Neuronal network dysfunction in a model for Kleefstra syndrome mediated by enhanced NMDAR signaling

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Kleefstra syndrome (KS) is a neurodevelopmental disorder caused by mutations in the histone methyltransferase EHMT1. To study the impact of decreased EHMT1 function in human cells, we generated excitatory cortical neurons from induced pluripotent stem (iPS) cells derived from KS patients. Neuronal networks of patient-derived cells exhibit network bursting with a reduced rate, longer duration, and increased temporal irregularity compared to control networks. We show that these changes are mediated by upregulation of NMDA receptor (NMDAR) subunit 1 correlating with reduced deposition of the repressive H3K9me2 mark, the catalytic product of EHMT1, at the GRIN1 promoter. In mice EHMT1 deficiency leads to similar neuronal network impairments with increased NMDAR function. Finally, we rescue the KS patient-derived neuronal network phenotypes by pharmacological inhibition of NMDARs. Summarized, we demonstrate a direct link between EHMT1 deficiency and NMDAR hyperfunction in human neurons, providing a potential basis for more targeted therapeutic approaches for KS.
Advances in human genetics over the past decade have resulted in the identification of hundreds of genes associated with intellectual disability (ID) and autism spectrum disorder (ASD)\(^1\). Within this group of genes the number of chromatin regulators is remarkably high\(^2\)–\(^4\). These ASD/ID-linked chromatin regulators are engaged in genome-wide covalent DNA modifications, postranslational modifications of histones, and control of genomic architecture and accessibility\(^5\) to control the expression of genes important for neurodevelopment and/or neuroplasticity\(^3\). Nevertheless, there is still a large gap between elucidating the genetic architecture of neurodevelopmental disorders (NDDs) and deciphering the cellular or molecular pathobiology\(^6\). In particular, we require better understanding of the relevance of genetic changes with respect to downstream functional consequences and whether there is overlap between patients within the clinical spectrum\(^6\).

Kleefstra syndrome (KS) (OMIM#610253) is an example of a rare monogenic NDD with ID, ASD, hypotonia, and dysmorphic features\(^7\)–\(^9\). KS is caused by heterozygous de novo loss-of-function mutations in the *EHMT1* gene (euchromatin histone lysine methyltransferase 1) or by small 9q34 deletions harboring the gene\(^7\). In a complex with EHMT2, EHMT1 methylates histone 3 at lysine 9 (H3K9me1 and H3K9me2), promoting heterochromatin formation leading to gene repression\(^10\). Constitutive and conditional loss of EHMT1 function in mice and Drosophila lead to learning and memory impairments\(^11\)–\(^13\). In addition, *Ehmt1*\(^+/−\) mice recapitulate the developmental delay and autistic-like behaviors that are observed in KS patients\(^14\),\(^15\).

At the cellular level, these mice show a significant reduction in dendritic arborization and number of mature spines in CA1 pyramidal neurons\(^11\). The dynamic regulation of H3K9me2 by EHMT1/2 is also involved in synaptic plasticity and learning and memory\(^16\)–\(^18\). EHMT1 and 2 are required for synaptic scaling, a specific form of homeostatic plasticity, by regulating the expression of brain-derived neurotrophic factor (BDNF)\(^16\). Yet it remains unknown how deficits caused by loss of EHMT1 mechanistically affect the development of human neuronal networks.

Human-induced pluripotent stem (iPS) cell technology\(^19\) enables us to study the specific role of individual cell types in developing neural circuits. Patient-derived neurons allow us to examine the early pathophysiology of NDDs using single-cell and neuronal network electrophysiological recordings to recapitulate disease progression\(^20\),\(^21\).

Here, we generated iPS cell lines from three patients with different *EHMT1* loss-of-function mutations to differentiate them into excitatory cortical neurons. Through in-depth characterization at single-cell and neuronal network level, we uncovered a robust and defined phenotype that was consistent across all patient lines and was also observed in neurons with CRISPR-engineered disruption of *EHMT1*. At the molecular and cellular level, we show that the electrophysiological phenotype is mediated by upregulation of NMDA receptor (NMDAR) subunit 1, which we also find in *Ehmt1*\(^+/−\) mice. We conclude by showing that pharmacological inhibition of NMDARs rescues the KS-associated network phenotypes. Therefore, our findings establish a direct link between EHMT1 deficiency in human neurons and NMDAR hyperfunction, providing new insights into the pathophysiology of KS.

**Results**

**Single-cell level characterization of KS neurons.** We generated iPS cell lines from two patients with KS and two healthy subjects (respectively KS\(_1\), KS\(_2\) and C\(_1\), C\(_2\)) (Fig. 1a, Supplementary Fig. 1, Material and Methods). One patient (KS\(_1\)) had a frameshift mutation in *EHMT1* leading to a premature stop codon (p. Tyr1061fs, patient 25 in ref. \(^22\)), while the other patient had a missense mutation in the Pre-SET domain (p.Cys1042Tyr, patient 20 in ref. \(^3\)), predicted to disrupt the conformation of this domain. As expected Western blot and real-time quantitative polymerase chain reaction (RT-qPCR) analyses revealed a 50% reduction of EHMT1 expression in KS\(_1\), while KS\(_2\) showed normal EHMT1 expression levels (Fig. 1b, Supplementary Fig. 2A). In addition to these lines, iPS cells were generated from an individual who has a mosaic microdeletion on chromosome 9q34 (233 kb) including *EHMT1*.\(^23\) We selected an iPS clone harboring the KS-causing mutation (KS\(_{MOS}\)) as well as a control clone not carrying the *EHMT1* deletion (CMOS) (Fig. 1a, Supplementary Figs. 1 and 2). This isogenic pair shares the same genetic background except for the KS-causing mutation, thereby reducing variability and enabling us to directly link phenotypes to heterozygous loss of *EHMT1*. Western blot analysis and RT-qPCR analysis showed a 40% reduction of EHMT1 expression in KS\(_{MOS}\) compared to CMOS (Fig. 1b, Supplementary Fig. 2A). All selected clones showed positive expression of pluripotency markers (OCT4, TRA-1-81, NANOG, and SSEA4) and single-nucleotide polymorphism (SNP) arrays were performed to confirm genetic integrity (Supplementary Fig. 1, Material and Methods).

We differentiated iPS cells into a homogeneous population of excitatory upper layer cortical neurons (iNeurons) by forced expression of the transcription factor transgene *Ngn2*.\(^24\) For all experiments, iNeurons were co-cultured with freshly isolated rodent astrocytes\(^25\) to facilitate neuronal and network maturation (Fig. 1c). All iPS lines were able to differentiate into MAP2-positive neurons at similar efficiency (Supplementary Fig. 2C, D). EHMT1 expression was reduced in neurons derived from KS\(_1\) and KS\(_{MOS}\) but not KS\(_2\) (Supplementary Fig. 2B) compared to controls. However, all KS iNeurons showed reduced H3K9me2 immunoreactivity, indicative of EHMT1 haploinsufficiency (Supplementary Fig. 2E). Twenty-one days after the start of differentiation (days in vitro, DIV), both, control and KS iNeurons showed mature neuronal morphology. We measured this by reconstruction and quantitative morphometry of DsRed-labeled iNeurons. We observed no significant differences between control and KS iNeurons in any aspect of neuronal somatodendritic morphology, including the number of primary dendrites, dendritic length and overall complexity (Fig. 1d, Supplementary Fig. 3A–F).

ID and ASD have been associated with synaptic deficits in rodents and humans\(^21\),\(^26\). We therefore investigated whether *EHMT1*-deficiency leads to impairments in synapse formation. To this end, we stained control and KS iNeurons for pre- and postsynaptic markers (i.e., synapsin1/2 and PSD95, respectively) at DIV 21. We observed that putative functional synapses were formed on control and KS iNeurons (Supplementary Fig. 3G), without any indications for differences in the number of synaptic puncta between the different iPS cell lines (Fig. 1e, Supplementary Fig. 3G). Furthermore, whole-cell patch-clamp recordings at DIV 21 of iNeurons grown in the presence of tetrodotoxin (TTX) also revealed no differences in the frequency or amplitude of AMPA receptor (AMPA)-mediated miniature excitatory postsynaptic currents (mEPSCs, Supplementary Fig. 3H). This indicates that KS iNeurons are generally not impaired in the AMPAR component of the excitatory input they receive (Fig. 1f, Supplementary Fig. 3H). At DIV 21 nearly 90% of control and KS patient iNeurons fired multiple APs, indicative of a mature state of their electrophysiological properties (Supplementary Fig. 3I). When recording intrinsic passive and active properties from control and KS patient iNeurons at DIV 21, we found no differences when comparing controls with KS lines. However, we did observe minor differences in AP decay time and Rheobase for
the specific lines KS2 and KS1, respectively. These differences were not observed between the isogenic lines (Supplementary Fig. 3J–N, Supplementary Data 1). Collectively, our data indicate that there were no significant differences between control and KS patient iNeurons with regard to neuronal morphology, excitatory synapses and intrinsic properties.

KS neuronal networks show an aberrant pattern of activity. Dysfunction in neuronal network dynamics has been observed in the brain of patients with psychiatric and neurological conditions27. In addition, neuronal network dysfunction has been identified in model systems for several ID/ASD syndromes20,28. Therefore, despite the normal properties of KS iNeurons on single cell level, we hypothesized that impairments during brain development caused by loss of EHMT1 would be reflected at the network level. To test this hypothesis, we examined and compared the spontaneous electrophysiological population activity of neuronal networks derived from controls and KS patients growing on microelectrode arrays (MEAs) (Fig. 2a). MEAs allow us to noninvasively and repeatedly monitor neuronal network activity through extracellular electrodes located at spatially separated points across the cultures.

First, we monitored the in vitro development of control neuronal networks on MEAs. We found that the activity pattern of control iNeuron networks changed progressively over several weeks (Fig. 2b–f), similarly to what was observed in rodent neuronal cultures29. In particular, during the first three weeks of
Fig. 2 Spontaneous electrophysiological activity of control- and KS patient-derived neuronal networks. **a** Representative image of a control-derived neuronal network on MEAs stained for MAP2 (red) and PAN Axon (green). **b** Representative raster plot of electrophysiological activity exhibited by control-derived neuronal network at different time points during development. **c–f** Quantification of network properties as indicated. **g** Raster plot of spontaneous electrophysiological activity exhibited by **g** control and **h** KS networks at DIV 28. 6 s of raw data showing a burst recorded from a representative channel are shown for each iPS line. **i–l** Quantification of network parameters as indicated. **m, n** Histogram showing the distribution of the network burst duration (bin size = 100 ms) and **n** the network inter-burst interval (bin size = 1 s). **o** Quantification of percentage of spike outside network burst and **p** quantification of coefficient of variability of the inter-burst interval. n = 23 for C1; n = 10 for C2; n = 10 for C莫斯; n = 15 for KS1; n = 15 for KS2; n = 12 for KSmos. Data represent means ± SEM. *P < 0.05, **P < 0.005, ***P < 0.0001, one-way ANOVA test and post hoc Bonferroni correction was performed between controls and KS patient-derived cultures and Mann-Whitney test was performed between two groups. Source data is available as a Source Data file.
differentiation, control iNeurons mainly showed sporadic APs, i.e., spikes (Fig. 2b–f, DIV 17), indicating they were immature and not yet integrated in a network. By week four electrical activity was organized into rhythmic, synchronous events (network burst), composed of many spikes occurring close in time and across all electrodes (Fig. 2b–f, DIV 24). This indicates that the iNeurons had self-organized into a synchronically connected, spontaneously active network. Both the firing rate, which is a general measure of total activity across the entire network, and network burst activity increased during development but plateaued by DIV 28 (Fig. 2c, d). After this time point neuronal network activity remained stable (Fig. 2c–i). We observed no difference in the overall level or pattern of activity between the C1, C2, and C MOS at DIV 28 (Fig. 2g–i). The highly reproducible network characteristics observed across all controls provided us with a consistent, robust standard to which we could directly compare KS-patient derived neuronal networks.

Next, we characterized the neuronal networks derived from KS patients. Similar to controls, network burst activity appeared by the fourth week in vitro (Fig. 2h). The global level of activity of KS networks was similar to controls (i.e., the firing rate, Fig. 2i). However, we found that network bursts occurred at lower frequency and with longer duration (Fig. 2j, k, m, Supplementary Fig. 4A, B). As a consequence of the lower network burst frequency, the inter-burst interval was longer (Fig. 2l, n, Supplementary Fig. 4C, D). We also observed that spike organization differed from controls, which was indicated by the smaller percentage of spikes occurring outside the network bursts (Fig. 2o). A final aspect is that KS networks also exhibited an irregular network-bursting pattern, illustrated by the statistically larger coefficient of variation (CV) of the inter-burst interval (Fig. 2p). Interestingly, the increased burst duration phenotype observed at the network level was also present at single-cell level (Supplementary Fig. S3O). Indeed, whole-cell voltage-clamp recordings of spontaneous excitatory postsynaptic currents (sEPSCs), showed that the activity received by KS-derived neurons was composed by longer burst durations than in control. Taken together, our data show that KS neuronal networks consist of fewer and irregular network bursts, and the bursts themselves were longer in duration than in control networks.

CRISPR/Cas9 deletion of EHMT1 recapitulates KS phenotypes.

To further address whether heterozygous loss of EHMT1 is causing the observed KS patient-derived network phenotypes, we expanded our analysis and generated a second set of isogenic human iPS cells. We made use of CRISPR/Cas9 gene editing technology to generate an isogenic control and EHMT1 mutant iPS cell line with a premature stop codon in exon 2 (C CRSIPR and KSCRISPR, Fig. 3a, Supplementary Fig. 5F, G). Western blot and RT-qPCR analysis revealed that EHMT1 expression was significantly reduced in KSCRISPR iPS and iNeurons compared to C CRSIPR (Fig. 3b, c, e, Supplementary Fig. 5F, G). Both, C CRSIPR and KSCRISPR iPS cells differentiated equally well to iNeurons (Supplementary Fig. 5H). Furthermore, KSCRISPR iNeurons showed reduced H3K9me2 immunoreactivity compared to C CRSIPR iNeurons (Supplementary Fig. 5I). We observed no differences in the formation of functional synapses, based on immunocytochemistry and mEPSC recordings between C CRSIPR and KSCRISPR, corroborating our results with the other KS cell lines (Fig. 3c, f, Supplementary Fig. 3H). At the network level, C CRSIPR showed a control-like network phenotype (Fig. 3d, g–k). KSCRISPR networks exhibited a phenotype similar to the other KS patient networks with less frequent network bursts, longer duration and in an irregular pattern. This establishes a causal role for EHMT1 in the observed neuronal network phenotypes.

Our results demonstrated that EHMT1 deficiency causes a reproducible neuronal network phenotype. We observed non-significant iPS cell line-specific variability in the functional network properties of, both, the control and KS groups, so that the multiple descriptive parameters extracted from the raw MEA recordings clearly delineated control from KS networks. This was confirmed in an unbiased discriminant analysis of network parameters, where control and KS networks clearly clustered away from each other. This separation was not observed when the analysis was performed on single-cell parameters (i.e., morphology and intrinsic properties, Supplementary Fig. 6A–F). Our direct comparison of iNeurons derived from iPS cells with a frameshift, missense or deletion in EHMT1 showed that the phenotype is due to aberrant EHMT1 enzymatic activity rather than the disrupted protein.

KS iNeurons show increased sensitivity to NMDAR antagonists.

KS patient-derived neuronal networks showed an aberrant pattern of activity, mainly characterized by network bursts with longer durations than controls. Previous studies on rodent-derived neuronal networks have shown that burst duration is directly influenced by AMPARs and NMDARs. Specifically, previous reports used receptor-type specific antagonists to show that AMPAR-driven bursts have short durations while NMDAR-driven bursts have comparatively longer durations. We therefore hypothesized that increased NMDAR activity contributed to the lengthened bursts in KS networks. To test this, we pharmacologically blocked either AMPARs or NMDARs and compared the effect on control and KS neuronal network activity at DIV 28. In accordance with previous work, we found that acute treatment with an AMPAR antagonist (NBQX, 50 µM) abolished all network burst activity, whereas inhibiting NMDARs (D-AP5, 60 µM) only slightly decreased the network burst activity (Fig. 4a, c) for control networks. This indicated that network burst activity is mainly mediated by AMPARs. In particular, we found it to be mediated by GluA2-containing AMPARs, since the network bursts were not blocked with Naspm (10 µM), an antagonist that selectively blocks GluA2-lacking AMPARs (Supplementary Fig. 7B, pre-D-AP5). Similar to controls, in KS networks NBQX completely abolished network burst activity (Fig. 4b, d). However, in stark contrast to controls, D-AP5 robustly suppressed the bursting activity in EHMT1 deficient lines (Fig. 4b, d, Supplementary Fig. 7A). Interestingly, the suppression of network burst activity by D-AP5 in KS networks was transient. Network burst activity showed ~50% recovery after 30 min and had returned to baseline (i.e., pre-D-AP5 levels) after 24 h (Fig. 4e, Supplementary Fig. 7C). The early stages of homeostatic plasticity, specifically synaptic upscaling, are initiated by global neuronal inactivity and characterized by insertion of GluA2-lacking AMPARs (i.e., Ca2+-permeable AMPARs, CP-AMPARs) into the synapse to restore activity. To determine the nature of the recovery, we first blocked KS networks with D-AP5, and added Naspm after 1 h. Naspm completely blocked the reinstated network bursting activity, indicating the recovery in KS networks was due to synaptic insertion of GluA2-lacking AMPARs (Fig. 4e, Supplementary Fig. 7B). Of note, although in control networks burst activity was not suppressed by D-AP5, we did observe that Naspm had a small but significant effect on the burst rate. This indicates that NMDAR blockage in controls also induced less pronounced synaptic insertion of GluA2-lacking AMPARs. After 24 h, the network activity in KS networks was again completely suppressed with NBQX but only partially with Naspm, suggesting that GluA2-lacking AMPARs were actively exchanged with GluA2-containing AMPARs (Supplementary Fig. 7C). Collectively, our results demonstrate that NMDAR inhibition with...
D-AP5 induces synaptic plasticity in KS networks, allowing reinstating network burst activity through the incorporation of GluA2-lacking AMPARs, which later on are replaced by GluA2-containing AMPARs.

Intrigued by these findings, we sought an alternative way to block network bursting. The classic method for inducing synaptic upscaling, with the sodium channel blocker TTX, would necessarily prevent observation of early stages of the dynamic recovery on MEAs. To circumvent this issue, we used the antiepileptic drug Retigabine, which is a voltage-gated K+ channel activator that effectively hyperpolarizes the resting membrane potential in neurons

We reasoned that Retigabine would have the combined effect of acutely hampering neurons in reaching AP threshold while leaving the voltage-gated Na+ channels unaffected. Thus, while simultaneously strengthening the Mg2+ block on NMDARs, we could still observe any (re)occurring network activity on the MEA. Indeed, when we applied Retigabine (10 μM) to the networks, they were temporarily silenced, with no discernible spiking or bursting activity. Within 100 min, there was again a Nasm-sensitive recovery mediated by GluA2-lacking AMPARs, which later on are replaced by GluA2-containing AMPARs.

NMDARs are upregulated in KS iNeurons. The results from our pharmacological experiments suggested that NMDAR expression might be increased in KS iNeurons relative to controls. To test this hypothesis, we measured the transcripts of the most common NMDAR and AMPAR subunits by RT-qPCR for C莫斯 and KSamos iNeurons (Supplementary Fig. 7E). We were intrigued to find a fourfold upregulation of GRIN1 mRNA, which encodes NMDAR subunit 1 (NR1), the mandatory subunit present in functional NMDARs. We found no significant changes in any other NMDAR (GRIN2A, GRIN2B, GRIN3A) or AMPAR (GRIA1, GRIA2, GRIA3, and GRIA4) subunit that we analyzed. We further corroborated these results with Western blot analysis, which revealed significantly increased NR1 expression for KSMOS and KSCRISPR iNeurons compared to C莫斯 and CCRISPR (Fig. 5a, Supplementary Data 1). Our previous functional data indicated that the reduction in methyltransferase activity of EHMT1 was directly responsible for the phenotypes we observed at the network level. Therefore, we used chromatin immunoprecipitation qPCR (ChIP-qPCR) to investigate whether the increased GRIN1 expression correlated with reduced H3K9me2 at the GRIN1 promotor. Our results showed that for KSMOS and KSCRISPR iNeurons H3K9me2 occupancy was reduced at the GRIN1 promoter. In accordance with our previous study in Ehmt1−/− mice, we also found that the occupancy at the BDNF promotor was reduced in KSamos and KSCRISPR iNeurons (Fig. 5b). Next, using immunocytochemistry, we found that NR1 was significantly increased in KSamos and KSCRISPR iNeurons compared to Cmos and CCRISPR iNeurons (Fig. 5c). Finally, we investigated whether the increased NR1 level also resulted in increased synaptic NMDAR activity. To this end we infected control or KS iNeurons at 7 DIV with an adeno-associated virus (AAV2) expressing mCherry-tagged channelrhodopsin (±80% infection efficiency). We recorded from uninfected cells in voltage clamp at a holding potential of −70 mV (AMPAR) or +40 mV (NMDAR) and measured (blue)light-evoked synaptic responses by exciting
Altered neuronal network activity in Ehmt1+/− mice. Having established that EHMT1-deficiency alters neuronal network activity due to N1R upregulation in KS iNeurons, we next set out to measure neuronal network activity in Ehmt1+/− mice, a validated mouse model that recapitulates the core features of KS. Similar to what we found in iNeurons, primary cultures of cortical neuronal networks derived from Ehmt1+/− mice showed network bursts with lower frequency and longer duration compared to cultures from littermate controls. The MFR was unaltered (Fig. 6a). Using whole-cell voltage clamp recordings in acute brain slices, we measured the ratio between AMPAR- and NMDAR-mediated currents from cortical Layer 4 to Layers 2/3 synapses. We found that the NMDAR/AMPAR ratio was significantly increased in cortical networks of Ehmt1+/− mice compared to WT littermates (Fig. 6b). We found no changes in the kinetics of NMDAR-mediated currents, suggesting that there is no difference in the expression of NMDAR subunits 2A or 2B between WT and Ehmt1+/− mice (Supplementary Fig. 6B).

Finally, we found no changes in the frequency or amplitude of AMPAR-mediated mEPSCs suggesting that the increased NMDAR/AMPAR ratio in Ehmt1+/− mice is due to increased NMDAR activity (Fig. 6c).

NMDAR inhibition rescues KS neuronal network phenotypes. Our previous experiments showed that by inhibiting NMDARs in KS networks for 24 h, we could shift the balance so that neuronal networks were progressively driven by GluA2-containing AMPARs, similar to controls (Supplementary Fig. 7C). Based on these results, we reasoned that the phenotypes in KS networks could be rescued by chronically inhibiting NMDARs in mature neuronal networks. We chose to potentiate the channel pore of NMDARs, and thereby primarily inhibiting postsynaptic calciump flux, with the selective, noncompetitive open-channel blocker MK-801. The immediate effects of MK-801 (1 µM) on KS network activity were similar to D-AP5, where network bursting was transiently suppressed and then recovered by insertion of GluA2-lacking AMPARs, which were again completely inhibited by Nastp (Fig. 7a, Supplementary Fig. 8B). Next, we treated CMOS and KSMOS networks beginning at DIV 28 with MK-801 for 7 days. We found that chronically blocking NMDARs in both CMOS and KSMOS networks reversed the major network parameters that we measured in KSMOS iNeurons, bringing them closer to controls (Fig. 7b–g). More specifically, we observed an increased burst frequency (Fig. 7d), with a parallel reduction in network bursting duration (NBD) was reduced (Fig. 7e) and the network bursting became more regular (Fig. 7g) showing values similar to control.
indicative of a shift towards AMPAR-driven networks. A discriminant analysis based on the aforementioned parameters showed that the KSMOS networks treated with MK-801 for 7 days became segregated from untreated KSMOS networks and closer to CMOS networks (Fig. 7h). Similar effects of MK-801 treatment were also observed in KS CRISPR (Supplementary Fig. 8C–G).

Discussion

In this study, we developed a human model of KS that enabled us to identify specific functional aberrations, from gene expression to neuronal network behavior, due to EHMT1 haploinsufficiency. We found that excitatory networks derived from KS patients showed a distinct and robust neuronal network phenotype with striking similarity across different types of mutations in EHMT1.

The phenotype was characterized by network bursts with a longer duration, lower frequency and more irregular pattern compared to controls. At the cellular level, we demonstrated that the network phenotype was mediated by NR1 upregulation.

Interestingly, the neuronal phenotype was consistent across species and model systems. Indeed, we found that network bursts also occurred with a longer duration and irregular pattern in dissociated neuronal networks from either embryonic rats (i.e., where Ehmt1 was downregulated through RNA interference29,37) or Ehmt1+/− mice. This indicates that some network parameters are consistently and similarly altered in divergent KS models. The appearance of a consistent phenotype both in a system where excitatory and inhibitory transmission were present and in a system where inhibition was absent (human iNeurons) indicates a major contribution from aberrant excitatory neurotransmission to KS pathobiology.

One major characteristic of KS networks was the longer duration of the network bursts. A change in burst length can be indicative of synaptic changes in GABARs, NMDARs, and/or AMPARs31. Given that inhibition was absent in our human model, we focused our analysis on NMDARs and AMPARs. We present several lines of evidence that suggest the long bursts exhibited by KS networks are mediated by upregulation of NR1. First, by acutely blocking AMPARs and NMDARs with chemical inhibitors, we show that network burst activity in KS networks is strongly dependent on NMDAR-mediated transmission, in
contrast to control networks, where network bursts are mainly dependent on AMPAR-mediated transmission. Second, we were able to reverse KS network phenotypes, including the long NBD, by blocking NMDAR activity. Third, we found that NR1 is upregulated in KS iNeurons at both, mRNA and protein level. Fourth, NR1 upregulation is paralleled by H3K9me2 hypo-methylation at the GRIN1 promoter. Fifth, NMDAR/AMPAR ratio was increased in KS iNeurons at both, mRNA and protein level. This cross-species comparison further validates that observed effects are due to decreased EHMT1 enzymatic function and show that development of network activity in human and mouse cortical networks may follow evolutionarily conserved and stable epigenetic programming.

Genetic evidence has directly implicated NMDARs in NDDs. For example, multiple heterozygous mutations in NMDAR subunit genes have been identified to be causal for ID, ASD, or epilepsy. NMDAR dysfunction is mainly attributed to hypofunction, but there are observations associating NMDAR hyperfunction to ID/ASD. Upregulated NR1 protein levels were found in the cerebellum of ASD patients and the NR2A and NR2B subunits of the NMDAR were found to be upregulated in the valproic acid animal model of autism. In the Rett syndrome mouse model, loss of MECP2 function resulted in developmental dysregulation of NMDAR expression. Of note, work in mouse models of NDDs show that changes in NMDAR expression are temporally and spatially restricted. This illustrates the importance of evaluating at what developmental age and in which brain regions changes in NMDAR expression occur, which is especially relevant for the design of rescue strategies. Our data shows that NR1 is upregulated in KS iNeurons of cortical identity and support the idea that dysfunctional glutamatergic neurotransmission plays a role in NDDs.

By blocking NMDARs in KS networks, we were able to induce the early phase of synaptic upscaling enabling the incorporation of GluA2-lacking receptors, followed by the insertion of GluA2-containing receptors. This plasticity mediated by AMPARs was more easily initiated in KS than in control networks and allowed KS networks to switch, at least temporarily, from a mainly AMPAR-dependent manner to NMDAR-mediated plasticity after a comparatively milder stimulus. This is in agreement with a recent study showing that inhibition of EHMT1/EHMT2 activity reinforces early LTP in an NMDAR-dependent manner. The authors showed that pharmacologically blocking EHMT1/2 before a mild LTP stimulus increased the LTP response, highlighting a role for EHMT1 and associated H3K9me2 in metaplasticity. A link between NMDARs and EHMT1 has also been shown in vivo where NMDAR activity regulates the recruitment of EHMT1/2 and subsequent H3K9me2 levels at target gene promoters in the lateral amygdala, in the context of fear learning. This, together with our data, suggests that there is a reciprocal interaction, between NMDAR activity and EHMT1 function, and a positive-feedback mechanism where EHMT1 methylates the NR1 promoter upon activation by...
NMDARs. If and under which circumstances such a feedback mechanism is active during development and/or in adulthood deserves further investigation.

At the molecular level we and others have shown that EHMT1 haploinsufficiency can cause modest transcriptional changes\cite{12,16,37,50}. We found that the upregulation of NR1 correlates with reduced H3K9me2 occupancy at the GRIN1 promoter. This suggests that during normal development EHMT1 is directly involved in the regulation of NR1 expression. However, we cannot exclude that EHMT1 also regulates NR1 expression via other, less direct mechanisms. For example, EHMT1 is a member of a large complex that includes the neuron-restrictive silencer factor (NRSF/REST) repressive unit, which is important for repressing neuronal genes in progenitors, including NR1\cite{31,51,53}. In addition, growth factors such as BDNF, which is increased upon EHMT1 deletion\cite{16}, have been shown to increase NR1 mRNA levels in cultured embryonic cortical neurons\cite{54}. Finally, we know from previous studies that EHMT1 regulates genome-wide H3K9me2 deposition, altering the expression of multiple genes\cite{12,16,37,50}.

An important aspect of our study is the identification of a robust and consistent network phenotype linked to KS on MEAs. We show that KS networks differed from controls based on a set of parameters describing the neuronal network activity and using discriminant analysis. Our analysis showed that individual unrelated controls clustered with little variation, which was exemplified by the fact our predictive group membership analysis was able to accurately assign control networks to the control group (Fig. S6). In contrast, we found that KS patient lines significantly differed from controls, including their respective isocontrols (CMOS vs. KMOS and CCRISPR vs. KSCRISPR). It should be noticed here that the 233 kB deletion in KS MOS incorporates another gene (CACNA1B) that might affect neuronal function, which might explain minor changes compared to the

**Fig. 7** NMDAR antagonist MK-801 rescues KS network phenotypes. **a** MK-801 (1 µM) effect on KS_MOS neuronal network activity (DIV 28). After 90 min, NAspm was added. Graph showing the effect of MK-801 (1 µM) treatment on the neuronal network burst frequency for CMOS and KS_MOS derived neuronal network 20 min after application. The values are normalized to the nontreated (NT) condition. **b** Representative raster plot showing the activity exhibited by KS patient-derived neuronal network (KS_MOS) grown on MEAs either nontreated or after one week of treatment with MK-801. Six second of raw data showing a burst recorded from a representative channel are shown for the NT and the treated conditions. **c** - **g** Quantification of network properties as indicated, n = 8 for CMOS; n = 6 for KS_MOS and n = 6 for KS_MOS treated with MK-801. **h** Canonical scores plots based on discriminant analyses for control (i.e., C_C, C_MOS, and C_CCRISPR), KS_MOS and KS_MOS treated with MK-801 (84% correct classification). Discriminant functions are based on using the following network activity parameters: firing rate, network burst rate, network burst duration, percentage of spike outside network burst and coefficient of variability of the inter-burst interval. Group envelopes (ellipses) are centered on the group centroids, n = 50 for controls; n = 6 for KS_MOS and KS_MOS treated with MK-801. Pie charts visualize accuracy of discriminant analyses functions by showing the relative distribution of lines per a-priori group after reverse testing for group identity. Data represent means ± SEM. *P < 0.05, **P < 0.0005, ***P < 0.0001, one-way ANOVA test and post hoc Bonferroni correction was performed between conditions. Source data is available as a Source Data file.
rescue memory deficits that reinstating EHMT1 function in adult rescue in mature networks agrees with previous data showing contextually de-aged networks. We find that this level of analysis on neuronal networks can potentially detect patient-specific phenotypic variance that arises early in development. It is foreseeable that, in future studies, an in-depth interrogation of the network activity for networks consisting of different human derived cell types or networks from brain organoids would allow measuring more complex neuronal signals on MEAs. This would be especially relevant for the stratification of genetically complex disorders (e.g., idiopathic forms of ASD) as these in vitro network phenotypes could then be used as endo-phenotype in pharmacological studies.

We show that it is possible to rescue the neuronal phenotype by blocking NMDARs in mature networks, a finding that has important clinical relevance. For example, NMDAR antagonists like ketamine and memantine have been used successfully in mouse models to treat RTT46,47 and other NDDs45,48 and led in some cases to improvements in small open-label trials for autism55–57. These data as well as ours, provide preclinical proof of concept that NMDAR antagonists could ameliorate neurological dysfunction and reverse at least some circuit-level defects. Furthermore, our observation that neuronal phenotypes can be rescued in mature networks agrees with previous data showing that reinstating EHMT1 function in adult flies is sufficient to rescue memory deficits.13,58 This adds to a growing list of genetically defined ID syndromes that might be amenable to postnatal therapeutic intervention58.

Summarized, our study shows that combining iPSC cell-derived human neuronal models with neuronal network dynamics is a promising tool to identify novel targets for possible treatment strategies for NDDs, such as ID and ASD.

Methods

Patient information and iPSC cell generation. In this study we used in total four control and four iPSC lines with reduced EHMT1 function. In contrast to a previous study59 we included patients in this study that present the full spectrum of KS associated symptoms, including ID and ASD. KS1 and KS2 originate from two individuals: a female KS patient with a frameshift and a missense mutation in the EHMT1 gene, respectively (patient 25 in ref. 52 and patient 20 in ref. 52). An isogenic pair of iPSC lines (KS1 and KS2) originated from an individual diagnosed with a mosaic heterozygous 233 kbp deletion of chromosome 9 which includes the EHMT1 gene (deletion starts after exon 4) and CACNA1B (deletion spans Exon 3: F-GAAGCAAAACCACGTCACTG; R-GTAGTCCTCAAGGGTGGGG). The donor vector was a piggyback construct containing a hygromycin resistant cassette as well as sequences that enable homology-directed repair (HDR). The genome-edited iPS cell line shows identical SNP profiles with the corresponding iPS cell line used for gene targeting. DNA fingerprinting performed by sequencing the top four off-target sites confirmed the correct targeting of the insertion of premature stop codons in Exon 2 of the EHMT1 gene. Cells were nucleofected using the Human Stem Cell Nucleofector® Kit 2 (Lonza, VPH-5022) in combination with the AMAXA-2b nucleofector, program F16. After nucleofection cells were resuspended in E8 flex (Thermo Fisher Scientific) supplemented with Revitacell (Thermo Fisher Scientific). When the iPSCs reached a confluence of about 40% split in 1:3 ratio and transferred to E8 medium and subsequently split once to remove any proliferating cell from the culture. From DIV 3 onwards half of the culture was kept for 7 days in E6 medium (Thermo Fisher Scientific). The medium was changed every 2 days. Cells were harvested after 7 days for RNA extraction with the GenElute Mammalian Total RNA kit (Sigma). cDNA synthesis was performed with Superscript III and used for qPCR according to manufacturer’s protocol with TaqMan assays from Life Technologies. Data analysis was performed with Scorecard software (online tool Life Technologies), comparing with a reference set of pluripotent stem cell lines.

Neuronal differentiation. iPSCs were directly derived into, excitatory cortical Layer 2/3 neurons by overexpressing the neuronal determinant Neurogenin 2 (NGN2) upon doxycycline treatment based on Zhang et al.24 and as we described previously.25 To support neuronal maturation, 4% prepared rat astrocytes26 were added to the culture in a 1:1 ratio two days after plating. At DIV 3 the medium was changed to Neurobasal medium (Thermo Fisher Scientific) supplemented with B-27 (Thermo Fisher Scientific), glutaMAX (Thermo Fisher Scientific), primocin (0.1 µg/ml), NT3 (10 ng/ml), BDNF (10 ng/ml), and doxycycline (4 µg/ml). G418 (1 mg/ml) was added once to remove any proliferating cell from the culture. From DIV 6 onwards half of the culture was refreshed three times a week. The medium was additionally supplemented with 2.5% FBS (Sigma) to support astrocyte viability from DIV 10 onwards. Neuronal cultures were kept in the whole differentiation process at 37°C/5% CO2.

Cortical cultures from mice. Primary cortical neurons were prepared from Ehm1−/− and Ehm1+/− mice from individual E16.5 embryos as previously described.16 Since the genotype was unknown at the time of harvest, each embryo was collected and the brains were processed separately. Each whole brain was kept on ice in 1 mL L-15 medium, organized separately in a 24-well plate, and tail clips were collected for genotyping.

Neuronal morphology reconstruction. To examine morphology of neurons cells on coverslips were transfected with plasmids expressing Discosoma species red (dsRed) fluorescent protein one week after plating. DNAin (MTI-GlobalStem) was used according to manual instructions. Medium was refreshed completely the day after DNAin application. After the treatment cells were cultured as previously described.

After 3 weeks of differentiation cells were fixed in 4% paraformaldehyde/4% sucrose (v/v) in phosphate-buffered saline (PBS) and mounted with DAKO. Transfected neurons were imaged using a Zeiss Axio Imager Z1 and digitally reconstructed using Neurolucida 360 software (MBF—Bioscience, Williston, ND, USA). For large cells multiple 2-dimensional images of these neurons were taken and the neurons were manually stitched together using the stitching plugin of FIJI 2017 software. The three-dimensional reconstructions and quantitative morphometrical analyses focused on the somatodendritic organization of the neurons. We defined origins for

other KS lines. When we performed predictive group membership analysis, we found that individual KS networks were mostly assigned to the corresponding patient line (Fig. S6), indicating that this level of analysis on neuronal networks can potentially detect patient-specific phenotypic variance that arises early in development. It is foreseeable that, in future studies, an in-depth interrogation of the network activity for networks consisting of different human derived cell types or networks from brain organoids would allow measuring more complex neuronal signals on MEAs. This would be especially relevant for the stratification of genetically complex disorders (e.g., idiopathic forms of ASD) as these in vitro network phenotypes could then be used as endo-phenotype in pharmacological studies.

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individual primary dendrites by identifying emerging neurites with diameters that were less than 50% of the diameter of the soma. Axons, which were excluded from reconstructions in further analyses, were visualized by their lush thin properties, far reaching projections and numerous directional changes. Neurons that had at least two primary dendrites and reached at least the second branching order were considered for analysis. For morphometrical analysis we determined soma size, number of primary dendrites, length and branching points per primary dendrite, and total dendritic length. To estimate the total span of the dendritic field (receptive area) of a neuron we performed convex hull 3D analysis. Note, that due to the two-dimensional nature of the imaging data, we collapsed the convex hull 3D data to two-dimensions, resulting in a measurement of the receptive area and not the volume of the receptive field. Furthermore, each individual axon was performed to investigate dendritic complexity in dependence form distance to soma. For each distance interval (10 µm each) the number of intersections (the number of dendrites that cross each concentric circle), number of nodes and total dendritic length was measured. Discriminant function analysis with canonical discriminant functions and reclassification of group membership based on parameters describing neuronal morphology were performed in SPSS.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde/4% sucrose (v/v) for 15 min and permeabilized with 0.2% triton in PBS for 10 min at RT. Non-specific binding sites were blocked by incubation in blocking buffer (PBS, 5% normal goat serum, 1% bovine serum albumin, 0.2% Triton) for 1 h at RT. Primary antibodies were diluted in blocking buffer incubated overnight at 4 °C. Secondary antibodies, conjugated to Alexa fluorochromes, were also diluted in blocking buffer and applied for 1 h at RT (see above). To distinguish the nucleus of the dendrite field (receptive area) of a neuron we performed convex hull 3D analysis. Note, that due to the two-dimensional nature of the imaging data, we collapsed the convex hull 3D data to two-dimensions, resulting in a measurement of the receptive area and not the volume of the receptive field. Furthermore, each individual axon was performed to investigate dendritic complexity in dependence form distance to soma. For each distance interval (10 µm each) the number of intersections (the number of dendrites that cross each concentric circle), number of nodes and total dendritic length was measured. Discriminant function analysis with canonical discriminant functions and reclassification of group membership based on parameters describing neuronal morphology were performed in SPSS.

MEAs and data analysis. All recordings were performed using the 24-well MEA system (MultiChannel Systems, MCS GmbH, Reutlingen, Germany). MEA devices are composed by 24 independent wells with embedded microwires (i.e., 12 electrodes/well, 80 µm in diameter and spaced 300 µm apart). Spontaneous electrophysiological activity of iPSC-derived neuronal network grown on MEAs was recorded for 20 min. During the recording, the temperature was maintained constant at 37 °C, and the evaporation and pH changes of the medium were prevented by inflating a constant, slow flow of humidified gas (5% CO2 and 95% O2) onto the MEA plate (with lid on). The signal was sampled at 10 kHz, filtered with a high-pass filter (i.e., Butterworth, 100 Hz cutoff frequency) and the noise threshold was set at ±4.5 standard deviations. Network burst analysis was performed off-line by using Multwell Analyzer (i.e., software from the 24-well MEA system that allows the extraction of the spike trains) and a custom software package named SPYCODE developed in MATLAB (The Mathworks, Natick, MA, USA) that allows the extraction of parameters describing the network activity. The mean firing rate (MFR) of the network was obtained by computing the firing rate of each channel averaged among all the active electrodes of the MEA. Burst detection: bursts were detected using a Burst Detection algorithm. 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. From the burst detection, the number of bursting channels (above threshold 0.4 burst/s and at least 5 spikes in burst with a minimal inter-spike-interval of 100 ms) was determined. Network burst irregularity: irregularity was estimated by computing the CV of the NBI, which is the standard deviation divided by the mean of the NBI. Discriminant function analysis with canonical discriminant functions and reclassification of group membership based on parameters describing neuronal network activity were performed in SPSS.
Pharmacological experiments. Control and KS patient neuronal networks were treated acutely with D-AP5 (60 μM), NBQX (50 μM), MK-801 (1 μM), Naspim (10 μM), 6R-Cl-NMethyl-ACl (10 μM) at DIV 28 after a 20-min recording of spontaneous activity. Then, the recording was paused, the compounds were added to the MEA, and the recording was restarted after 5 min. We recorded neuronal network activity for 60 min with D-AP5 or NBQX, 90 min with MK-801, 100 min with Retigabine, and for 20 min after the addition of Naspim. In experiments where we examined the effect of chronic NMDAR blockade, control and KS patient neuronal networks were treated with 1 μM MK-801 starting at DIV 28 and lasting 7 days total. MK-801 was replenished every two days, where it was freshly diluted to 1 μM in complete Neurobasal medium for the routine half-medium change. All experiments were performed at 37°C.

Single-cell electrophysiology. For single-cell recordings we used neurons derived from C1, C2, C3, C4, C5, C6, C7, C8, K1, K2, K3, K4, K5, and K6-2 after 3 weeks of differentiation. 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 40× water-immersion objective (Olympus Life Science, PA, USA), an infrared video camera (Nikon, GmbH, Tokyo, Germany), a cooled charge-coupled device camera (COOLLED pE-200; 10 ms, 470 nm). Stimulus strength, location of stimulus were computed with software developed in MATLAB (The Mathworks, Natick, MA, USA). Uninfected cell were recorded in voltage clamp at a holding potential of −60 mV. Only considered neurons with spontaneous synaptic activities that were not driven by depolarizing current injection just sufficient to produce a cell voltage response. Burst detection, burst frequency and duration were determined with burst detection software in Clampfit. AMPA amplitudes were quantified indicating multisynaptic input, or if the averaged amplitude was below 25 pA. AMPA amplitudes were quantified as the peak amplitude at −70 mV holding voltage, and the NMDA amplitudes were quantified as the average of the 5 ms trace 65 ms after the stimulus artifact.

Animals. For the animal experiments presented in this study, mice heterozygous for a targeted loss-of-function mutation in the Ehmt1 gene (Ehmt1+/− mice) and their WT littermates on C57BL/6 background were used, as previously described11. Animal experiments were conducted in conformity with the Animal Care Committee of the Radboud University Nijmegen Medical Centre, The Netherlands, and conform to the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive 2010/63/EU.

Statistics. The statistical analysis for all experiments was performed using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). We ensured normal distribution using a Kolmogorov–Smirnov normality test. To determine statistical significance for the different experimental conditions p values <0.05 were considered to be significant. Statistical analysis between lines were performed with two-way ANOVA and Post hoc Bonferroni correction. We analyzed significance between Control and Kleefstra groups by means of the Mann–Whitney U test. Data are presented as mean ± standard error of the mean (SE). Details about statistics are reported in Supplementary Data 1 (Excel file statistics).

Receptor expression and localization. All procedures related to the expression of the H4R were performed as described12. The expression construct was a TRC-dependent single copy expression vector that includes the human H4R cDNA inserted into the reverse orientation translations regulator cassette of the TRC expression vector (Stratagene). HEK293 cells were transfected with the expression vector. Cell lines were used at 90% confluence. The expression was determined by quantitative RT–PCR and Western blotting of total cell lysates. The data presented were obtained from at least three independent experiments with three technical replicates each. The expression level was determined using the ΔΔCt method analyses.

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Acute slice electrophysiology. We used litter-matched Ehmt1+/− and Ehmt1−/− mice of adolescent age (postnatal day 21–24). Acute slices were prepared as described earlier3. In brief, animals were deeply anesthetized with isoflurane, then quickly decapitated. 350-μm-thick coronal slices were cut using a microtome (HM650V, Thermo Scientific) in ice-cold “cutting and storage” ACSF containing 87 mM NaCl, 11 mM d-glucose, 7.5 mM sucrose, 3 mM NaH2PO4, 2 mM CaCl2, 1 mM MgCl2, and 26 mM NaHCO3, continuously oxygenated with 95% O2/5% CO2 and set to 37°C. The slices were then transferred to a recording setup as described above, and incubated in recording ACSF (124 mM NaCl, 10 mM d-glucose, 3 mM NaH2PO4, 1.25 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, and 26 mM NaHCO3 at 30 °C with added 100 mM Picrotoxin to block GABAergic transmission. A bipolar electrode (CE2C55, FHC) coupled to a SD9 stimulator (Grass Instruments, RI, USA) was inserted into layer 4 of the auditory cortex, and pyramidal cells were placed in layer 2/3 above the bipolar electrode (<200 μm lateral distance) using 3–6 MΩ borosilicate pipettes filled with a Cs+–based intracellular solution containing 115 mM CsMeSO3, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl2, 4 mM Na2ATP, 0.4 mM NaGTP, 10 mM Na-Phosphocreatine, 0.6 mM EGTA, and 5 mM QX-314. The cells were held in voltage-Clamp mode controlled by an SEC-05-X amplifier (NPI, Tamm, Germany), and low-pass filtered at 3 kHz and sampled at 20 kHz with a Power-1401 acquisition board and Signal software (CED, Cambridge, UK). Data were analyzed in Clampfit 10.7 (Molecular Devices).

H4R staining and quantification. H4R protein expression was determined in wild-type and Ehmt1−/− brains using Western blot analysis and the rabbit polyclonal anti-H4R antibody (ab17776, Abcam, Cambridge, UK). Protein expression levels were normalized to β-actin mRNA levels measured by qPCR and expressed as fold changes relative to the WT littermates. The data presented were obtained from at least three independent experiments with three technical replicates each. The expression level was determined using the ΔΔCt method analyses.
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Author contributions

M.F., H.v.B., D.S., and N.N.K. conceived and supervised the study. M.N. performed all animal work. M.F., K.L., J.K., and N.N.K. designed all the experiments. M.F., K.L., J.K., G.G., B.M., J.v.R., K.F., N.K., A.O., W.v.d.A., I.v.d.W., T.K.G., and C.S. performed all the experiments. H.v.B., T.K., and H.Z. provided resources. D.S., M.F., K.L., G.G., N.K., K.F., A.O., B.M., J.v.R., and M.N. performed data analysis. M.F., K.L., J.K., and N.N.K. wrote the paper. D.S., H.v.B., T.K., and M.N. edited the paper.

Competing interests

The authors declare no competing interests.

Additional information

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