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Distinct Pathogenic Genes Causing Intellectual Disability and Autism Exhibit a Common Neuronal Network Hyperactivity Phenotype

Graphical Abstract

Highlights
- KSS gene deficiency leads to hyperactive neuronal network functioning
- EHMT1-deficient neurons show altered excitatory-inhibitory balance
- KSS gene deficiency leads to increased neuronal excitability
- KSS target genes converge on neuronal excitability and synaptic function regulation

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In Brief
Frega et al. show that mutations in functionally distinct genes leading to Kleefstra syndrome converge at the molecular, cellular, and neuronal network levels. KSS gene deficiency leads to hyperactive neuronal network communication and altered excitatory-inhibitory balance. Common biological pathways related to ion-channel expression and synaptic communication underlie this functional convergence.
Distinct Pathogenic Genes Causing Intellectual Disability and Autism Exhibit a Common Neuronal Network Hyperactivity Phenotype

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SUMMARY

Pathogenic mutations in either one of the epigenetic modifiers EHMT1, MBD5, MLL3, or SMARCB1 have been identified to be causative for Kleefstra syndrome spectrum (KSS), a neurodevelopmental disorder with clinical features of both intellectual disability (ID) and autism spectrum disorder (ASD). To understand how these variants lead to the phenotypic convergence in KSS, we employ a loss-of-function approach to assess neuronal network development at the molecular, single-cell, and network activity level. KSS-gene-deficient neuronal networks all develop into hyperactive networks with altered network organization and excitatory-inhibitory balance. Interestingly, even though transcriptional data reveal distinct regulatory mechanisms, KSS target genes share similar functions in regulating neuronal excitability and synaptic function, several of which are associated with ID and ASD. Our results show that KSS genes mainly converge at the level of neuronal network communication, providing insights into the pathophysiology of KSS and phenotypically congruent disorders.

INTRODUCTION

Neurodevelopmental disorders (NDDs), including intellectual disability (ID) and autism spectrum disorder (ASD), are genetically and phenotypically heterogeneous. Despite the identification of Mendelian mutations in more than 800 genes that give rise to some type of NDD (Kochinke et al., 2016), our understanding of the key molecular players and mechanisms is still fragmented and needs conceptual advances. Furthermore, how mutations and DNA variants in distinct genes can, in some cases, lead to similar clinical phenotypes, is poorly understood (Kleefstra et al., 2014; Vissers et al., 2016). Recent studies have proposed that the genetic heterogeneity among NDDs is buffered at the level of molecular pathways where the effects of many different DNA variants converge (Chen et al., 2014; Gandal et al., 2018; Voineagu et al., 2011). However, we still have to resolve the exact nature of such converging pathways and how disruptions thereof give rise to commonality in terms of brain dysfunction and pathology.

In recent years, evidence has accumulated that synaptic processes and neuronal gene transcription through epigenetic modification of chromatin structure plays an important role in both normal cognitive processes and the etiology of NDDs (Gabriele et al., 2018; Kleefstra et al., 2014). Kleefstra syndrome (OMIM#610253) is an example of a rare NDD comprising ID, ASD, hypotonia, and dysmorphic features as major hallmark phenotypes (Kleefstra et al., 2006, 2009; Vermeulen et al., 2017). The canonical disease is caused by de novo loss-of-function mutations in the gene EHMT1 (Euchromatin Histone Lysine Methyltransferase 1, also known as GLP) (Kleefstra et al., 2006). Interestingly, however, we previously found de novo het erozygous mutations (all with predicted loss of function) in four other chromatin modifiers, i.e., SMARCB1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily B member 1; missense mutation), MLL3 (Histone-lysine N-methyltransferase 2C, or KMT2C; missense mutation), NR1I3 (Nuclear receptor, subfamily 1 group I member 3; missense mutation), and MBD5 (Methyl-CpG-binding domain protein 5; frameshift mutation), result in core clinical features highly reminiscent of Kleefstra syndrome and that we collectively refer to as the Kleefstra syndrome spectrum (KSS) (Kleefstra et al., 2012). The corresponding proteins are directly or indirectly involved in epigenetic regulation of gene expression and are also associated with other disorders that share certain cognitive features with KSS. For example, MBD5 deletions are associated with the chromosome 2q23.1 deletion syndrome resembling Smith-Magenis syndrome (Bonnet et al., 2013; Talkowski et al., 2011), missense mutations in SMARCB1 are associated with Coffin-Siris syndrome (Diets et al., 2019; Gossai et al., 2015),
and intragenic EHMT1 duplications are associated with schizophrenia (Kirov et al., 2012; Talkowski et al., 2012).

EHMT1 cooperates with its mammalian paralog EHMT2/G9a and exhibits enzymatic activity for histone 3 lysine 9 mono-and di-methylation (H3K9me1 and H3K9me2, respectively), which is known to promote a heterochromatiner structure and hence gene repression (Tachibana et al., 2002; Yamada et al., 2011). Loss of EHMT1 function in mice and Drosophila leads to learning and memory impairments (Balemans et al., 2010, 2013; Benevento et al., 2017; Iacono et al., 2018; Kramer et al., 2011; Schaefer et al., 2009). Additionally, Ehmt1 <sup>-/-</sup> mice recapitulate autistic-like features that are seen in patients with Kleefstra syndrome (Balemans et al., 2010). At the cellular level, these mice show a significant reduction in dendritic arborisation and the number of mature spines in CA1 pyramidal neurons (Balemans et al., 2013), together with a reduced ability to establish synaptic scaling, a specific form of homeostatic plasticity (Benevento et al., 2016). Furthermore, EHMT1 deficiency alters cortical neuronal network activity during development (Martens et al., 2016), but the underlying mechanisms remain to be determined.

Each of the KSS gene products functions to epigenetically regulate transcription, while protein-protein interaction data and genetic interaction studies in Drosophila indicate that the corresponding proteins are engaged in shared biological processes (Kleefstra et al., 2012). A recent study in Drosophila has strengthened this notion by showing that two of the KSS genes, EHMT1 and KMT2C (MLL3), are required for short-term memory and share direct and indirect gene targets (Koemans et al., 2017). Collectively this leads to the hypothesis that the epigenetic modifiers associated with KSS coalesce on gene networks for molecular or cellular pathways that affect neuronal function in the same way. Yet, this hypothesis is seemingly confounded by the fact that the modifiers have distinct and in some cases even antagonistic functions (Koemans et al., 2017). For example, EHMT1 and MLL3 directly modify histones (Barski et al., 2007). But the H3K9me1 and H3K9me2 marks catalyzed by EHMT1 repress gene transcription, while H3K4 methylation by MLL3 results in transcriptional activation. Furthermore, SMARCB1 is part of an ATP-dependent chromatin remodeling complex (Nakayama et al., 2017; Wilson and Roberts, 2011), MBD5 binds to heterochromatin (Laget et al., 2010), and NR113 is a nuclear hormone receptor (Cho et al., 2005).

In this study, we combined molecular, cellular, and electro-physiological approaches to address the question of whether a loss in any KSS gene similarly affects neuronal function. We directly compared monogenic loss of four KSS genes (Ehmt1, Smarcb1, Mll3, or Mbd5) in developing neuronal networks. We show that despite several functional and molecular changes unique to each respective KSS gene knockdown, all of the KSS-gene-deficient neuronal networks were hyperactive during the course of development and showed an altered organization compared to wild-type networks. In the context of integrated analysis of NDDs caused by haploinsufficiency in interrelated chromatin pathways, our results may provide a first explanation for why core clinical features are shared by KSS patients and other phenotypically congruent, but genetically distinct, disorders involving ID and ASD.

RESULTS

Knockdown of KSS Genes Leads to Hyperactive Neuronal Network Activity during Development

In a previous study, we reported that EHMT1 deficiency affects neuronal network activity during early development (Martens et al., 2016). We showed a delay in network formation that was followed by an increased network burst irregularity later in development. We also observed that EHMT1-deficient networks were still in an unstable dynamic state late in development.

Here, we investigated whether the loss of function of individual KSS genes (Ehmt1, Smarcb1, Mll3, or Mbd5) leads to neuronal circuitry impairments in vitro. NR113 was not included since we found it not to be expressed in primary rat cortical neurons (data not shown), and EHMT1-deficient cultures were included in this study for a proper and comprehensive comparison between the KSS genes (same developmental period and culturing methodology). To compare neuronal networks during development, we used rat cortical cultures in which Ehmt1, Smarcb1, Mll3, or Mbd5 were downregulated through RNA interference that allows standardization across conditions (e.g., starting always from the same cell density). Cultures were infected at day in vitro (DIV) 2 with lentiviruses expressing previously validated short hairpin RNAs (shRNAs) targeting Ehmt1 (Benevento et al., 2016; Martens et al., 2016) or newly designed shRNAs targeting Smarcb1, Mll3, or Mbd5. Two independent shRNAs per gene were selected that reduced the respective expression levels by at least 50% (see Benevento et al., 2016; Figures S1A and S1B). For each of the generated viruses, we found no detrimental effect on neuronal density, viability, and cell type development (see Figure S1C). We recorded spontaneous electrical activity in all cultures by growing them on micro-electrode arrays (MEA) (Figure S2A). At the single-channel level (black box, Figure S2A), control neuronal networks (non-infected or GFP-infected) exhibit random events in the form of action potential (AP) spikes (highlighted in blue, Figure S2A) and bursts (highlighted in pink, Figure S2A). Together, these parameters are indicative of the overall spontaneous firing activity (i.e., firing rate, highlighted in gray, Figure S2A). When bursts appear simultaneously in most of the channels (defined as 80% of the active channels), they form a synchronous event, called a network burst (green box, Figure S2A). Typically, the pattern of activity in control neuronal networks develops following a stereotyped pattern (Figure S2B; Chiappalone et al., 2006; Martens et al., 2016). We found that early in development (i.e., DIV 10), neuronal networks displayed spontaneous electrophysiological activity comprised of random spikes and bursts. During the second week in vitro, network bursts appeared, indicating that neurons start to functionally organize into a network. During development, the overall firing and network burst activity increased together with a reduction of the random spiking activity (Figures S2C–S2E). Furthermore, the neuronal network synchronous activity developed from a stochastic toward a typical regular pattern. During the third week in vitro, the firing and network burst frequency plateaued, and from this point on, the neuronal network activity remained stable. This stable state of activity indicates a functionally “mature” neuronal network.
To investigate whether KSS genes share a similar function during neuronal network formation and hence show common alterations at the neuronal network level when knocked down, we examined the electrophysiological activity of KSS-gene-deficient networks and compared them to control cultures at DIV 10 (“immature state”) and DIV 20 (“mature state”) (see raster plots in Figures 1A1–1D1).

We show that EHMT1-, SMARCB1-, and MLL3-deficient neuronal networks were phenotypically similar during development. At DIV 10, these networks exhibited a higher level of random spiking activity, whereas the spike and network burst rates were similar compared to controls (Figures 1A2–1A4, 1B2–1B4, and 1C2–1C4). As the networks matured, the activity of EHMT1-, SMARCB1-, and MLL3-deficient networks strongly increased. At DIV 20, these networks exhibited a higher level of activity (i.e., firing rate and/or network burst rate) compared to controls, indicating that the mature networks were in a hyperactive state (Figures 1A5, 1A7, 1B5, 1B7, 1C5, and 1C7; Table S1).

Although MBD5-deficient networks were also hyperactive, their developmental trajectory differed from the other KSS genes. At DIV 10, MBD5-deficient networks already showed an increase in overall activity expressed as mean firing rate (MFR; Figure 1D2), albeit with immature characteristics (i.e., more random spikes; Figure 1D3). The level of synchronous activity exhibited by controls and MBD5-deficient networks was similar at DIV 10 (Figure 1D4). Interestingly, whereas control neuronal networks increased their firing rate during development, MBD5-deficient neuronal networks did not. In fact, at DIV 20, MBD5-deficient networks exhibited less overall activity compared to controls (MFR; Figure 1D5) but with no differences in the network burst rate and a significantly higher number of random spikes (Figures 1D6 and 1D7). This indicates that MBD5-deficient networks, although more active early in development, failed to organize properly by DIV 20.

Overall, we found prominent differences in the activity patterns exhibited by KSS-gene-deficient neuronal networks compared to controls. Furthermore, our data indicate that shRNA-mediated knockdown of the KSS genes results in hyperactivity during development. The network phenotypes were all recapitulated with a second independent shRNA targeting each gene, indicating specificity (Figures S2F and S2G; see Benevento et al., 2016).
KSS Gene Deficiency Alters Neuronal Network Burst Activity

Since our results showed that KSS gene deficiency leads to network hyperactivity, we next investigated if the typical pattern of network burst activity was also affected by studying network burst duration (NBD), network inter burst interval (NIBI), and network burst regularity. Whereas most of the network bursts (90.0%) in control neuronal networks lasted less than 200 ms (Figures 2A and 2F), we found that in KSS-deficient neuronal networks, NBDs were differently distributed (Figures 2A–2J). In particular, EHMT1-deficient networks showed NBDs longer than controls (48.2% of NBDs > 200 ms; Figure 2G).

SMARCB1-deficient networks exhibited NBDs both longer and shorter than controls (15.5% of NBDs > 600 ms and 31.9% of NBDs < 200 ms), indicated by multiple peaks in the distribution (Figure 2H). The NBD distribution of MLL3- and MBD5-deficient networks was shifted to shorter durations compared to controls, indicated by the percentages of NBDs shorter than 200 ms (92%, 98.7%, and 94% for control, MLL3-, and MBD5-deficient networks, respectively; see distribution plot in Figures 2F, 2I, and 2J).

Then, we studied how each KSS gene deficiency affected the NIBI. The majority (90.1%) of NIBIs in control neuronal networks occurred within a range of 5–20 s (Figure 2K). In contrast, we observed that KSS-gene-deficient networks showed NIBIs

Figure 2. KSS Gene Deficiency Alters Neuronal Network Burst Activity

(A–E) Representative raster plots showing 30 s of recording of the electrophysiological activity of controls (A), EHMT1-deficient (B), SMARCB1-deficient (C), MLL3-deficient (D), and MBD5-deficient (E) cultures at DIV 20. Inset represents 5 s of recording displaying a network burst.

(F–J) Distribution of the duration of the network burst (i.e., NBD) exhibited by controls (F), EHMT1-deficient (G), SMARCB1-deficient (H), MLL3-deficient (I), and MBD5-deficient (J) networks (bin size of 1 ms). Pie diagrams display the percentage of network bursts with durations in three ranges: 0–0.2 s (light gray), 0.2–0.6 s (dark gray), and 0.6–2 s (black).

(K–O) Distribution of intervals between consecutive network bursts (i.e., NIBI) exhibited by controls (K), EHMT1-deficient (L), SMARCB1-deficient (M), MLL3-deficient (N), and MBD5-deficient (O) networks (bin size of 1 s). Pie diagrams display the percentage of NIBIs belonging to three intervals: 0–5 s (light gray), 5–20 s (dark gray), and 20–60 s (black).

(P) Coefficient of variation of the NIBI indicates the regularity of the network burst appearance in EHMT1-, SMARCB1-, MLL3-, and MBD5-deficient cultures as compared to controls at DIV 20.

(Q) Heatmap showing the relative values of the parameters describing the phenotype exhibited by EHMT1-, SMARCB1-, MLL3-, and MBD5-deficient neuronal network as compared to control. The scale of the relative values is indicated from 0 (blue) to 3 (red), where 1 indicates the control (white).

Control, n = 17; EHMT1, n = 12; SMARCB1, n = 9; MLL3, n = 18; MBD5, n = 11. Data represent mean ± SEM. *p < 0.05 (Mann-Whitney test was performed between two groups and one-way ANOVA test and post hoc Bonferroni correction was performed between all genotypes). AC, active channel; BC, bursting channel; BD, burst duration; CV, coefficient of variability; IBI, inter burst interval; NBD, network burst duration; NBR, network burst rate; NIBI, network inter burst interval; MBR, mean burst rate; MF, mean frequency intra burst; MFR, mean firing rate; PRS, percentage of random spike.
shorter than controls (55.3%, 41.9%, 59.9%, and 45.9% of NI-BIs < 5 s for EHMT1-, SMARCB1-, MLL3-, and MBD5-deficient networks, respectively; Figures 2L–2O).

Finally, we investigated whether KSS gene deficiency affected the typical regular network burst pattern exhibited by control neuronal networks. To determine the regularity, we computed the coefficient of variation of the NIBIs. We found that all KSS-gene-deficient networks, except MLL3, exhibited an irregular network burst pattern, as indicated by the higher coefficient of variation of the NIBIs compared to controls (Figure 2P).

In summary, our data indicate that KSS-gene-deficient neuronal networks become hyperactive during development and showed impairments in the pattern of network burst activity later in development (Figure 2Q; Table S2).

**EHMT2-Deficient Networks Show a Different Phenotype Compared to KSS-Gene-Deficient Networks**

EHMT2 is a paralog of EHMT1 but has not been associated with KSS. To investigate whether the network phenotypes are specific to KSS gene deficiency, we knocked down *Ehmt2* using validated shRNAs (Benevento et al., 2016) in developing neuronal cultures. In contrast to KSS-gene-deficient networks, EHMT2-deficient neuronal networks exhibited significantly lower MFRs both at DIV 10 and DIV 20 (Figures S2H–S2L). This further confirms our previous observations that loss of EHMT1 or EHMT2 in neurons can generate distinct phenotypes (Benevento et al., 2016; Iacono et al., 2018).

**Deficiency of KSS Genes Leads to Increased Neuronal Excitability**

The increased neuronal network activity we found at DIV 20 might be caused by altered intrinsic neuronal parameters resulting in hyperexcitability of the individual neurons and/or changes in extrinsic parameters related to synaptic signaling. Supporting this notion, intrinsic parameters linked to neuronal excitability have recently been shown to be regulated, at least in part by epigenetic modifications via DNA methylation (Meadows et al., 2016). Using whole-cell patch-clamp recordings of individual neurons at DIV 20, we measured intrinsic passive and active electrophysiological properties (Figures 3A–3F; Table S3).

In EHMT1-deficient neurons, we found a hyperpolarizing shift of the AP threshold combined with unaltered resting membrane potentials ($V_{\text{m}}$) (Figures 3B and 3D). At standard holding potentials (~60 mV), however, these changes did not result in a reduction in the AP firing rheobase (Figures 3A and 3E), since the EHMT1-deficient neurons also showed lower membrane input resistances ($R_{\text{in}}$; Figure 3C).

Similar to EHMT1-deficient neurons, SMARCB1- and MLL3-deficient neurons both showed a hyperpolarizing shift of the AP threshold at unaltered (MLL3) or depolarized $V_{\text{m}}$ (SMARCB1) (Figures 3B and 3D). In addition, SMARCB1-deficient neurons showed an unchanged $R_{\text{in}}$ and MLL3-deficient neurons showed an increased $R_{\text{in}}$ (Figure 3C). These alterations may underlie our finding that both of these KSS gene deficiencies share a reduced AP firing rheobase (Figures 3A and 3E), which supports increased neuronal excitability.

Neurons in MBD5-deficient networks were the only ones that showed no change in the AP threshold (Figure 3D). Even though at ~60 mV the rheobase remained unchanged (Figure 3E), the generally depolarized $V_{\text{m}}$ (Figure 3B) in combination with an increased $R_{\text{in}}$ (Figure 3C) still implies an increased excitability of MBD5-deficient neurons due to a higher responsiveness to incoming excitatory (depolarizing) synaptic current at $V_{\text{m}}$.

For all tested KSS genes, we thus found changes in intrinsic properties that directly (AP threshold) or indirectly ($V_{\text{m}}$, $R_{\text{in}}$, $t$) affect the generation of APs. Therefore, we compared the AP waveforms across genotypes (Figure 3F; Table S3). Whereas APs generated by EHMT1-deficient neurons showed no significant changes in their AP waveform, SMARCB1- and MLL3-deficient neurons both showed broader APs, mediated by a slower rising phase (i.e., rise time, MLL3-deficient neurons only) and/or slower repolarization phase (i.e., decay time, SMARCB1- and MLL3-deficient neurons). Contrasting these, APs in MBD5-deficient neurons were significantly shorter, due to a faster repolarization phase.

Taken together, these results indicate that alterations in intrinsic passive and active properties in KSS-gene-deficient neuronal networks is genotype specific but as a whole imply different levels of increased neuronal excitability.

**KSS Gene Deficiency Leads to Altered Excitatory and Inhibitory Synaptic Inputs**

In addition to increased intrinsic excitability, the hyperactivity observed in KSS-gene-deficient networks could also be explained by a change in excitatory/inhibitory (E/I) balance. To investigate this, we measured synaptic properties in our cultures, with and without KSS gene knockdown. We first measured miniature inhibitory postsynaptic currents (mIPSCs) in EHMT1-deficient networks at DIV 20 (Figure 3S). We found a significant reduction in mIPSC frequency, but not in mIPSC amplitude, when compared to control cultures. We previously showed that knockdown of EHMT1 did not affect miniature excitatory postsynaptic current (mEPSC) frequency or amplitude in rat neuronal networks (Benevento et al., 2016). Therefore, our combined results suggest that the E/I balance is shifted in favor of excitation due to reduced inhibitory synaptic input.

Because mIPSC frequency is known to correlate with the number of synapses and release probability of a neuron, we counted the number of synapses in both control and KSS-gene-deficient neuronal networks (Figures 3G and 3H; Table S3). First, we quantified the number of inhibitory synapses on individual dendrites by counting the number of colocalizing presynaptic vesicular GABA transporter (VGAT) and postsynaptic Gephyrin puncta. We found a significant reduction in the number of inhibitory synapses for all KSS genes when compared to control cultures (Figures 3G and 3H). Thus, KSS gene deficiency likely has a strong effect on the formation and/or maintenance of inhibitory synapses. Next we quantified excitatory synapses by counting the number of presynaptic vesicular glutamate transporter (VGLUT) and postsynaptic density-95 protein (PSD95) puncta colocalizing. We found a significant reduction in the number of excitatory synapses in SMARCB1-, MLL3-, and MBD5-deficient neuronal networks but not in EHMT1-deficient networks (Figures 3G and 3H), in line with our previous reports (Benevento et al., 2016; Martens et al., 2016).
In summary, we show that in EHMT1-deficient networks, the E/I balance is strongly shifted to increased excitation due to reduced inhibitory synaptic input. SMARCB1-, MLL3-, and MBD5-deficient cultures showed a reduction in inhibitory input, which was also accompanied with a reduction in excitatory input.

KSS Deficiency Causes Deregulation of Genes Controlling Neuronal and Synaptic Processes

Next, we investigated the molecular changes that could underlie the hyperactivity that we observed in KSS-gene-deficient networks. To address this, we performed RNA sequencing (RNA-seq) on KSS-gene-deficient neuronal networks at DIV 20.
Using DESeq2 (Love et al., 2014), in all KSS gene-deficient networks, we detected differentially expressed (DE) genes (q value < 0.1), as compared to control cultures. Knockdown of Ehmt1 gave rise to more upregulated than downregulated genes. In contrast, we detected more downregulated genes than upregulated genes in SMARCB1-, MLL3-, and MBD5-deficient networks (Figure 4A; Table S4). This observation indicates that SMARCB1, MLL3, and MBD5 regulate gene transcription in an opposite direction, as compared to EHMT1. This is consistent with SMARCB1 and MLL3 being known to be transcriptional activators and EHMT1 to be a transcriptional repressor (Tachibana et al., 2005). In addition, EHMT1- and MBD5-deficient networks showed a lower number of total DE genes compared to SMARCB1- and MLL3-deficient networks (Figure 4A). To gain an overview of the global gene expression pattern, we performed a principal-component analysis (PCA) on DE genes obtained from all pairwise comparisons (3,083 genes; Figure 4B). PCA allowed discrimination of KSS-gene-deficient networks, with DE genes of SMARCB1- and MLL3-deficient networks being close to each other, on the opposite end of DE genes of EHMT1-deficient networks. The DE genes of MBD5-deficient cultures were found to be closest to the control. Furthermore, the comparisons
of DE genes between each pair of the KSS-gene-deficient networks using the scatterplot analysis (Figure S4) showed that DE genes of SMARCB1- and MLL3-deficient networks had the highest correlation ($r^2 = 0.90$). These data indicate that knockdown of different KSS genes resulted in distinguishable gene expression patterns, where those of SMARCB1- and MLL3-deficient networks were most similar and those of EHMT1-deficient cultures were most different.

Interestingly, Gene Ontology (GO) annotation of DE genes detected from EHMT1-/-, SMARCB1-/-, and MLL3-deficient networks showed high similarity in their associated biological functions (biological processes [BPs]), particularly in ion transport and chemical synaptic transmission (Figure 4C). DE genes detected in MBD5-deficient networks were associated with apparently different biological functions including cell adhesion, nucleosome assembly, and protein translation. However, GO annotation assessed for cellular components (CC) revealed that DE genes detected from all knockdown conditions were very similar, mainly associated with axon, dendrite, synapse and postsynaptic density (Figure 4D). These results indicate that the similar neuronal structures are affected by KSS gene knockdown through distinct molecular mechanisms. A closer examination of DE genes that are known to play roles in synaptic and ion channel functions showed that most of these genes were affected by knockdown of all four KSS genes, but the regulation was different, with opposite expression patterns of SMARCB1, MLL3, and EHMT1 knockdown and a unique pattern of MBD5 knockdown (Figure 4E). In addition, we identified 34 DE genes represented in all knockdown conditions (Figure S5A). Also here the expression patterns of these 34 DE genes were mostly in opposing directions between SMARCB1/MLL3- and EHMT1-deficient networks (Figures S5B, S5C, and S5E; Table S4). Remarkably, this small number of 34 DE genes revealed an enrichment of GO terms related to learning, memory, neurons, and dendrites (Figure S5D). Of interest, many of the 34 DE genes have previously been associated with cognitive disorders, seizures or epilepsy, ASD, motor abnormalities, and sleep disturbances (Table S4), which is a constellation of symptoms seen in KSS.

Taken together, these data show that KSS target genes share similar functions in regulating neuronal structures and activity, with a prominent enrichment for genes that directly affect neuronal excitability (e.g., potassium and sodium channels) and synaptic function, including several GABA and glutamate receptors (Figure 4E). However, KSS genes regulate distinct sets of individual target genes through different transcriptional or functional mechanisms. The difference at the functional level was most apparent for MBD5, which was consistently the most dissimilar of the KSS genes, from the functional level (Figures 1 and 2Q) to gene expression level.

**Increased Cell Excitability and Reduced Inhibition in Ehmt1+/− Mice**

Having established that loss of EHMT1 leads to increased cell excitability and reduced synaptic inhibition in vitro, we aimed to corroborate these results by measuring intrinsic and synaptic properties in acute hippocampal brain slice preparations of Ehmt1+/− mice.

First, we examined the development of synaptic inputs by recording mIPSCs and mEPSCs at postnatal day (P) 7, P14, and P21 in Ehmt1+/+ and Ehmt1+/− mice, revealing a reduction in mIPSC amplitudes at all investigated time points (Figures 5B, 5E, and 5H; Table S5) in CA1 pyramidal neurons. We found an increase of mIPSC frequency at P7, but a strong reduction at P21 (Figures 5C, 5F, and 5I) leading to a general reduction of inhibitory connectivity at P21, consistent with our observation in dissociated rat cortical neurons. Recording of the paired pulse ratio (PPR) at P21 revealed an increased PPR specifically at 50 ms inter-stimulus interval (ISI) following stimulation in stratum radiatum but not stratum oriens (Figures S6A–S6C), indicating that these interneurons have a reduced probability of release onto CA1 pyramidal cells. Interestingly, mEPSC amplitude and frequency were unaltered between Ehmt1+/+ and Ehmt1+/− mice (Figures 5J–5R). In addition, recording of the PPR following stimulation of the Schaffer collaterals, the main excitatory input to CA1 pyramidal neurons, showed no changes in the probability of release at P21 (Figure S6D). These data confirm our in vitro data and suggest that EHMT1 plays a role in controlling E/I balance by regulating inhibitory inputs onto CA1 pyramidal cells. This is in line with the expression pattern of EHMT1, which next to excitatory neurons (Balemans et al., 2013), we also found to be expressed in both parvalbumin and somatostatin positive cells (Figure S6E).

In analogy to the primary neuronal cultures, we then investigated the intrinsic excitability of CA1 pyramidal neurons by means of their intrinsic electrophysiological properties. An input/output curve with increasing amounts of injected current revealed an increased excitability of CA1 pyramidal neurons (Figures 6A and 6B), which was accompanied by a reduced rheobase (Figure 6F). This reduction of the rheobase can be specifically attributed to a hyperpolarization of the AP threshold (Figure 6D), since other intrinsic properties remained unchanged (Table S5). These results indicated that CA1 pyramidal cells were intrinsically more excitable in Ehmt1+/− mice compared to Ehmt1+/+ mice.

Since CA1 pyramidal cells show a reduced inhibitory synaptic connectivity and an increased intrinsic excitability, we hypothesized that these cells should display higher level of spontaneous spiking activity. To investigate this, we performed cell-attached patch-clamp recordings of CA1 pyramidal neurons to record basal AP frequency. In standard recording solution (3 mM KCl), CA1 pyramidal cells are inactive. Elevating KCl concentration to 7 mM resulted in AP firing in all recorded cells and revealed a higher AP frequency in Ehmt1+/− compared to Ehmt1+/+ mice (Figures 6G and S6H). These data indicate that the combination of reduced inhibitory synaptic inputs and an increased intrinsic excitability may result in increased basal activity of CA1 pyramidal neurons in Ehmt1+/− mice.

**DISCUSSION**

**KSS-Gene-Deficient Networks Share a Common Mode of Failure**

Previously we showed that EHMT1 deficiency transiently delays the appearance of spontaneous network activity, eventually...
resulting in an irregular network bursting pattern (Martens et al., 2016). Here, we show that following excessive random spiking activity in immature cultures, the irregular network burst pattern is generally accompanied by a more frequent network burst rate (i.e., network hyperactivity) in mature EHMT1-deficient networks. The network phenotypes after loss of SMARCB1, MLL3, or MBD5 showed striking similarities, resulting in an irregular network burst pattern and/or hyperactivity. Our results therefore imply that the KSS-gene-deficient networks share a common mode of failure when establishing network communication (Figure S2L). This implied common mode of failure was reflected in different compositions of altered parameters of network activity in a genotype-specific manner. For example, hyperactivity was observed as an increase in firing rate in EHMT1-, SMARCB1-, MLL3-, and MBD5-deficient neuronal networks, while network burst rate was altered in MLL3-deficient networks. The developmental trajectory for MBD5-deficient networks was representative for the genotype-specific differences too. While the other knockdowns showed hyperactivity late in development, MBD5-deficient networks were excessively active at an early, immature stage (DIV 10). The functional convergence we observed at the neuronal network level is in line with a recent study in Drosophila showing similar deficits in short-term memory between flies lacking EHMT1 and MLL3 in mushroom bodies (Koemans et al., 2017). Together, our data indicate that neuronal circuits represent logical loci for the manifestation of a disease, in which changes in diverse genes, protein networks, cell types, or developmental stages may elicit similar or specific changes in circuit function.

In general, hyperactivity in networks can be mediated by two major factors: (1) changes in synaptic signaling between neurons resulting in altered E/I balance and (2) changes in intrinsic electrophysiological properties of the neurons within the networks resulting, e.g., in hyperexcitability (Suresh et al., 2016). At the single-cell level, we found in SMARCB1-, MLL3-, and MBD5-deficient cultures a strong reduction in both excitatory and inhibitory synaptic inputs. The reduction (~50%) was similar for excitatory...
and inhibitory synapses, suggesting that the E/I balance was not changed by those knockdowns. EHMT1-deficient cultures, however, showed a strong decrement in inhibitory synaptic input, without excitatory synaptic input being affected. This was the case in vitro as well as in the Ehmt1+/−/C0 mice. Indeed, we found mIPSC amplitude to be decreased at all investigated time points in the Ehmt1+/−/C0 mice, whereas the mIPSC frequency was strongly reduced at P21. This effect on frequency can be explained by fewer inhibitory synapses and by a reduced release probability of inhibitory synapses following stimulation in stratum radiatum but not stratum oriens. The specific effect of loss of Ehmt1 on inhibition is relevant because imbalanced E/I is associated with ASD in humans and rodent models (Del Pino et al., 2018; Fenton, 2015; Nelson and Valakh, 2015; Selten et al., 2018). In particular, a loss in the efficiency of inhibitory synaptic strength has been observed in many NDDs, including Rett syndrome and Fragile X syndrome (Braat and Kooy, 2015; Chao et al., 2010; Moskalyuk et al., 2019; Olmos-Serrano et al., 2010; Telias et al., 2016; Wood et al., 2009). The changes in excitatory and inhibitory inputs observed in KSS-gene-deficient neurons imply alterations in proteins directly or indirectly linked to synapse function. In line with this concept, for all KSS gene knockdowns we found a multitude of DE genes linked to both glutamatergic and GABAergic synaptic transmission, including up- and downregulation of glutamate and GABA receptors, adhesion molecules, and postsynaptic density proteins. Presumably, multiple genes are responsible for the observed functional changes, and at the same time, a portion of the underlying transcriptional changes could be subtractive or antagonistic in nature, resulting in limited functional consequences for synaptic transmission. Further studies would be required to identify direct versus indirect targets, for example, through the identification of the underlying epigenetic changes.

In addition to the shift in E/I balance that we found in EHMT1-deficient neurons, neuronal hyperexcitability could contribute to increased and/or irregular network burst activity in KSS-gene-deficient networks (Suresh et al., 2016). Enhanced neuronal excitability can be mediated by either a passive or active intrinsic property or a combination of both. These properties encompass increased membrane input resistance or a hyperpolarized shift in the threshold for generating APs, particularly in combination with a depolarized membrane potential. Indeed, at the single-cell level, across all four investigated KSS genes we found changes in the intrinsic neuronal properties that imply increased excitability. However, the increased excitability in KSS-gene-deficient neurons was genotype specific, both in terms of contributing properties and extent of increase (i.e., mild for EHMT1- and MBD5-deficient networks and more pronounced for SMARCB1- and MLL3-deficient ones). Furthermore, we found robust genotype-specific alterations in the AP kinetics, ranging from relatively slow (SMARCB1 and MLL3) to fast (MBD5) repolarization kinetics. The various changes in passive and active parameters that result in increased neuronal excitability in KSS-gene-deficient networks suggest that ion channel expression is altered. In particular, reduced expression of different classes of voltage gated potassium (Kv) channels, such as Kv1.1 (KCNA1) or Kv2.1 (KCNB1), and/or increased expression of voltage gated sodium (NaV) channels, such as SCN1A, SCN2A, or SCN3A, has been shown to increase somatodendritic excitability and AP kinetics (Guan et al., 2007; Mohapatra et al., 2009; Speca et al., 2014).
Furthermore, dysregulation or dysfunction in several classes of Kᵥ and Naᵥ channels, including those mentioned above, have been found to be associated with NDDs (de Kovel et al., 2017), epilepsy, and ASD (Weiss et al., 2003). Strikingly, in all KSS-gene-deficient networks we found altered regulation of a battery of genes coding for different types of ion channels, including several classes of Kᵥ and Naᵥ channels. However, the diversity and similarities in the intrinsic electrophysiological parameters are also reflected by changes in gene expression. Neurons haploinsufficient for the repressive regulator EHMT1 almost exclusively show upregulation of genes coding for Kᵥ and Naᵥ channels, whereas neurons deficient for SMARCB1 and MLL3 consistently show downregulation in these genes. The opposing up- and downregulation of genes for ion channels illustrates that the detected hyperexcitability is likely to be the combined consequence of complex changes in ion channel composition that can either be dominated more by increased Naᵥ channel expression (EHMT1), by reduced Kᵥ channel expression (SMARCB1 and MLL3), or by a complex combination of both (MBD5).

Molecular Convergence in KSS

An intriguing finding that we uncovered by comparing RNA-seq of each knockdown is a set of 34 commonly dysregulated transcripts. The list comprises genes that are associated with cognitive disorders, epilepsy, or ASD, and most code for proteins involved in synaptic function or ion channels (Table S4). Interestingly, four of these genes have been identified as hub genes in co-expression networks analyzed from the cortical tissue of ASD patients: Scamp5, Slc12a5, SynJ1, and Unc13a (Gupta et al., 2014; Lombardo et al., 2017). The majority of genes in the list are upregulated after Ehmt1 knockdown but downregulated by Smarcb1, Mll3, or Mbd5 loss of function. Accordingly, EHMT1 enzymatic activity generally represses transcription, while SMARCB1 and MLL3, and according to our data MBD5, function as activators. The divergent effects on mRNA expression may explain some phenotypic differences we observed. There are four transcripts upregulated by Ehmt1 knockdown that are directly involved in high-frequency neuronal activity: the protein products of Scamp5 and Unc13a (aka Munc13-1) maintain high rates of vesicular endo- and exocytosis (Betz et al., 1998; Zhao et al., 2014), while Scn8a codes for the sodium channel Naᵥ1.6, whose channel properties support high firing rates (Raman et al., 1997). Finally, Grin1 is an interesting transcript since enhancing NMDAR activity has been directly implicated in lengthening burst duration (Suresh et al., 2016). Accordingly, we found enhanced Grin1 expression in EHMT1-deficient cultures (long bursts) and reduced Grin1 expression in SMARCB1-, MLL3-, and MBD5-deficient networks (shorter bursts; Figures 2F–2J). Interestingly, we recently used induced pluripotent stem cells to investigate how EHMT1 deficiency affects human neurons (Frega et al., 2019). Even if the methodologies used are very different (i.e., homogeneous population of excitatory neurons in human model versus various types of inhibitory/excitatory neurons and glia in rodent model), we found similar phenotypes. In particular, we found network burst with longer durations and higher irregularities were exhibited at the neuronal network level and increased NMDAR expression was found at the molecular level. This cross-species comparison further corroborates our results and indicates that NMDAR could be a specific target for treatment.

The 34 overlapping genes may be inexorably linked (representing a neuronal co-expression module), and at least some may be direct, common targets of the pathogenic epigenetic modulators found in KSS. It would therefore be useful to decipher the epigenetic marks that control expression, especially since it is not clear which transcriptional changes are causal versus those that are collateral. Importantly, our data suggest that the molecular pathophysiological mechanism underlying KSS may not depend on whether the parallel transcriptional changes are a gain or loss. Instead, the implication is that a dysfunction in dynamic transcriptional regulation during development leads to disease and consequently hinders proper neuronal specialization or cortical patterning, as has been suggested to occur in autism (Voineagu et al., 2011).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.12.002.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nael Nadif Kasri (n.nadif@donders.ru.nl).

All shRNA’s generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

For the slice electrophysiology experiments presented in this study, male mice heterozygous for a targeted loss-of-function mutation in the Ehmt1 gene (Ehmt1+/− mice) and their wild-type (WT) littermates on C57BL/6J background were used at postnatal day (P) 7, 14 and 21, as previously described (Balemans et al., 2010). Mice were kept in standard Macrolon type III cages with an artificial light-dark cycle of 12 hours (lights go on at 7:00 am). Per cage 3–6 animals were housed in presence of sawdust bedding, a mouse igloo, and nest building material. Food and water were available ad libitum. Room temperature was always kept stable at 21°C with controlled humidity (Balemans et al., 2010). For the shRNA knockdown experiments presented in this study, pregnant WT Wistar WU rats from Charles River were sacrificed after which embryos (E18) were removed for generating primary cultures (see section Primary neuronal cell culture) (Charles River).
Animal experiments were conducted in conformity with the Animal Care Committee of the Radboud University Nijmegen Medical Centre, the Netherlands, under DEC application number 2015-0038, and conform to the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive 2010/63/EU.

**Primary neuronal cell culture**

One day before plating, glass coverslips (14 mm; Menzel GmbH) treated with 65% nitric acid (Sigma-Aldrich) were coated with 0.0125% polyethyleneamine (PEI; Sigma-Aldrich) overnight. On the day of plating, MEAs were coated with 100 μL poly-D-lysine solution (Sigma-Aldrich, Cat.No. P7280; 100 μg/ml in 50 mM borate buffer, pH 8.5) placed directly over the array in a drop for 3 hr at 37°C in a humidified cell culture incubator. Then, MEAs were washed 3X with sterile water and air-dried in a laminar flow hood. Briefly, each pregnant Wistar rat was deeply anesthetized using isoflurane (Pharmachemie B.V. Haarlem, Cat.No. 45112106) and sacrificed by cervical dislocation. Embryos (E18) were quickly and aseptically removed by cesarean section. Whole brains were removed from 2 embryos, the meninges were stripped away, and the cerebral cortices were dissected out as described (Frega et al., 2017). The tissue was placed directly into a 15 mL conical tube and digested in 2 mL papain solution (prepared as 200 U papain [Sigma-Aldrich, Cat.No. P4762], 1.6 mg L-cysteine [Sigma-Aldrich, Cat.No. C7880] in 10 mL Segal’s medium) at 37°C for 45 min in a water bath. The digestion was then inactivated by adding 8 mL room-temperature seeding medium (Neurobasal medium [Invitrogen, Cat.No. 21103] supplemented with 10% FBS [Sigma-Aldrich, Cat.No. F7524], 2% B-27 [Invitrogen, Cat.No. 17504], 1% GlutaMAX [Invitrogen, Cat.No. 35050] and 1% Pen/Strep solution [Sigma-Aldrich, Cat.No. P4333]). The cortices were dissociated and filtered through a 70-μm cell strainer placed atop a 50 mL conical tube. The collected cells were spun at 200 x g for 8 min, the medium was aspirated, and the cell pellet was gently resuspended using a pipettor with 1 mL tip and 800 μl seeding medium. The suspension was then diluted to 10 mL with seeding medium and mixed thoroughly before counting. Viable cells were counted using a hemacytometer and trypan blue exclusion. We consistently obtained 2 - 3 x10⁶ cells/ml and > 95% viability at the time of plating. Dissociated cells were plated on the PEI-coated glass coverslips at a final density of 1200 cells/mm². For the MEAs, cells were carefully seeded onto the center of the array in a 50 μl droplet at a concentration of 1200 cells/μl. After settling for 3 hr in an incubator, 450 μL prewarmed seeding medium was slowly added, followed by 500 μL culture medium (i.e., seeding medium without serum). At DIV2, cells were transduced with a lentivirus expressing GFP or an shRNA targeting the mRNA of interest. Non-infected and GFP-only infected cells were used as controls. Since we did not observe differences between these conditions, results from both were pooled together. Beginning at DIV 3, and every 2 days thereafter, half the medium was removed and replenished with freshly-prepared, prewarmed culture medium.

Toxicity of shRNA infection was assessed by checking viability (with Propidium Iodide (PI) staining [ThermoFisher Scientific, cat.no. BMSS00PI], Figure S1) after infection of the cells with different dilutions of the virus, as explained in detail in Martens et al. (2016). Then, we followed a stringent quality check on neuronal densities for all of our experimental assessments. In particular, we always performed visual inspection of the neuronal density immediately after plating and during the entire development. If neuronal clustering was observed, cultures were discarded. On the functional level, to be sure that any abnormal activity is not due to changes in plating or developmental conditions, within each batch we always included control networks next to the gene-deficient ones. Batches in which control networks showed an abnormal cell density or pattern of activity were rigorously discarded from the analysis.

**Cell lines**

HEK293T cells (ATCC Cat.No CRL-3216) were cultured thawed and cultured in high-glucose DMEM (Sigma Cat.No D0819) supplemented with 10% Sodium-Pyruvate (Sigma Cat. No. S8636), 1% Pen/strep and 10% FCS in 10 cm Petri dishes. One to two passages after thawing, cells were split using 0.05% Trypsin-EDTA (GIBCO Cat.No. 25300054) in 1:10 dilution for the generation of lentiviral particles as described before (Kasri et al., 2008) and in the RNA interference section.

**METHOD DETAILS**

**RNA interference**

For RNAi knockdown experiments, DNA fragments encoding short hairpin RNAs (shRNAs) directed against rat Ehmt1 (Benevento et al., 2016), Ehmt2 (Benevento et al., 2016), SmarcB1, Mll3 or Mbd5 mRNA were cloned into the pTRIPΔU3-EF1α-EGFP lentiviral vector (Kasri et al., 2008; Nadif Kasri et al., 2009). Used hairpin sequences encompassed: SmarcB1 hp#1: GGAGATTGCCATCCGAAAT, Smarcb1 hp#2: GCCCTCTTCAGCACACAT, Mll3 hp#1: GCCCTCATTACACCAAT, Mll3 hp#2: GGCCAAGACCTGTGTA, Mbd5 hp#1: CGGGAAATGGTCTGAAAAGGT, Mbd5 hp#2: CTGAAGGACACGACTTTAAC. Empty vector expressing GFP only was used as control vector. Lentiviral particles were prepared, concentrated, and tittered as described previously (Kasri et al., 2008). In brief, lentiviruses were generated by co-transfecting the transfer vector, the psPAX2 packaging vector (Addgene #12260), and the VSVG envelope glycoprotein vector pmD2-G (Addgene plasmid #12259) into HEK293T cells, using calcium phosphate precipitation. Supernatants of culture media were collected 48-h after transfection and filtered through a 0.45 μm syringe filter. Viral particles were then stored at ~80°C until use. Efficiency of shRNA was assessed by qPCR and or western blot in case of EHMT1 and EHMT2 (Benevento et al., 2016). Cortical neurons were plated in a 6 well and infected at DIV 2, cell lysed and RNA extracted at DIV 21.
Reverse transcription quantitative polymerase chain reaction

RNA was extracted using the NucleoSpin® RNA kit (Macherey-Nagel cat. no. 740955.50) according to the manufacturer’s protocol. Reverse transcription was performed using iScript cDNA Synthesis Kit (Biorad cat.no. 1708891). qPCR and melting curve analyses were performed using GoTaq® qPCR Master Mix (Promega A600) on a 7500 Real-Time PCR System (Life Technologies). Fold change was calculated between KSS-gene deficient networks and GFP-shRNA controls via the ΔΔCt method. The primers used for qPCR are listed in Table S6.

RNA-Seq

RNA-Seq using total RNA was executed as described in Oti et al. (2015). In summary, 500 ng of total RNA was used to obtain double-stranded cDNA (ds-cDNA) that was subsequently purified with MinElute Reaction Cleanup Kit (QIAGEN #28206). For the library construction, the KAPA Hyper Prep Kit (Kapa Biosystems #KK8504) standard protocol with a modification of a 15-minute USER enzyme (Biolab # M5505L) incubation step for the library amplification and 3ng ds-cDNA as the starting material were used. The library quantification was performed with the KAPA Library Quantification Kit (Kapa Biosystems #KK4844) followed by pair-ended sequencing with NextSeq500 (Illumina).

Immunocytochemistry

Primary neurons were fixed with 4% paraformaldehyde/4% sucrose in phosphate buffered saline (PBS) for 15 min. After fixation cells were washed and permeabilized with PBS and 0.2% Triton. Cells were blocked in blocking buffer (PBS, 5% normal goat serum, 1% bovine serum albumin, 0.2% Triton) for one hour, followed by incubation with primary antibodies in blocking buffer overnight at 4°C. Cells were washed with PBS the next day and incubated with secondary antibodies for one hour at room temperature. Cells were washed with PBS again to remove antibody excess and DNA was stained using Hoechst (1:10000). Finally, cells were mounted using DAKO (DAKO, S3023) fluorescent mounting medium and stored at 4°C. Epifluorescent pictures were taken at a 63x magnification using the Zeiss Axio Imager 2 equipped with apotome. All conditions within a batch were acquired with the same settings (laser power % and exposure time) in order to compare signal intensities between different experimental conditions. Number of puncta was quantified via manual counting using ImageJ (scale 1 pixel = 0.072 μm). Due to the large protein size of MLL3, knockdown efficiency was determined first by staining for EHMT1, SMARCB1, MLL3 and MBD5 and compare the corrected total cell fluorescence (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)) to GFP-shRNA treated cultures using ImageJ. Primary antibodies: guinea pig anti-VGAT (1:500, Synaptic Systems 131 004); mouse anti-Gephyrin (1:500, Synaptic Systems 147 111); rabbit anti-VGLUT (1:1000, Synaptic Systems 135 302); mouse anti-PSD95 (1:50, Thermo Scientific MA-045); Guinea pig anti-MAP2 (1:1000, Synaptic systems 188004); Mouse anti-SMARCB1 (1:500, Abnova H00006598-M01); Rabbit anti MBD5 (1:500, Proteintech 15961-1-AP); Mouse anti-EHMT1 (1:1000, Abcam ab41969); Rabbit anti-MLL3 (1:1000, Millipore ABE1851); Rabbit anti-GABA (1:1000, Sigma A2052). Secondary antibodies: goat anti-guinea pig Alexa Fluor 647 (1:2000, Invitrogen A-21450); goat anti-rabbit Alexa Fluor 647 (1:2000, Invitrogen A-21245); goat anti-mouse Alexa Fluor 568 (1:2000, Invitrogen A-11031) and goat anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen A-11034).

MEA recordings

The neuronal network activity was recorded for 30 min by means of micro-electrode Arrays (MEAs), devices made up of 60 planar microelectrodes (TiN/SiN, 30 μm electrode diameter, 200 μm spaced) arranged over an 8x8 square grid (except the four electrodes at the corners), supplied by Multi Channel Systems (MCS, Reutlingen, Germany). In addition, we used the 6-wells MEAs (6 independent wells, each one with 9 recording and 1 reference embedded electrodes) to perform virus dose responses experiments. After 1200x amplification (MEA 1060, MCS), signals were sampled at 10 kHz using the MCS data acquisition card. Recordings were performed outside the incubator at temperature of 37°C and to prevent evaporation and changes of the pH medium, a constant slow flow of humidified gas (5% CO2, 20% O2, 75% N2) was inflated onto the MEA.

Whole patch clamp recordings in neuronal cultures

Experiments were performed in a recording chamber on the stage of an Olympus BX51WI upright microscope (Olympus Life Science, PA, USA) equipped with infrared differential interference contrast optics, an Olympus LUMPlanFL N 40x water-immersion objective (Olympus Life Science, PA, USA) and kappa MXC 200 camera system (Kappa optronics GmbH, Bleichen, Germany) for visualization. Through the recording chamber a continuous flow of carbonated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 1.25 NaH2PO4, 3 KCl, 26 NaHCO3, 11 Glucose, 2 CaCl2, 1 MgCl2 (adjusted to pH 7.4), warmed to 30°C, was present. Patch pipettes (6-8 MΩ) were pulled from borosilicate glass with filament and fire-polished ends (Science Products GmbH, Hofheim, Germany) using the PMP-102 micropipette puller (MicroData Instrument, NJ, USA). Pipettes were filled with a potassium–based solution containing (in mM) 130 K-Gluconate, 5 KCl, 10 HEPES, 2.5 MgCl2, 4 Na2-ATP, 0.4 Na3-ATP, 10 Na-phosphocreatine, 0.6 EGTA (adjusted to pH 7.25 and osmolarity 304 mOsmol) for recordings of intrinsic electrophysiological properties in current clamp mode. For recordings of miniature inhibitory postsynaptic currents (mIPSCs) in voltage clamp mode pipettes were filled with a cesium–based solution containing (in mM) 115 CsMeSO3, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na2-ATP, 0.4 Na3-ATP, 10 Na-phosphocreatine, 0.6 EGTA (adjusted to pH 7.2 and osmolarity 304 mOsmol). Recordings were made using a SEC-05X amplifier (NPI Electronic GmbH, Tamm, Germany) and recorded with the data acquisition software Signal (CED, Cambridge, UK). Recordings were not corrected for a liquid junction.
potential of approximately –10 mV. Intrinsic electrophysiological properties were analyzed using Signal and MATLAB (MathWorks, MA, USA), while mIPSCs were analyzed using MiniAnalysis (Synaptosoft Inc, Decatur, GA, USA).

Intrinsic electrophysiological properties were recorded in current clamp mode, where the resting membrane potential (V_{rmp}) was determined after achieving whole-cell configuration. Cells were selected on a V_{rmp} of –55 mV or lower. All further recordings were performed at a holding potential of –60 mV. Passive membrane properties were determined via a 0.5 s hyperpolarizing current of –25 pA. Action potential (AP) characteristics were determined from the first AP elicited by a 0.5 s depolarising current injection just sufficient to reach AP threshold. The mIPSCs were measured in voltage clamp mode, in the presence of 1 μM tetrodotoxin (TTX; Tocris, Bristol, UK), 5 μM 6-cyano-7-nitroquinoline-2,3-dione (CNQX) and 100 μM 2-amino-5-phosphonovaleric acid (APV) at a holding potential of +10 mV.

Acute slice electrophysiology

Mice of both genders were taken at ages indicated in the text ± 1 day P7 (P6-8), P14 (P13-15) or P21 (P20-22) and anesthetized using isoflurane before decapitation. Ventral slices (350 μm) were cut using a HM650V vibratome in ice cold artificial cerebrospinal fluid (ACSF) containing (in mM): 87 NaCl; 11 Glucose; 75 Sucrose; 2.5 KCl; 1.25 NaH2PO4; 0.5 CaCl2; 7 MgCl2; 26 NaHCO3, continuously oxygenated with 95% O2/5% CO2 at 30°C. Before recording, slices were transferred to the recording setup and incubated in recording (ACSF) containing (unless otherwise stated) (in mM): 124 NaCl, 1.25 NaH2PO4, 3 KCl, 26 NaHCO3, 10 Glucose, 2 CaCl2, 1 MgCl2 and continuously oxygenated with 95% O2/5% CO2 at 30°C and incubated 15 minutes prior to recording. Cells were visualized with an upright microscope (Olympus). Patch pipettes (3-5 MΩ) were made from borosilicate glass capillaries and filled with intracellular solution containing (for voltage clamp, in mM): 115 CsMeSO3; 20 CsCl; 10 HEPES; 2.5 MgCl2; 4 Na2ATP; 0.4 Na3GTP; 10 Na-phosphocreatine (pH 7.2-7.3, 290 mOsm) or (for current clamp, in mM): 130 K-Gluconate; 5 KCl; 10 HEPES; 2.5 MgCl2; 0.6 EGTA; 4 Na2ATP; 0.4 Na3GTP; 10 Na-phosphocreatine (pH 7.2-7.3, 290 mOsm). Traces were recorded using a Multiclamp 700B amplifier (Molecular Devices, Wokingham, United Kingdom), sampled at 20 kHz and filtered at 2 kHz. Cells were excluded from analysis if the access resistance exceeded 25 MΩ. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of Tetrodotoxin (1 μM), 6-Cyano-7-nitroquinoline-2,3-dione (CNQX, 5 μM) and D-(-)-2-Amino-5-phosphonovaleric acid (APV 100 μM). Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of TTX and Picrotoxin (PTX, 100 μM). Paired pulse ratio (PPR) was in the presence of CNQX and D-APV, the recording ACSF contained 4 mM CaCl2 and 4 mM MgCl2, and was calculated as peak2/peak1 after correcting for any residual current at the second pulse. Spontaneous action potential (AP) frequency was calculated from the total number of APs during a 10 minute recording. Intrinsic properties were analyzed as follows: Resting membrane potential was recorded directly after break in. All other properties were recorded at a holding potential of –60 mV. Input resistance was calculated from 6 responses to increasing negative current injections (5 pA per step) (Tricoire et al., 2011). Rise and decay time were calculated at 20%–80%/80%–20% of the amplitude, respectively. The adaptation ratio was calculated as the 8th/3rd inter-spike interval. Miniature recordings were analyzed using Mini Analysis Program (Synaptosoft, Decatur, GA, USA). Other traces were analyzed using Clampfit 10.2. All drugs were purchased from Tocris (Abingdon, United Kingdom).

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA-Seq data analysis

Reads were aligned using the STAR version 2.5.2b (Dobin et al., 2013) to the RNOR 6.0.88 Rat Genome. UCSC genome browser tracks were obtained by generating bigwig files (using wigToBigWig) and uploading them to the UCSC genome browser (Kent et al., 2002). Count files (Read counts per gene) were then used in differential expression analysis performed with the DESeq2 (Love et al., 2014) package. Differential gene expression of KSS knockdown was calculated in comparison to GFP control knockdown (See Table S4). If not otherwise stated, all data analyses were performed with an adjusted P value below 0.1 (p < 0.1) to define differentially expressed genes. Principle component analysis (PCA) plots were created with the DESeq2 package. GOTERM function gene annotation analyses were performed with DAVID (Huang et al., 2009). Scatterplots were made with all genes (FPKM < 1000) and differentially expressed genes. Principle component analysis (PCA) plots were created with the DESeq2 package. GOTE R function gene annotation analyses were performed with DAVID (Huang et al., 2009). Scatterplots were made with all genes (FPKM < 1000) and the ggpplot library (https://ggplot2.tidyverse.org/). Venn overlap diagram was created using the Venny (version 2.1) online tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html). RNaseq data are deposited to database: GEO: GSE120061.

MEA data analysis

Data analysis was performed offline using a custom software package named SpyCode (Bologna et al., 2010) developed in MATLAB© (The Mathworks, Natick, MA, USA). Spike detection. Spike were detected by using the Precise Timing Spike Detection algorithm (PTSD). Briefly, spike trains were built using three parameters: (1) a differential threshold set to 8 times the standard deviation of the baseline noise for each channel; (2) a peak lifetime period (set at 2 ms); (3) a refractory period (set at 1 ms). Burst detection. Burst were detected using a Burst Detection algorithm18. The algorithm is based on the computation of the logarithmic inter-spike interval histogram in which inter-burst activity (i.e., between bursts and/or outside bursts) and intra-burst activity (i.e., within burst) for each recording channel can be easily identified, and then, a threshold for detecting spikes belonging to the same burst is automatically defined. The number of the active channels (AC, above threshold 0.1 spikes/sec) and the number of bursting channels (BC, above threshold 0.4 burst/min and at least 5 spikes in burst with a minimal
inter-spike-interval of 100 ms). Furthermore, the mean firing rate (MFR), the burst frequency (MBR, mean burst rate), the burst duration (BD), the inter burst interval (IBI), the mean spike frequency intra burst (MFB) and the percentage of spike outside burst (PRS, percentage of random spike) of the network were obtained by computing the parameters of each channel and averaging among all the active electrodes of the MEA. **Network burst detection.** Synchronous event were detected looking for sequences of closely-spaced single-channels bursts. A network burst is identified if it involves at least the 80% of the network active channels. The distributions of the network burst duration (NBD, s) and Network Inter Burst Interval (NIBI, interval between two consecutive network burst, s) were computed using bins of 1 ms and 1 s respectively. **Network burst irregularity.** Irregularity was estimated by computing the coefficient of variation (CV) of the network inter burst intervals (NIBI), which is the standard deviation divided by the mean of the NIBI.

Criteria we used to identify insufficient quality of neuronal network activity were: i) wells with a firing frequency lower than 0.1 spikes/s; ii) wells that do not show network burst; iii) wells with low connectivity (< 80%); iv) networks with decreasing firing rate over development. If control networks within one batch showed one of the conditions reported above, the entire batch was discarded.

Mann-Whitney test was performed between two groups and one-way ANOVA test and post hoc Bonferroni correction was performed between all genotypes. Data are expressed as Mean ± standard error of the mean (SEM). N is indicated as amount of recorded MEA wells/amount of independent neuronal preparations. Details about mean, SEM, n and P values are reported in the Supplemental Information.

**Statistics**

The statistical analysis of all the reported data were performed using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA) and MATLAB® (The Mathworks, Natick, MA, USA). We determined normal distribution using a Kolmogorov-Smirnov normality test. Statistical analysis between all genotypes were performed with one-way ANOVA and Post hoc Bonferroni correction. We analyzed by differences between two groups (control and one genotype) by means of the Mann-Whitney-U-Test. P values < 0.05 were considered significant. Data are expressed as Mean ± standard error of the mean (SEM). If not mentioned differently in the corresponding figure legend, than n indicates the amount of recorded cells/amount of independent neuronal preparations. Details about mean, SEM, n and P values are reported in the Supplemental Information.

**DATA AND CODE AVAILABILITY**

The accession number for the RNA sequence reported in this paper can be found in database: GEO: GSE120061. All raw data composing the figures in this paper can be accessed via Mendeley Data: https://doi.org/10.17632/2v25frvmvs.1.