion. For the volume measurements, this allows for the analysis of the deformations needed to take each individual mouse's anatomy into this final atlas space, the goal being to model how the deformation fields relate to genotype. The jacobian determinants of the deformation fields are then calculated as measures of volume at each voxel. Significant volume differences can then be calculated by warping a pre-existing classified MRI atlas onto the population atlas, which allows for the volume of 182 different segmented structures encompassing cortical lobes, large white matter structures (i.e. corpus callosum), ventricles, cerebellum, brain stem, and olfactory bulbs (Dorr et al., 2008; Richards et al., 2011; Steadman et al., 2014; Ullmann et al., 2013) to be assessed in all brains. Further, these measurements can be examined on a voxel-wise basis to localize the differences found within regions or across the brain. Multiple comparisons in this study were controlled for using the False Discovery Rate (Genovese et al., 2002).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- the current understanding of *Syngap1* neurobiology is reviewed
- four species-conserved *Syngap1* phenotypes are described
- new findings are presented that strengthen these phenotypes
- molecular and cellular functions of *Syngap1* are discussed in a phenotypic context
- major unexplored areas of *Syngap1* neurobiology are discussed

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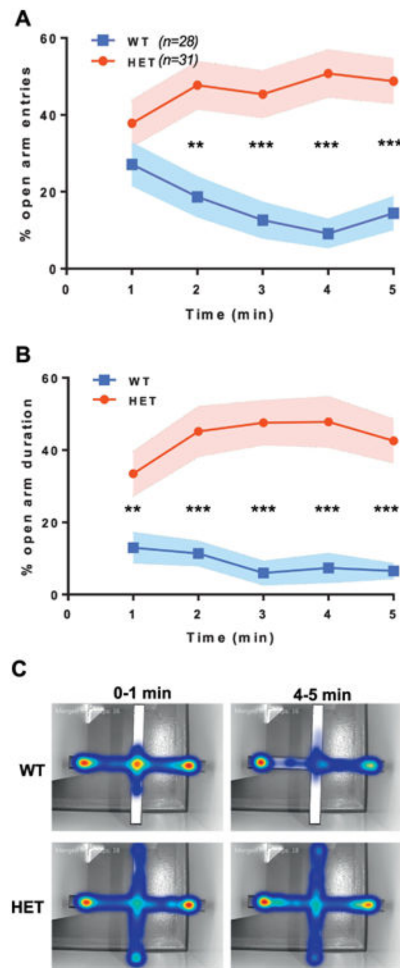

Figure 1. Evidence that *Syngap1* heterozygosity disrupts innate fear within the Elevated Plus Maze

The data shown here reflect a re-analysis of an EPM experiment previously published by our group (Ozkan et al., 2014). Mice were run in a standard 5 min elevated plus maze paradigm. Percent open arm entries were binned for each minute and calculated. **A)** Open arm entries for EMX1-WT and EMX1-Syngap1 Hets over five one-minute bins. Large overall differences in open arm entries between genotypes ($F(1,57)=29.511$, $p=1.197E-6$); RMANOVA. Posthoc comparisons show all bins, except for the first, were significantly different from each other; $^{**}p<0.01$, $^{***}p<0.001$. **B)** Open arm duration for EMX1-Syngap1 Hets over five one-minute bins. Large overall differences in open arm entries between genotypes ($F(1,57)=44.577$, $p=1.098E-8$); RMANOVA. Posthoc comparisons show all bins, including the first, were significantly different from each other; $^{**}p<0.01$, $^{***}p<0.001$. **C)** Collapsed, integrated heat maps for EMX1-WT and EMX1-Syngap1 Hets showing the cumulative location of animals at two times during the test.

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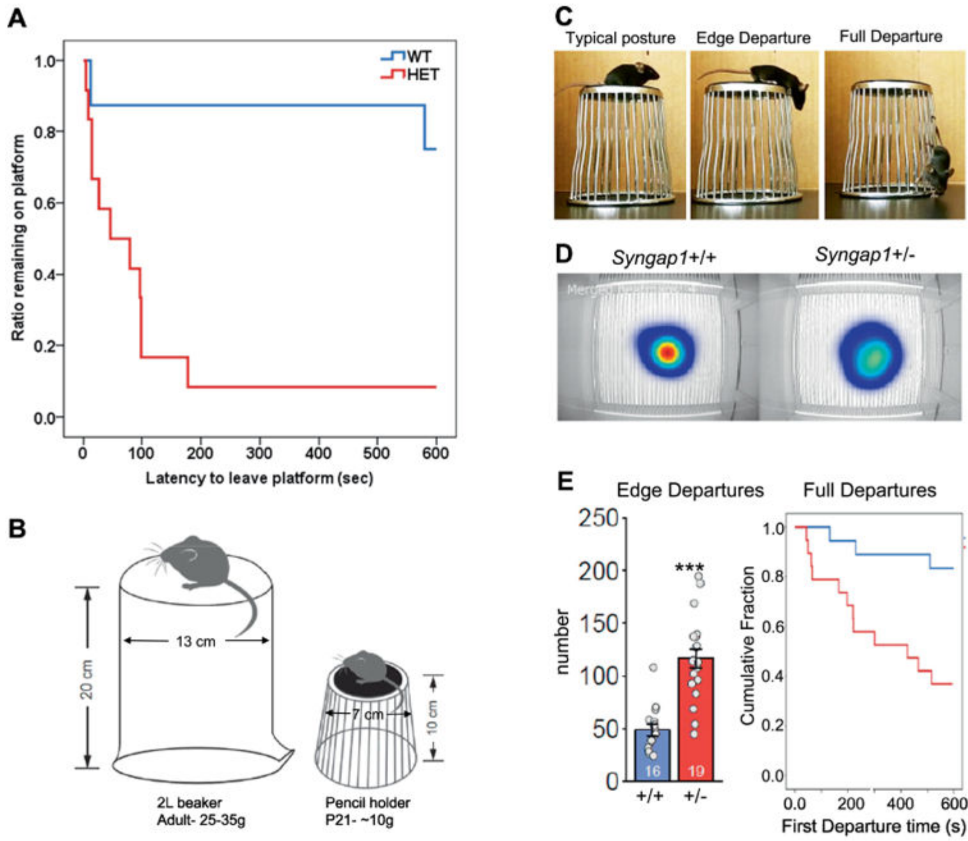

Figure 2. Modeling height-related risk-taking in *Syngap1* mice

A) Cliff avoidance task in adult conventional *Syngap1* animals. We calculated the fraction of animals remaining on the platform over the course of a ten-minute testing period; chi square, $X^2(1)= 9.061$, $p<0.003$. **B)** Modifications to cliff avoidance apparatus made for testing young mice. **C)** Risk-taking platform with quantifiable risk-taking postures in PND21 mice. **D)** Top-view heat map showing cumulative location of nose around the platform for each genotype. **E)** Quantification of risk-taking behaviors. *left,* number of edge departures during the test; t-test, $p<0.001$. *right,* cumulative fraction remaining on the apparatus as animals make their first full departure from the platform; chi square, $X^2(1)= 8.59$, $p<0.005$.

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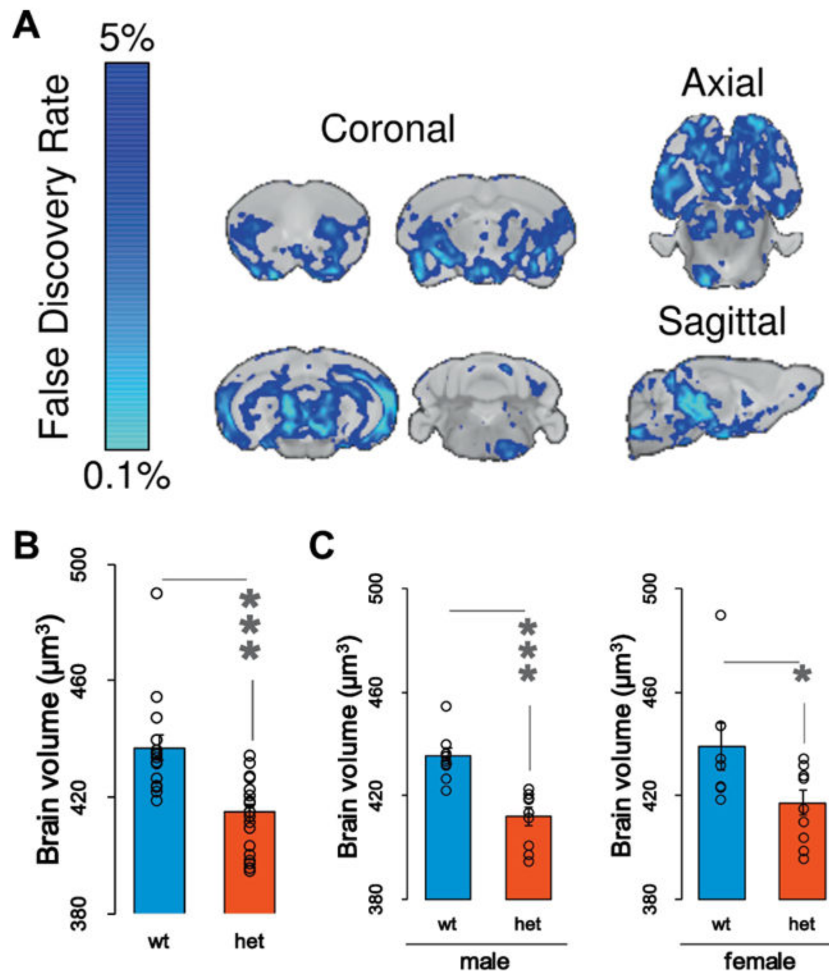

Figure 3. Brain volume measurements in *Syngap1* mice

A) Various MRI sections depicting absolute changes in brain volume in *Syngap1* Het mice (n=18) relative to *Syngap1* WT (n=16) at PND90. Blue color represents significance of reduced volume across brain areas thresholded at 5% FDR. **B)** Total brain volume in WT and Het mice. Bar graphs and relative data points depicting the absolute volume change in both mutants and wildtypes [t(27.680) = 4.319 p = .0002]; **C)** Evaluation of total brain volume analyzing male and female separately. Male [t(16) = 4.977, p = .00014]; Female [t(14) = 2.251, p =.041]

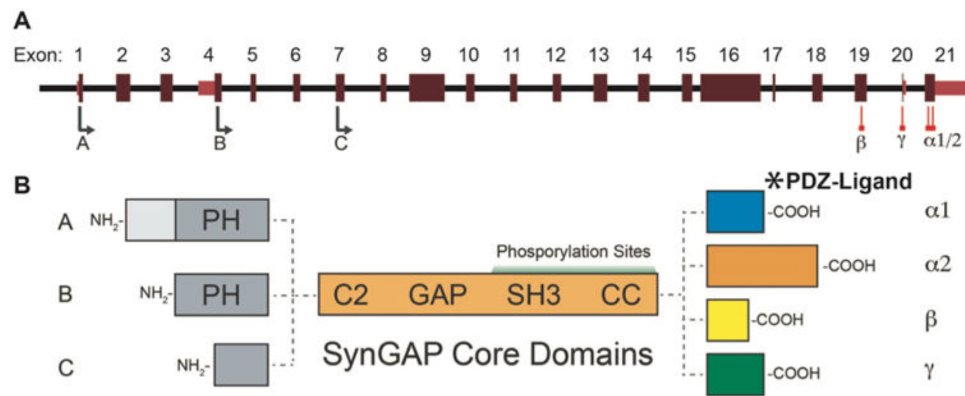

Figure 4. *Syngap1* alternative splicing and resulting isoforms

A) Map showing alternative use of exons in N and C-terminal isoforms. N-terminal variants are constituted via use of different start codons in exon 1, 4 or 7. Exon 4 is present only in B-SynGAP. C-terminal isoforms originate from use of different splice acceptors in exon 19 and 21. Exon 20 is included only in γ isoform. **B)** Schematics of SynGAP isoforms & protein domains. A and B isoforms include full pleckstrin homology (PH) domain. In C-SynGAP, this domain is truncated. Core regions common to all isoforms include C2, GAP, Src Homology 3 (SH3) and coiled-coil (CC) domains. Multiple phosphorylation sites are present downstream of the GAP domain. In the C-terminus, α1 isoforms contain a type-1 PDZ ligand. Structure/function relationships of α2, β, γ isoforms remain largely unknown

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