Trisomy 21 impairs synchronized activity and connectivity in developing human down syndrome cortical excitatory neuron networks

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Summary

Down syndrome (DS) is the most common genetic cause of intellectual disability, affecting one in 700 live births worldwide, and is caused by trisomy of the human chromosome 21 (Hsa21). Here, we investigated whether trisomy 21 alters the development of human excitatory cortical neuronal networks, and whether that could contribute to the neurodevelopmental phenotypes of DS. By recapitulating cerebral cortex development *in vitro* with Trisomy 21 and control isogenic and non-isogenic human induced pluripotent stem cells (hiPSC), we assessed the impact of Hsa21 triplication on activity and connectivity in developing excitatory cortical neural networks. Our analysis revealed a significant decrease in neuronal activity in TS21 neurons early in development. Unlike euploid cortical excitatory neurons, TS21 neurons failed to develop synchronised bursting activity *in vitro* and *in vivo* following transplantation into the mouse forebrain. Viral transynaptic tracing identified significant reduction of neuronal connectivity in TS21 neuronal networks *in vitro*, suggesting that reduced network connectivity contributes to the reduction of synchronised bursting. Furthermore, TS21 neurons displayed significantly reduced expression of voltage-gated potassium channels, with single-neuron recordings confirming the absence of hyperpolarization-activated currents. This points to a functional loss of the potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 (HCN1) in TS21 neurons. Together, these findings indicate that Hsa21 trisomy leads to alterations in ion channel composition, spontaneous synchronised activity, and neuronal connectivity in cortical excitatory neuron networks, which likely contribute to the neurodevelopmental features of DS.

Keywords: Neuroscience, Down Syndrome, neuronal activity, voltage-gated potassium channels

Introduction

Down syndrome (DS) is the most common chromosomal abnormality in humans, associated with a number of neurodevelopmental and neurological features, including microcephaly and intellectual disability[1]. Trisomy of Hsa21 (TS21) alters the expression of > 200 Hsa21 protein coding genes[2], resulting in a gene dosage imbalance believed to underlie several of the phenotypes observed in DS[3,4]. Trisomy 21 in humans commonly results in a range in developmental and morphological changes in the forebrain, including a decrease in neural progenitor cells[5], altered cortical lamination[6] and hippocampus morphology, a reduction of dendritic spines[7,8], and overall reduced brain volume[9]. In adulthood, individuals with DS face a high risk of early-onset Alzheimer's disease, largely due to the presence of the APP gene Hsa21, leading to a functional triplication of the APP locus[10]. Functionally, altered neuronal activity[11,12] and synchronization[13] are observed in the brains of individuals with DS, reflecting an altered network architecture characterised by increased local connectivity[14,15]. However, the developmental origins and cellular mechanisms underlying the changes in synchronised brain activity in DS are currently not well understood.

Several mouse models for human TS21 have been developed, which use either syntenic regions of mouse chromosomes or parts of human chromosome 21, many of which partially replicate some aspects of human DS neuropathology[16-21]. Human induced pluripotent stem cells (hiPSCs) and their directed differentiation to model the development of different regions of the CNS provide alternative systems to study human-specific aspects of neurodevelopmental disorders[22,23]. Several TS21 hiPSC lines have been generated[24-29] and used to investigate various features of AD pathology and development[30]. In our previous work, DS donor hiPSC- derived neurons showed normal *in vitro* single cell electrophysiological properties, but increased production of Aβ peptides, increased amyloid plaques and mislocation of

phosphorylated tau, reminiscent of AD pathology[29]. We also showed reductions in developing cortical neuron network activity *in vivo*[31]. However, in an another study, TS21 human neurons exhibited reduced synaptic activity, despite maintaining normal excitability[28]. Furthermore, it remains unclear whether and how synchronized network activity, which is essential for the formation and refinement of functional networks[32], is affected. Specifically, it is not yet known whether the reduced activity of pyramidal neurons is an intrinsic property of the ecitatory neuron network itself or is influenced by hyperactive interneurons[33] and/or astrocytes[34].

Here, we differentiated isogenic and non-isogenic TS21 and control hiPSCs to cortical excitatory neurons to study the effects of the extra Hsa21 copy on neuronal network activity and connectivity during early development. We identified impaired neuronal network activity development in TS21 neuronal networks *in vitro* and *in vivo* after transplantation into the somatosensory cortex of mice. We furthermore found that TS21 neurons were severely under-connected compared to control neurons. Using gene expression analysis, we discovered a downregulation of multiple voltage gated potassium channels and the hyperpolarization-activated cyclic nucleotide–gated channel HCN1 in TS21 neurons. Collectively, the ion channel changes, reduced neuronal connectivity, and alterations in network activity synchronization are likely to contribute to the functional deficits observed *in vivo,* which underlie the cognitive and intellectual features of Down syndrome.

Results

DS and control hiPSCs successfully differentiate into cortical-like excitatory neurons

We differentiated two TS21, DS hiPSC lines TS21-1, (DSIPS)[27], TS21-2, (Tho3B)[31] and two WT, control lines WT-1, (NDC1.2)[35], WT-2, (Tho3F)[31] towards a cortical forebrain identity as previously described[36]. Notably, TS21-2 and WT-2 are an isogenic pair, from the same donor, where the third copy of Hsa21 was lost in WT-2 during re-programming[31]. Immunofluorescence staining for the neural progenitor marker Pax6 and the proliferation marker Ki67 confirmed robust neuronal induction and abundant mitotically active neural progenitor cells **(Fig 1A)** that gave rise to Tbr1, Ctip2 **(Fig 1B)**, and Tuj1 **(Fig 1C)** deep-layer cortical neurons at day 50 in both control and TS21 lines.

Whole cell electrophysiological recordings on day 53/54 showed a large proportion of TS21 and control neurons firing action potentials after current injection (**SuppFig 1 A, D**). We did not detect significant differences in resting membrane potential (WT-1: n=20, -36±14mV; TS21-1: n=21, -31±14mV; WT-2: n=22, -32±13mV; TS21-2: n=19, -27±8mV; mean±SD, Fig 1B), evoked action potential amplitude (WT-1: n=14, 14±10mV; TS21-1: n=18, 16±11mV; WT-2: n=17, 19±13mV; TS21-2: n=13, 15±11mV; mean±SD, **SuppFig 1C**) or in voltage gated Na^+ currents (**SuppFig 1E**), indicating no differences in whole cell properties at this early developmental timepoint.

Excitatory network activity development is impaired in DS neurons

Human cortical neural networks undergo stereotypical activity changes during development, with an early phase of uncorrelated firing, followed by a phase of highly synchronised bursting, and lastly the emergence of highly structured, complex firing patterns later in development[37]. These complex patterns are similar to synchronised

bursting *in vivo* [38-40] and in cortical brain slices[41], and are believed to play a role in the maturation of neuronal circuits.

To test if synchronised bursting also occurs in TS21 cortical excitatory networks *in vitro*, we loaded cortical excitatory neurons derived from the two WT (WT-1, WT-2) and two TS21 hiPSC lines (TS21-1, TS21-2) with the membrane permeable calcium sensitive dye Oregon Green 488 BAPTA-1, AM (OGB), and recorded fluorescence changes over two minutes. Changes in the fluorescence signal (ΔF/F) served as a readout for neuronal activity and allowed the simultaneous recording of the neuronal activity of hundreds of neurons at single-cell resolution. As previously reported[37], we found that both WT lines acquired spontaneous synchronized bursting activity between day 50 and 60 **(SuppFig 2 A, B)**, which was sensitive to the reversible effect of the Na^+ channel blocker tetrodotoxin (TTX) (SuppFig 2 C, left).

Changes in network synchronicity were quantified by calculating power spectra for the average network activity (blue) and the average activity of individual neurons (red) (**SuppFig 2 C, D, E)**. In a synchronized network, the network power spectrum for a given frequency is close to the power spectrum of a single neuron. In unsynchronized networks the network power spectrum will be close to its own randomized power spectrum (cyan). Control synchronized networks had network power spectra overlapping with the power spectrum of individual neurons at 0.1Hz, indicating a high degree of synchronization at this frequency. Voltage-gated sodium channel blockade with tetrodotoxin (TTX) led to a complete loss of synchronized activity and the network power spectrum overlapped with its own, randomized power spectrum. After removal of TTX, neuronal firing returned to levels similar to before TTX application and showed a high degree of synchronicity (**SuppFig 2 C, right**).

Glutamate receptors are important mediators of excitatory network activity. Therefore, we tested if synchronized neuronal network activity is α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or N-methyl-D-aspartate (NMDA) receptor-dependent[37,42] by recording neuronal activity of WT neurons in the presence of the AMPA receptor antagonist CNQX (**SuppFig 2D**) or the NMDA receptor antagonist AP5 (**SuppFig 2E**). Both drugs led to a complete and reversible loss of synchronicity, although non-synchronized, spontaneous single neuron firing was still present under both conditions. We further noted a small decrease in the average neuronal activity, as indicated by a decrease in the power spectrum (red). Taken together, these results replicate previous findings[37], demonstrating that spontaneous synchronized network activity is caused by excitatory network activity and is AMPA and NMDA receptor dependent.

To test if this stereotypic, synchronized bursting behavior is also present in TS21 neuronal networks, we compared spontaneous network activity of TS21 and WT control neurons over a 30 day period. As reported previously[37], WT neurons showed strong but uncorrelated or weakly synchronized activity at day 50. By day 60, most of the neurons exhibited a high degree of synchronized bursting, which disappeared at day 70 (**Fig 2 A, C**). The phase of synchronized bursting observed at day 60 in WT networls was completely absent in TS21 networks, and synchronized firing did not initiate in TS21 networks up to day 80 (**Fig 2 B, D**), the last point at which we assayed activity. The loss of synchronized activity was caused by the extra copy of Hsa21, as isogenic control (WT-2) networks developed synchronized activity similar to the independent control line (WT-1) (**Fig 2 A, C**).

We further calculated the mean integral of the power spectra over 0.1 and 1 Hz and noticed a small but significant decrease of averaged neuronal network activity in both TS21 lines (TS21-1: n=17, 0.025±0.009; TS21-2: n=24, 0.016±0.014), compared to

WT neurons at day 50 (WT-1: n=22, 0.112±0.082; WT-2: n=25, 0.131±0.103; WT-1 vs. WT-2: ns, WT-1 vs. TS21-1: **, WT-1 vs. TS21-2: ***, WT-2 vs. TS21-1: ***, WT-2 vs. TS21-2: ***, TS21-1 vs. TS21-2: ns, Tukey's multiple comparisons test, *P <0.05) (**SuppFig 3 A, B**). Taken together, these results demonstrate that TS21 neurons do not undergo normal network activity development and lack a phase of spontaneous synchronized bursting activity present in WT neurons.

Transplantation and *in vivo* two-photon Ca2+ imaging confirms impaired network synchronization in TS21 neurons

Given that the *in vitro* networks are almost entirely excitatory, with only a small number of interneurons present at later time-points, and are not integrated within wider CNS networks, we engrafted control and TS21 neurons into the mouse forebrain and longitudinally followed their activity over time. Using this approach, we have previously found that the human neurons receive synaptic inputs from the host neurons[31]. To specifically record from the engrafted neurons, we transduced neural progenitor cells and already present immature neurons from each genotype with a lentiviral vector expressing the red fluorescence protein tdTomato and the genetically-encoded calcium sensor GCaMP6s[43]. We then transplanted this mixture of neural progenitor cells and immature (d36-38) neurons into the somatosensory cortex of immunodeficient adult mice and implanted a small cranial window, for *in vivo* imaging, on top of the cortex[31] **(Fig3 A)**. Cortical identity of transplanted neurons was confirmed by expression of the transcription factor Tbr1 (expressed in Layer 6 projection neurons) and the human-specific nuclear marker hNu 5 to 6 months post transplantation (**Fig 3 B**).

To confirm that the transplanted neurons were functional, we performed patch-clamp recordings in acute brain slices 5 months after transplantation. Grafted cells were identified based on fluorescence and selected for patching based on the presence of

pyramidal cell morphology. We did not detect significant changes in the resting membrane potential (WT-1: n=18 cells, -54±7 mV; TS21-1: n=21 cells, -49±13 mV; WT-2: n=27 cells, -57±9 mV; TS21-2: n=16 cells, -55±11 mV, mean±SD, **SuppFig 4 A**) or the input resistance (WT-1: n=18 cells, 1.34±0.63 GΩ; TS21-1: n=21 cells, 1.83±1.20 GΩ; WT-2: n=27 cells, 0.97±0.75 GΩ; TS21-2: n=16 cells, 1.35±1.07 GΩ, mean±SD, **SuppFig 4 B**) between all four lines. However, a significant decrease in membrane capacitance was present when comparing disomic WT-1 with trisomic TS21-1 neurons (WT-11: n=18 cells, 19.44±9.41 pF; TS21-1: n=21 cells, 13.06±7.07 pF; WT-2: n=27 cells, 24.58±11.96 pF; TS21-2: n=16 cells, 20.9±13.2 pF, mean±SD (**SuppFig 4 C).**

Neurons from all four lines fired action potentials after current injection (**SuppFig 4 D-I).** The frequency of evoked action potentials upon incremental steps of current injection was similar between WT-1 vs TS21-1 and WT-2 vs TS21-2, with the exception of at +50 pA between WT-2 vs TS21-2 (Tukey's multiple comparisons test after two-way ANOVA, interaction $F_{12,321} = 0.3128$, $P = 0.9869$; $^{*}P < 0.05$; *ns*, not significant, **SuppFig 4 L**). Evoked action potential amplitudes (WT-1: n=18 cells, 91.3±11.1 mV; TS21-1: n=14 cells, 77.0±16.4 mV; WT-2: n=24 cells, 87.9±16.0 mV; TS21-2: n=10 cells, 82.7±10.8 mV, mean±SD, **SuppFig 4 J**) and widths (WT-1: n=18 cells, 2.20±0.74 ms; TS21-1: n=16 cells, 1.59±0.48 ms; WT-2: n=24 cells, 2.13±0.99 ms; TS21-2: n=10 cells, 1.48±0.42 ms, mean±SD, **SuppFig 4 K**) were slightly reduced in TS21 neurons, which could be attributed to differences in neuronal maturation or electrogenesis between cells from the two genotypes.

Finally, we recorded spontaneous network activity in the grafts by *in vivo* imaging of GCaMP6-expressing neurons (**Fig 3 C).** While neurons in all grafts had spontaneous baseline neural activity at 3 months post-transplantation, we found a significant reduction in mean pixel-wise correlation across entire ROIs in both TS21 lines (**Fig 4**

D), indicating decreased synchronized neuronal activity between multiple somas and neuropil. To exclude that this synchronization deficit was not due to a delay in functional maturation of TS21 networks, we repeated recordings of spontaneous activity 5 months after transplantation (**Fig 4 F**). Again, we observed a strong synchronization defect in TS21 neurons. This deficit in network synchronization was associated with the presence of an extra copy of Hs21 and was reversed by normalization of the Hsa21 copy number in the isogenic control (WT-2 cell line).

Reduced connectivity in TS21 neuronal networks

To test whether changes in connectivity could underlie the synchronized network activity defect present in TS21 neural networks, we assayed the connectivity of DS and control neurons using a pseudotyped, monosynaptic spreading rabies virus[22,44]. To do so, we transduced neurons with low titer lentivirus (LV-SYN-HTG) expressing a histone H2B-GFP fusion, the TVA receptor and a glycoprotein (H2B-GFP::TVA::G), under the control of the human synapsin 1 promoter. After 10 days we transduced neurons expressing TVA with a pseudotyped mCherry-expressing rabies (Rabies-mCherry) virus. Since high levels of rabies virus can have negative effects on cell survival, we limited rabies spread to 7 days. Primary infected neurons were double positive for H2B-GFP and mCherry. Monosynaptically connected neurons to those primary infected neurons were positive for mCherry only (**Fig 4 C**).

To score connectivity, we used an automated high-throughput imaging system to take images of the middle of the culture dish in an unbiased way (**Fig 4 A**), scoring average connectivity as the number of mCherry positive neurons relative to GFP positive neurons. We calculated a connectivity value consisting of the ratio of the red to green fluorescence pixel values, with higher values indicating higher connectivity[22]. This approach normalised for differences in primary infection levels of the lenti- and rabies virus. Using this metric, there was a significant decrease in

connectivity among TS21 neurons compared to control neurons (WT-1: 1.18±0.04; TS21-1: 1.02±0.04; WT-2: 1.20±0.06; TS21-2: 1.04±0.06; mean±SD; n=4 wells) (**Fig 4 B**). This connectivity defect was specific to TS21 neurons as it was not present in disomic isogenic control neurons (WT-2), which had connectivity rates indistinguishable from the unrelated euploid (disomic) control line (WT-1). Furthermore, the observed decrease in connectivity in TS21 neurons was not due to the inability of the rabies virus to transfer between TS21 neurons, as we were able to detect clusters of mCherry-only positive neurons in all four lines, but to a much lower extent in TS21 neuronal cultures (**Fig 4 C**).

The mechanism underlying retrograde monosynaptic rabies spread is not fully understood and it is conceivable that virus spreading is influenced by neuronal activity. In that case, our observed decrease in connectivity in trisomic neurons could be explained by their lower basal network activity (**Supp Fig 3 B**). We tested this hypothesis by adding the sodium channel blocker tetrodotoxin (TTX**; SuppFig 2 C**) to the culture medium 24h after rabies virus infection and for the 7 days thereafter. We did not detect a significant difference in connectivity rates between TTX (0.88±0.02) and mock treated neurons (0.85±0.02), indicating no effect of neuronal activity on rabies virus spreading between neurons (**Supp Fig 5**). Taken together, these results indicate that the extra copy of Hsa21 reduces neuronal connectivity among TS21 neurons.

Voltage gated potassium (K+) channel expression is altered in TS21 neurons

Ionotropic glutamate receptors mediate synaptic transmission, plasticity and memory function[45]. Several DS mouse models show deficits in hippocampal dependent learning, as well as LTP and LTD induction, indicating a role of AMPA and NMDA receptors in DS[46-49]. Since we found that spontaneous, synchronised network activity is AMPA and NMDA receptor dependent **(SuppFig 2 D, E),** we asked if the lack of

synchronicity in TS21 neurons could be caused by dysregulation or loss of ionotropic glutamate receptors. We performed an RNAseq analysis on day 50 and found 235 genes that were significantly upregulated and 160 genes that were significantly downregulated in trisomic versus WT control neurons.

We first asked if genes located on Hsa21 are among those significantly upregulated genes in TS21 neurons. Indeed, we found a significant upregulation of many Hsa21 located genes including Amyloid Precursor Protein (APP), Down Syndrome Cell Adhesion Molecule (DSCAM), the Glutamate Ionotropic Receptor Kainate Type Subunit 1 (GRIK1) and the Potassium Voltage-Gated Channel Subfamily J Member 6 (KCNJ6) (**SuppFig 6 A**), which have all been implicated in DS pathology[10,50]. Next, we asked if there are changes in the expression levels of the AMPA and NMDA receptor subunits. Whilst we did not detect changes in most AMPA and NMDA receptor subunits, we found a significant upregulation of GRIN2A in TS21 neurons. GRIN2A is located on Hsa16 and encodes for the NMDA type subunit 2A (NR2A), which is predominantly expressed in the adult brain, particularly in neurons[51-53]. This increase in NR2A subunit expression is in line with a previous report that showed increased NR2A expression in a DS mouse model leading to changed Ca^{2+} dynamics in those neurons[54] (**SuppFig 6 B, C)**.

Next, we performed a gene ontology (GO) analysis and found that the GO terms “synaptic transmission” and “Voltage gated potassium channel activity” were highly overrepresented amongst downregulated genes in TS21 neurons. This was reflected in a marked downregulation of voltage gated (KCNA4, KCNQ5, KCNJ10, KCNH3, KCNH7, KCNJ3) and calcium activated K+ channels (KCNN1) in TS21 neurons (**Fig 6 A**).Importantly, a similar transcriptional dysregulation can be found in excitatory neurons of the DS midgestational cortex (Lattke et al, submitted). We also found a significant downregulation of the potassium/sodium hyperpolarization-activated cyclic

nucleotide-gated channel 1 (HCN1), a mediator of rhythmic activity[55]. To further test the role of HCN1 in DS, we performed electrophysiological recordings of WT control and TS21 grafted neurons *ex vivo*. Intriguingly, we found that hyperpolarization-activated currents, elicited after current injection, were significantly reduced in TS21 neurons **(Fig 5 B-D)**. Those currents showed a strong correlation between membrane hyperpolarization and current voltage **(SuppFig 7),** suggesting that they are mediated by HCN channels, since membrane hyperpolarization is both necessary and sufficient to activate these channels, further implicating the HCN1 channel in DS pathology.

Discussion

In this study, we have longitudinally assayed the development of spontaneous neuronal network activity and connectivity of developing isogenic TS21 and control hiPSC-derived cortical forebrain neurons, both *in vitro* and *in vivo*. We found a modest decrease of spontaneous firing activity in TS21 neurons early in development, and a marked reduction[32] of synchronized bursting activity in trisomic neuronal networks. Synchronized bursting is found in many different model systems[37-41,56,57], suggesting a universal feature of neuronal network development and maturation. Interestingly, EEG recordings and event-related brain potentials of individuals with DS also exhibit altered cortical network activity and weaker cortical synchronization[11-13], and decreased synchronization was also found in the neocortex[18] and in primary hippocampal neurons of DS mice[17]. Our results suggest that the changes in synchronization observed in TS21 neurons are intrinsic to the excitatory neuronal network and unlikely to be mediated by the low number of interneurons[58] or astrocytes[34], at least in these early stages of development. In support of this, we recently generated a single-cell multiomic atlas of the DS midgestational cortex that revealed broad changes in genomic programs, including downregulation of membrane potential and synaptic function genes, primarily affecting the excitatory neuron lineage (Lattke et al., under revision).

To investigate whether the reduction of synchronised neuronal network activity could result from altered connectivity between neurons, we used a retrograde, pseudotyped rabies virus[22,37,44], and found a significant decrease in connectivity between trisomic neurons. Importantly, trisomic neurons were not impaired in forming connections *per se*, nor was the virus spread inhibited by the extra Hsa21 copy. *In vivo,* rabies spread is tightly correlated with synaptic inputs and only occurs between chemical synapses and not via gap junctions or local spreading of the virus[59], which raises the question of whether the lower connectivity rates in trisomic neurons could

be explained by their reduced spontaneous activity. However, a complete block of spontaneous activity with TTX in control neurons did not alter the connectivity rate compared to untreated neurons, indicating that the decrease in connectivity observed in trisomic neurons is independent of neuronal activity. We also considered whether changes in synaptic numbers or functional properties could explain the deficit in connectivity in Ts21 neurons. A reduction of total synapse number has previously been reported in a DS mouse model[19], but a study using hiPSC-derived cortical neurons did not detect differences in synapse numbers and further showed functional synapse formation in both trisomic and control neurons[29]. Importantly, we also did not detect changes in the synaptic properties of trisomic neurons.

The finding of reduced connectivity between T21 cortical neurons is in line with a human study of MR functional connectivity showing a simplified brain network architecture in the DS brain, dominated by a high degree of local connectivity and a lack of long-range connectivity[15]. Whilst we did not detect topological changes, we found a pronounced decline of interconnected neurons in trisomic networks, which could interfere with normal neuronal synchronization during development.

Finally, to further explore mechanisms of reduced synchronicity, we performed RNA sequencing of *in vitro* neurons. Our gene expression analysis found a strong upregulation of KCNJ6, as well as a downregulation of many voltage gated K+ channels, potentially implicating those channels in DS pathology. Potassium channels are important mediators of neuronal excitability and action potential shape. In particular, the K+ channel KCNJ6 is located on Hsa21 and leads to changes in synaptic plasticity in a DS mouse model[20]. Furthermore, KCNJ6 currents are elevated in hippocampal neurons of DS mice due to impaired GABAergic inhibition[21]. Single neurons from DS mice further exhibit reduced K+ currents related to changes in A-type, M-type and delayed rectifier channels[17], which could impact network activity. We also found a downregulation of the HCN1 channel. HCN channels, which

upon activation conduct a mixed Na^+/K^+ current, thus depolarizing the membrane towards firing threshold of action potentials, have been implicated in several neuronal functions, including the regulation of dendritic integration and synaptic transmission, as well as the generation of neuronal and network rhythmic oscillations in several brain regions[55]. Various HCN channel subtypes are differentially expressed at distinct developmental periods and contribute to network activity maturation[60]. In cortical neurons, hyperpolarization-activated currents are mainly mediated by the HCN1 subtype[61] and HCN1 channels are important to regulate dendritic excitability[62], and misregulation can contribute to multiple neurological disorders[63]. Interestingly, Aβ production is dependent on HCN1 channel activity *in vitro*[64] and a recent meta-analysis found a significant downregulation of HCN1 in the human AD brain[65], thus implicating HCN1 expression in AD pathology. Consistent with this evidence, we found a reduction of HCN-mediated hyperpolarization-activated currents in TS21 neurons *ex vivo*, which could potentially alter cortical network activity and development in TS21 neurons.

In summary, our data from isogenic control and DS neurons reveal a fundamental difference in the development of DS excitatory neuronal networks and a direct influence of the extra Hsa21 copy in channel composition, neuronal network activity development and connectivity. This hampered neural network synchronization and connectivity is intrinsic to the excitatory neuron population and likely contributes to the neurodevelopmental and neurological features of DS.

Materials and Methods

Generation of iPSCs

Skin biopsies were collected from donor individuals with DS by the University of Cambridge Dept. of Psychiatry, following approval of the Cambridgeshire Research Ethics Committee, with informed consent from the donor or representative. Samples were designated anonymous identifiers following collection. Skin punch biopsies were subdivided and cultured at 37°C in 5% CO2 in DMEM + Glutamax (ThermoFisher) supplemented with 10% fetal calf serum, penicillin (50 U ml^{-1}), and streptomycin (50 µg ml^{-1}) and 1mM Sodium Pyruvate (Sigma) until fibroblast outgrowth was observed. When plates were confluent, fibroblasts were trypsinized and transferred to T75 dishes for further expansion. Cellular reprogramming of fibroblasts was carried out using Cytotune 2.0 Sendai Reprogramming kits (ThermoFisher) according to the manufacturer's protocol, and established using feeder free conditions in Essential-8 media (ThermoFisher).

Cortical neuron differentiation

All cells were maintained at 5% CO2 at 37°C in a humidified incubator. WT1: NDC1.2[35], DS1: DSIPS[27], WT2: (Tho3F), DS 2: (Tho3B)[31] iPSC were cultured in Essential-8 medium (ThermoFisher) as feeder free cultures on Geltrex (ThermoFisher) coated plates. Neuronal induction was performed as described previously[36]. In brief, iPSCs were harvested with Accutase (Innovative Cell Technologies) as single cell suspension and plated at a density to reach 100% confluency in 24h and neuronal induction was started (day 0). Essential-8 medium was changed to neuronal induction medium consisting of neuronal maintenance medium supplemented with 10µm SB43152 (Tocris) and 1µm Dorsomorphin (Tocris). The medium was changed daily until day 12. On day 12 the neuroepithelial sheet was lifted with Dispase (ThermoFisher), broken into smaller clumps and plated on laminin (Sigma) coated plates in neuronal induction medium. On day 13 the medium

was changed to neuronal maintenance medium supplemented with 20 ng ml^{-1} FGF2 (PeproTech). Medium was changed every other day and FGF2 was withdrawn from the medium on day 17. Cells were split with Dispase at a 1:2 ratio when neuronal rosettes started to meet. On day 25 cells were dissociated with Accutase (ThermoFisher) and re-plated on laminin coated plates. Until day 34 cells were expanded at a 1:2 ratio when they reached 90% confluency. On day 36 neurons were plated on laminin coated plates at 85000 cells/cm^2 and used for subsequent experiments.

Neuronal maintenance medium (NMM) (1L) consists of 500ml DMEM:F12+glutamax (ThermoFisher), 0.25ml Insulin (10mg ml^{-1}, Sigma), 1ml 2-mercaptoethanol (50mM ThermoFisher), 5ml Non-essential amino acids (100X ThermoFischer), 5ml Sodium Pyruvate (100mM, Sigma), 2.5ml Pens/Strep (10000U/µl, ThermoFisher) , 5ml N2 (ThermoFisher), 10ml B27 (ThermoFisher), 5ml Glutamax (100X, ThermoFisher) and 500ml Neurobasal (ThermoFisher) medium.

Immunofluorescence staining

Cells were washed with PBS and fixed with 4% paraformaldehyde for 10min at room temperature (RT). They were washed (5min 3 X TBS, 3 X TBS + 0.3% Triton-X100 at RT) and blocking was performed with 5% normal goat serum in TBS + 0.3% Triton-X100 for 1h at RT. Cells were incubated with primary antibodies overnight at 4C using the following antibodies: TBR1 (1:1500, Abcam: ab31940), CTIP2 (1:1000, Abcam: ab18465), TUJ1 (1:1000, Biolegend: 8012021), GFP (1:1000, Abcam ab13970), mCherry (1:1000, Clontech: 632543). On the next day, cells were washed (5min 3 X TBS, 3 X TBS + 0.3% Triton-X100 at RT) and incubated for 1h at RT with Alexa-dye conjugated secondary antibodies (Thermo Scientific) diluted 1:500 in TBS + 0.3% Triton-X100. Cells were washed 6 X for 5min with TBS and 4',6-Diamidino-2-Phenylindole (DAPI, 5µg/ml final concentration) was added to the second wash step.

Imaging was performed using an inverted Leica TCS SP8 scanning confocal microscope (Leica Microsystems) or Opera Phenix High-Content Screening System (Perkin Elmer).

In vitro calcium imaging

Neurons were switched to complete BrainPhys (BP) medium (STEMCELL Technologies) and network activity was assessed between day 50 and day 80. 6 to 8 independent wells were assayed per line and the same wells were assayed over this time period. Neurons were loaded with the calcium indicator Oregon Green 488 BAPTA-1, AM (OGB, ThermoFisher). OGB was dissolved in DMSO and 0.4% w/v Pluronic F-127 (Sigma-Aldrich) to a concentration of 0.8mM OGB. This solution was further diluted with BP medium to a final concentration of 3.2µm and neurons were incubated with OGB for 1h at 37°C and 5%CO_2. Neurons were washed 3 times with BP medium and network activity was assayed 30min afterwards. The cells were imaged at 37°C and 5%CO_2 on a Deltavision wide field fluorescence microscope (GE Healthcare Lifescience) equipped with an EMCCD camera. Four two-minute time lapse movies at 10Hz were captured per well. Time lapse movies were analyzed using a custom written Matlab (Mathworks) script. For pharmacological experiments cells were recorded in BP medium containing 1µM tetrodotoxin (TTX, Tocris Bioscience), 50µm 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Bioscience), or 50µm DL-2-Amino-5-phosphonopentanoic acid (AP5, Tocris Biocience). After the recording session drugs were removed by three washes with BP medium and the same locations were imaged 45min later.

Lentiviral generation

Third generation lentivirus was produced by calcium phosphate transfection of the Lenti-X 293T cell line (Clontech) with pBOB-synP-HT (Addgene plasmid # 30456) expressing a histone:GFP, TVA and rabies glycoprotein separated by 2A sites from the human synapsin promoter. Cells were co-transfected with the packaging

plasmids plasmids pRSV-Rev (Addgene plasmid # 12253) and pMDLg/pRRE (Addegene plasmid # 12251) and the VSVG envelope plasmid pMD2.G (Addgene plasmid # 12259). Medium was replaced 16h after transfection. The virus supernatant was harvested after 8h replaced with new medium and harvested again after 12h. Both collections were pooled, debris was removed with a 0.45µm filter and virus supernatant was stored at -80°C for subsequent use.

Monosynaptic rabies tracing

Neurons were grown in BP medium as described above. On day 40 they were infected with low titer SYN-HTG lentivirus. Medium was exchanged the next day to remove the lentivirus. On day 50 neurons were infected with mCherry expressing pseudotyped rabies virus provided by Marco Tripodi, MRC Laboratory of Molecular Biology, Cambridge UK. Neurons were fixed 6 days later and immunofluorescence stainings for GFP and mCherry were performed as described above. To test the effects of neuronal activity on virus spread, neurons were infected with low titer SYN-HTG lentivirus on day 38. Rabies infection was performed on day 48. 24h after the infection 1µM TTX was added to the medium. Medium was changed every other day with TTX supplemented until fixation on day 54.

RNAseq library preparation and analysis

Neurons were grown in neuronal maintenance medium. On day 50 they were harvested with Accutase, homogenized with a QIAshredder (Quiagen) and RNA was isolated using the RNeasy Mini Kit (Quiagen) following the manufacturer's protocol. RNA quality was assayed on a 2200 TapeStation Instrument (Agilent) and sequencing libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina) following the manufacturer's protocol. 50bp single read ends were performed on a HiSeq4000 (Illumina) sequencer. Each sample library comprised 91-109 million reads, of which ~83% were mapped to the GRCh37 genome using STAR[66]. DESEq2[67] was run on counts data for genes with >1 count. Differentially

expressed (DE) genes were selected using a Benjamini-Hochberg (BH) adjusted p-value of 0.05; for Figure X, the gene counts are normalized for library size using DESeq2. goseq[68] was used for gene ontology (GO) analysis of DE genes. The list of downregulated or upregulated genes were compared individually against the list of all detected genes and significantly overrepresented GO terms were selected using a BH adjusted p-value of 0.05.

Cell Transplantation

Animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986, UK. Immunodeficient mice (JAX™ NSG, Charles River) aged between 3-5 months were given 3% isoflurane mixed with oxygen as induction anaesthesia, followed by an intraperitoneal injection of ketamine (0.083 mg/g) and xylazine (0.0078 mg/g). Craniotomies were performed over the right somatosensory cortex, as previously described[69]. Neural cortical progenitors were allowed to differentiate for 31 days, before being transduced with a lentiviral vector carrying either GFP or GCaMP6s plus tdTomato genes, under the human Synapsin-1 promoter. Between days 35 and 38, cells were dissociated and injected into the right somatosensory cortex with a glass needle using a micro syringe pump (UMP-3, World Precision Instruments), at the following stereotactic coordinates: anterior-posterior = -1.8 mm; medial-lateral = +2.8 mm; dorsal-ventral = -0.5 mm from bregma. Approximately 40.000 cells were injected in 1 µl of sterile cortex buffer solution. A 5-mm diameter glass coverslip was placed over the craniotomy and sealed with cyanoacrylate tissue adhesive. The exposed skull was covered in dental cement and a metal plate placed on the left side of the skull, for positioning at the 2-photon microscope stage.

***In vivo* Imaging**

Animals were imaged in a custom built 2-photon microscope (Prairie Technologies) equipped with a tunable Ti:saphire laser (Coherent) and a 20x water immersion

objective (NA 1.2 Olympus). Animals were tightly head-fixed to the microscope stage and lightly anesthetized using isoflurane at a concentration between 0.5-1% mixed with oxygen. Body temperature was maintained by placing the animals on a heat pad. Transplanted cells were first identified by their tdTomato signal using a 1040 nm laser beam, and single plane images were taken. GCaMP6 was then excited using a 920 nm pulsed laser beam, and five-minute time lapse movies were acquired at 3 Hz in a single plane. Data was acquired for approximately 60 minutes per animal. Time lapse movies were corrected for possible within-scan motion using the moco plugin in ImageJ (NIH). A pixel-wise correlation analysis was performed on all calcium movies in the following way: a median projection over time of the calcium imaging movie was subtracted from each frame in the movie to provide a ΔF time-series. The ΔF time-series of each pixel was then correlated with every other pixel in the movie. The mean and the standard-deviation of the distribution of correlation values was then taken for each pixel.

Electrophysiology

Acute coronal brain slices (350 µm) were maintained at room temperature (22-25 °C) in external solution (NaCl 125 mM, KCl 2.5 mM, NaH_2PO_4 1.25 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, D-glucose 10 mM and D-mannitol 15 mM, pH adjusted to 7.4 and equilibrated with 95% O_2 and 5% CO_2). Human neurons were identified in the somatosensory cortex by excitation of GFP. Whole-cell current-clamp recordings were performed at 35 °C using borosilicate glass micropipettes with a resistance of 4-8 MΩ filled with intracellular solution (K^+ gluconate 130 mM, KCl 10 mM, EGTA 0.5 mM, NaCl 1 mM, $CaCl_2$, 0.28 mM, $MgCl_2$ 3 mM, Na_2ATP 3 mM, GTP 0.3 mM, phosphocreatine 14 mM and HEPES 10 mM, pH adjusted to 7.2). Current-voltage relationships were evoked by stepwise current injection from -80 up to +50 pA in steps of 10 pA.

Immunofluorescence

At the end of the *in vivo* experiments, animals were given an overdose of ketamine and xylazine, and were intracardially perfused with 4% paraformaldehyde (PFA). Brains were dissected and fixed overnight in 4% PFA solution. 40 µm-coronal brain sections were cut using a vibratome (Leica Biosystems) and stained free-floating. Briefly, sections were permeabilized and blocked in a 10% donkey serum in PBS with 0.5% Triton X-100 for 1h at room temperature, followed by overnight incubation at 4 °C with anti-human nuclei antibody (1:200, Millipore: MAB 1281) and anti-Tbr1 antibody (1:200, Abcam: ab31940) in 5% donkey serum in PBS with 0.1% Triton X-100. Sections were subsequently washed and incubated for 2h at room temperature with Alexa Fluor conjugated secondary antibodies (1:1000, Thermo Fisher). Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific), before sections were washed and mounted onto glass slides using ProLong® Gold Antifade Mountant (Thermo Fisher Scientific). Sections were imaged with an inverted scanning confocal TCS SP8 microscope (Leica Microsystems) using a 40x objective (NA 1.45).

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Author contributions

F.J.L and M.P. conceived, planned and analyzed the *in vitro* aspects of the manuscript. V.D.P and R.R. conceived and planned the live imaging, characterization, and analysis of transplanted donor-derived neurons and contributed to the study design; M.P performed and analysed iPSC differentiations, immunohistochemistry, RNA extractions, calcium imaging experiments, pseudotyped rabies virus labelling, *in vitro* electrophysiology recordings, and prepared the relevant figures. A.S. analyzed the gene expression and prepared the relevant figures. H.R. developed and provided calcium imaging analysis scripts. R.R. and V.D.P. performed the grafting and the two-photon imaging experiments, analyzed the data, and prepared the related figures and text; M.A.S. performed *ex vivo* electrophysiology recordings and analysis from graft tissue generated by V.D.P. and R.R. S.J.B.

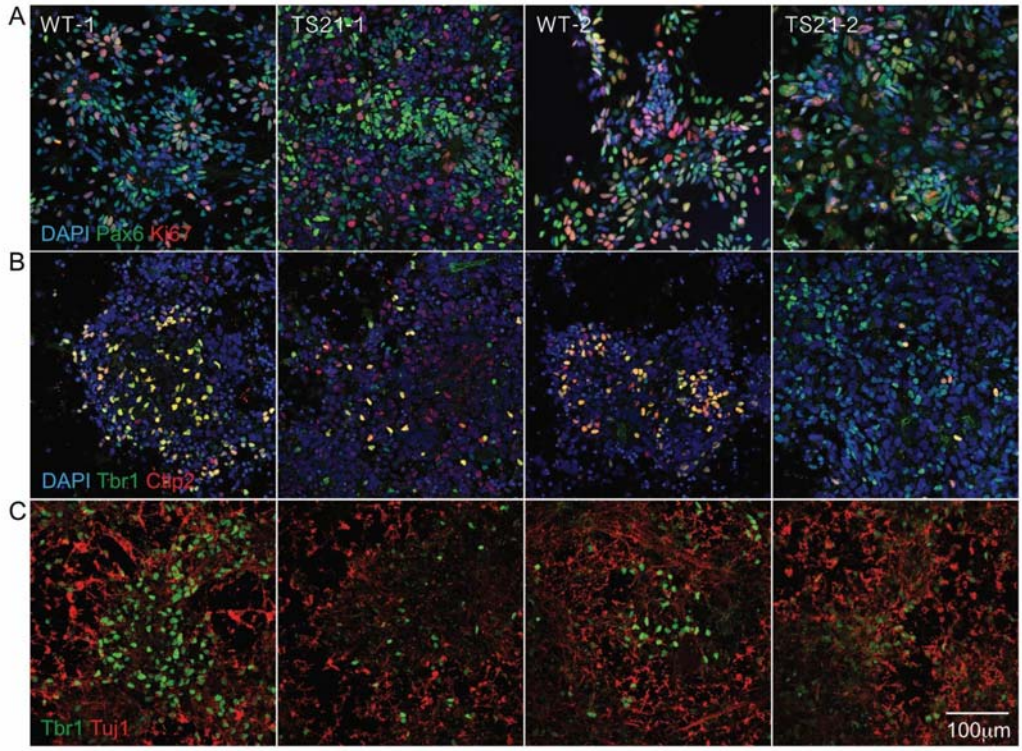
analyzed the *in vivo* calcium imaging data and prepared the relevant figures and text with input from R.R. and V.D.P.; F.J.L led the project; and F.J.L and M.P. wrote the paper with contributions from all authors. All authors approved the final manuscript.

Declaration of Interests

FJL is an employee and shareholder in Talisman Therapeutics. MP is an employee of F. Hoffmann-La Roche Ltd.

Figures

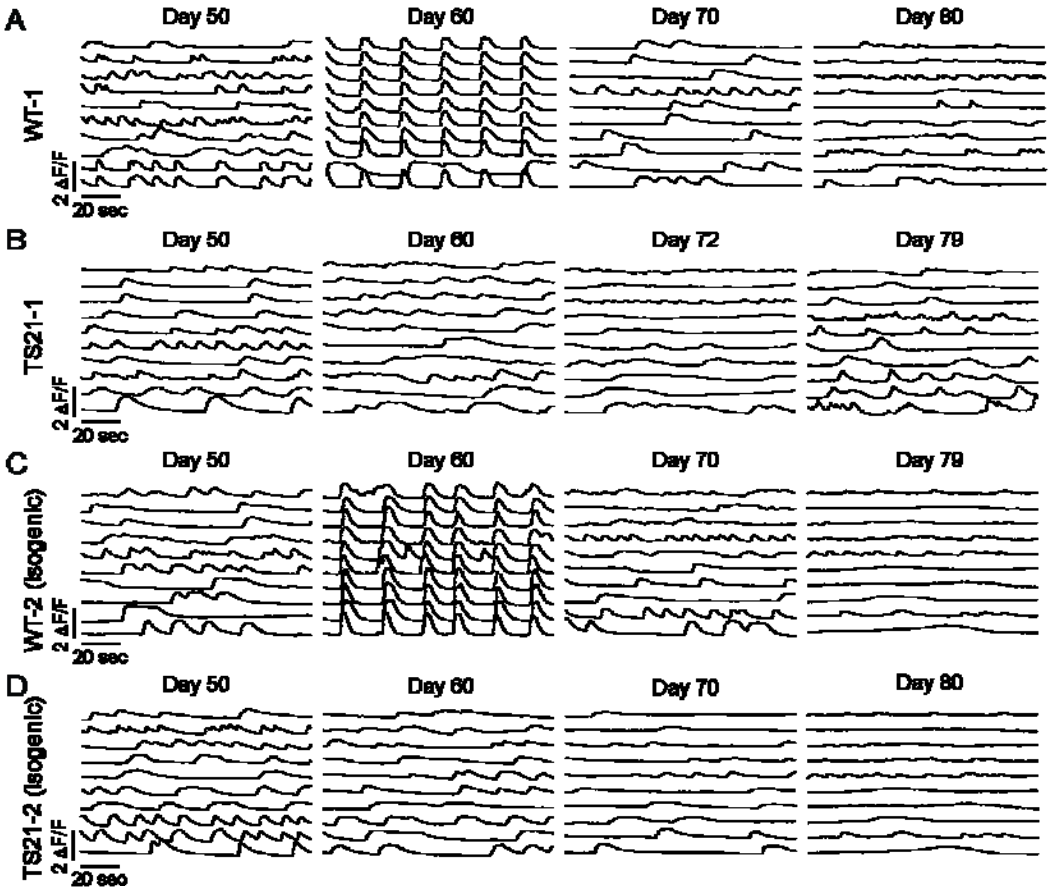
Figure 1



***In vitro* differentiation of control (WT) and trisomic (TS21) iPSCs into neural progenitor cells and cortical excitatory neurons**

A: Confocal image stacks of neural progenitor cells of two control (WT-1, WT-2) and two trisomic (TS21-1, TS21-2) cultures at day 35 – 37. B: Confocal image stacks of WT and TS21 trisomic neurons at day 50, immunofluorescence staining for the deep layer marker Tbr1 and Ctip2. C: Confocal image stacks of WT and TS21 neurons at day 50 immunofluorescence staining for the deep layer marker Tbr1 and the pan neuronal marker Tuj1.

Figure 2

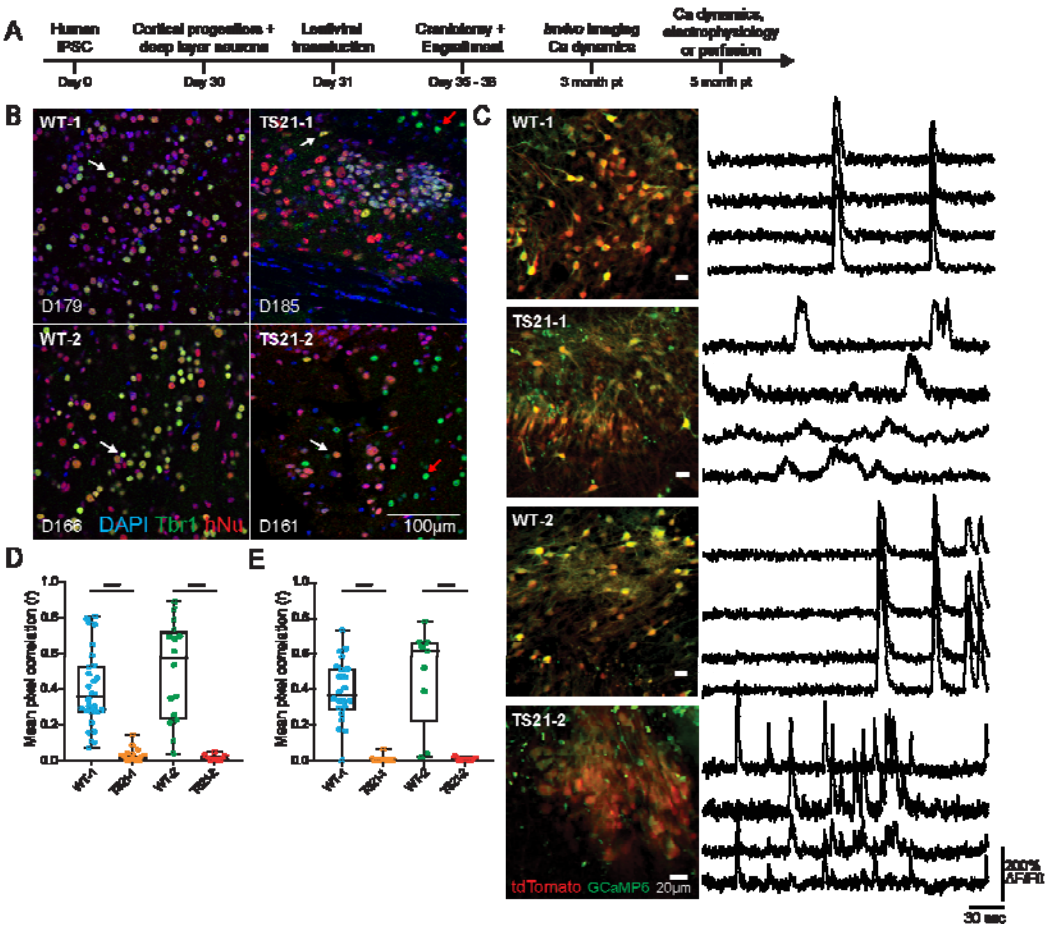


Altered spontaneous network activity development in TS21 neuronal networks *in vitro*

A, B, C, D: Traces of fluorescence changes (ΔF/F) in WT (WT1-1, WT-2) and trisomic

(TS21-1, TS21-2) neurons loaded with the calcium indicator OGB at different developmental stages. Each trace shows a 2 minutes time series of the activity of a single neuron. Note the absence of synchronized activity at day 60 in both trisomic lines compared to WT control lines. Importantly, synchronized network activity is reinstated in the isogenic, disomic control line WT-2.

Figure 3

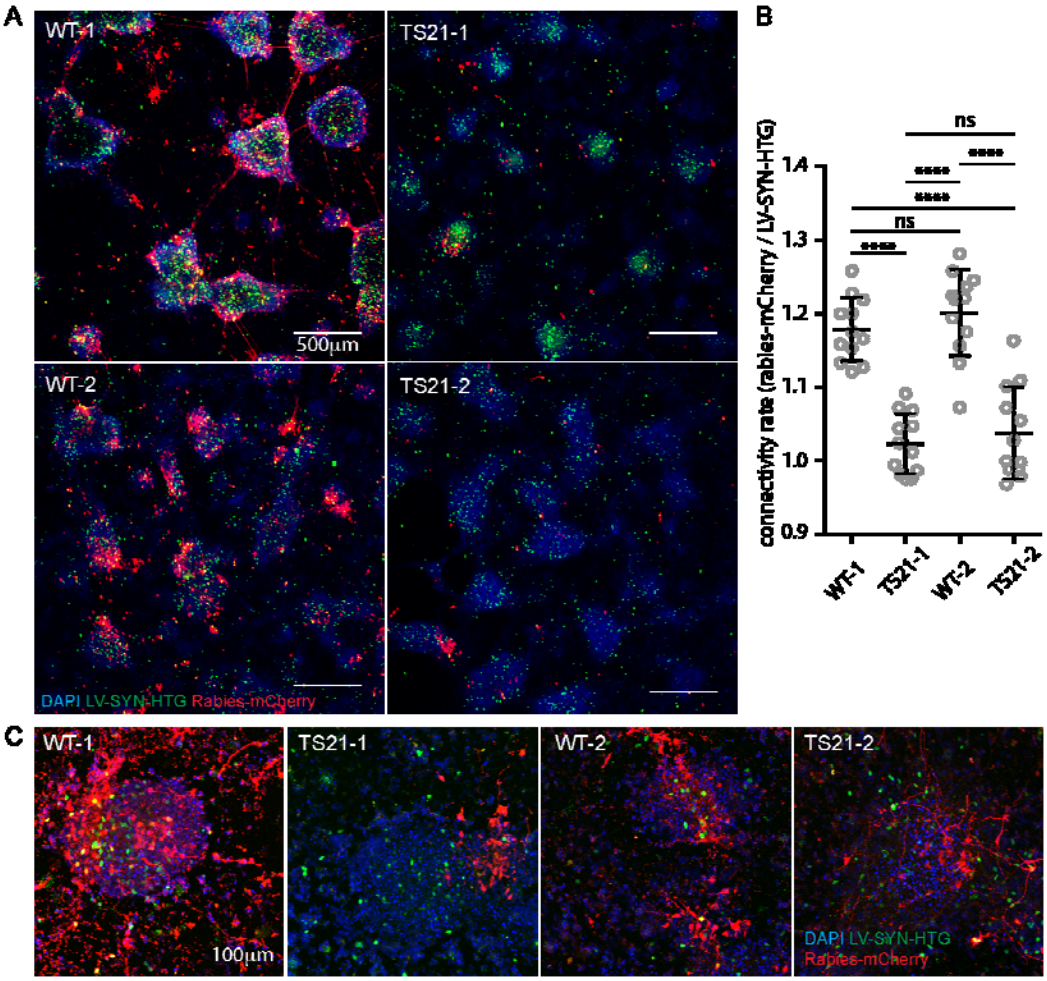


Altered network activity in trisomic (TS21) grafted neurons

A: Schematic of experimental design. B: iPSC derived human cortical neurons transplanted into the somatosensory cortex of mice. Representative confocal images of a graft section immunostained for the deep layer marker Tbr1 and the human specific nuclear marker (hNu). White arrows indicate human neurons double positive for Tbr1 and hNu. Red arrows indicate Tbr1 positive mouse neurons. Days post-transplantation are indicated. C: Left: example images of a two-photon time-series of tdTomato and GCaMP6 expressing neurons 3 months post transplantation. Right: Traces of fluorescence changes (ΔF/F0) in control and trisomic neurons. Note the absence of synchronized activity in both cell lines of TS21 neurons compared to WT neurons. D: Mean pixel correlation (r) measured 3 months after graft implantation for WT-1 (n = 28 ROIs), TS21-1 (n = 26 ROIs), WT-2 (n = 16 ROIs) and TS21-2 (n = 12

ROIs) grafts. Mann-Whitney U-test, ****P < 0.0001. E: Mean pixel correlation (r) measured 5 months after graft implantation for WT-1 (n = 22 ROIs), TS21-1 (n = 12 ROIs), WT-2 (n = 9 ROIs) and TS21-2 (n = 11 ROIs) grafts. At least 3 mice per line per condition (3 mpt and 5 mpt). Mann-Whitney U-test, ****P < 0.0001. Median, interquartile range, minimum and maximum values are indicated. Each data point represents an ROI.

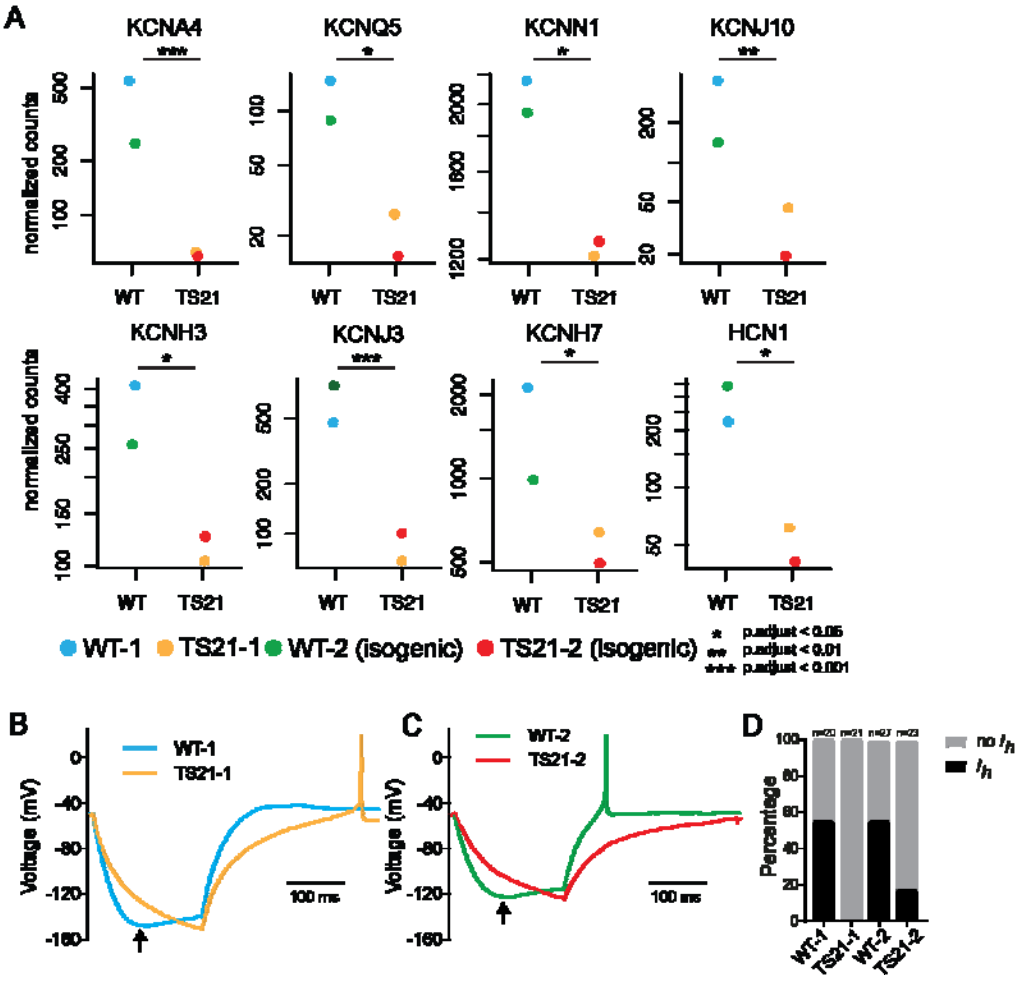
Figure 4



Reduced connectivity in trisomic (TS21) neurons

A: Representative images of WT and trisomic neurons, co-infected with a lentivirus expressing TVA and nuclear GFP (TVA-NLS-GFP) and a pseudotyped mCherry expressing rabies virus (Rabies-mCherry). B: Graph showing the relative red to green pixel values of WT and trisomic neuronal cultures. Note the significant decrease in TS21 neurons compared to WT neurons indicating a lower level of connectivity. C: Confocal image stacks of neurons in A taken at higher resolution. Note that connected neuronal clusters (red neurons connected to the ones with green nuclei) can be found in WT and trisomic neurons.

Figure 5

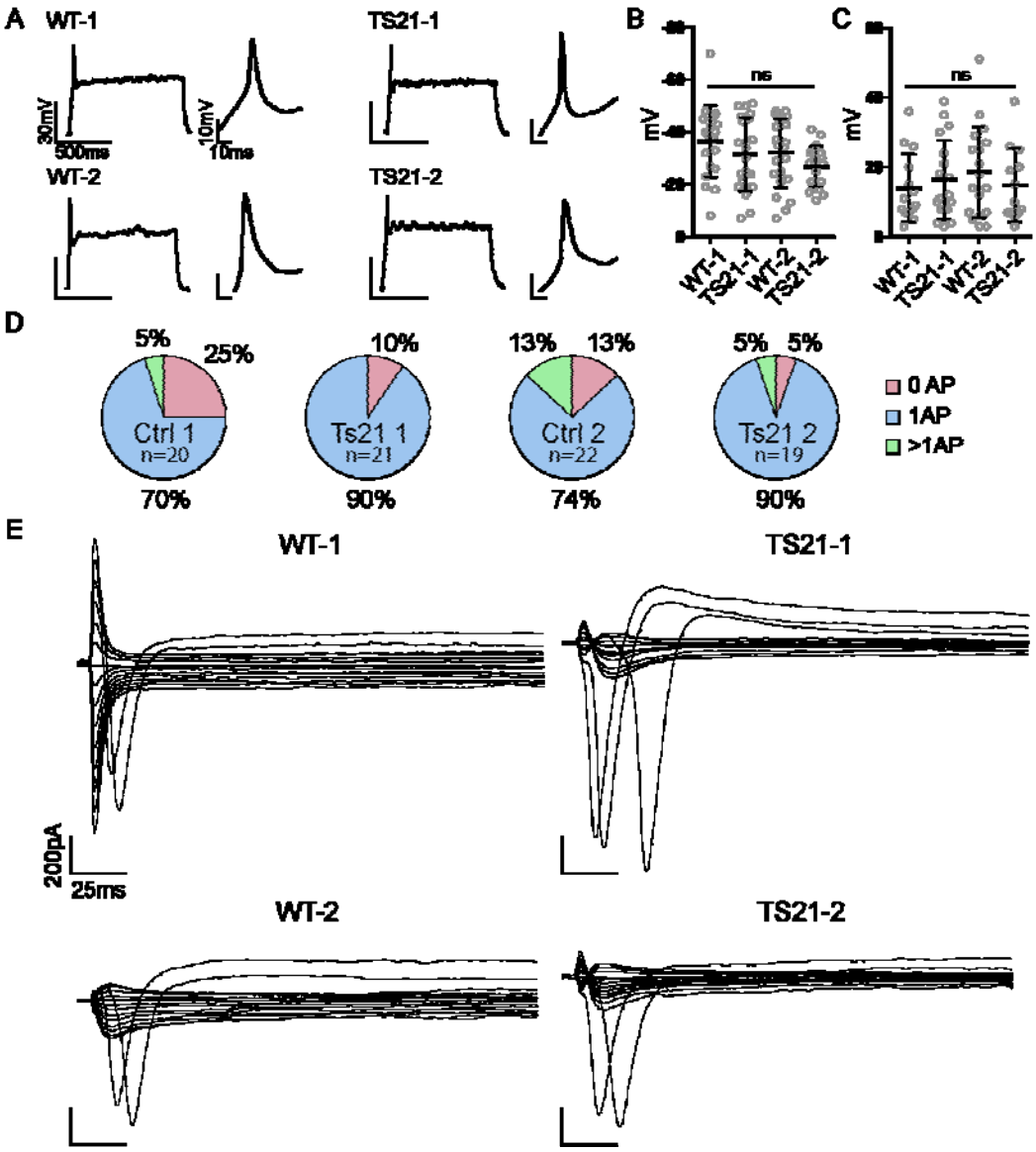


Decreased potassium channel expression in TS21 neurons

A: Gene expression analysis of K+ and K+/Na+ channel expression in TS21 and WT neurons, note that all 8 channels show a significant down-regulation in TS21 neurons. B, C: Detail of hyperpolarization activated currents elicited *in vivo* after current injection in current clamp mode. Arrows indicate the depolarising sag D: Percentage of cells in which hyperpolarization activated currents (I_h) could be measured per cell line (WT-1 55.0% vs. TS21-1 0%, Fisher's exact test ****P < 0.0001; WT-2 55.56% vs. TS21-2 17.39%, Fisher's exact test ** P < 0.01).

Supplemental Figures

S1

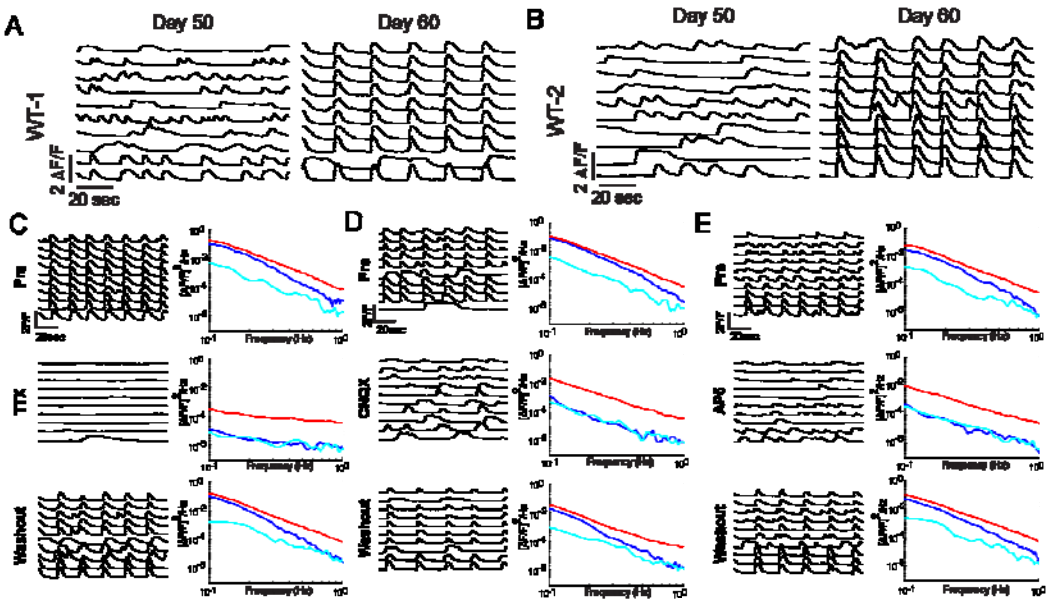


Electrophysiological characterization of TS21 and WT control neurons at day 53/54 shows unaltered whole cells properties in trisomic neurons

A: Whole cell electrophysiological recordings of TS21 and WT neurons at day 53/54. Neurons fire a single action potential (AP) after current injection. Inset shows a higher magnification of the AP. B: Resting membrane potential, C: AP amplitude after current injection. D: Number of action potentials fired after current injection in

current clamp mode. E: normal Na+ currents in TS21 and control neurons when voltage clamped.

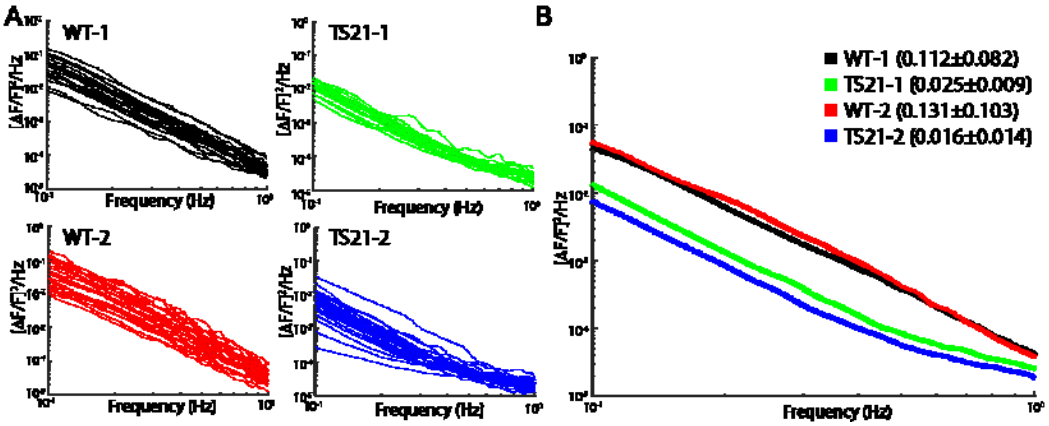
S2



Spontaneous synchronized network activity development is AMPA and NMDA receptor dependent

Traces of fluorescence changes (ΔF/F) in WT neurons loaded with the calcium indicator OGB at day 50 and 60. A: WT-1 , B: WT-2 (disomic isogenic). C, D, E: Left: Synchronized network activity is NMDA and AMPA receptor dependent. WT-1 neuronal networks were treated with three different drugs, during their synchronized phase. Pre: activity before pharmacological treatment, drugs (TTX, AP5, CNQX), Washout: 1h after pharmacological treatment. Left: ΔF/F traces of single neurons loaded with OGB, Right: corresponding power spectra of the 50 most active neurons, red line: average power of all neurons, blue line: power spectrum of the network, cyan line: randomized network power. C: TTX, synchronized network activity is reversely blocked by the Na channel blocker TTX, D: CNQX, synchronized network activity is reversely blocked by the AMPA receptor antagonist CNQX. E: AP5, synchronized network activity is reversely blocked by the NMDA receptor antagonist AP5.

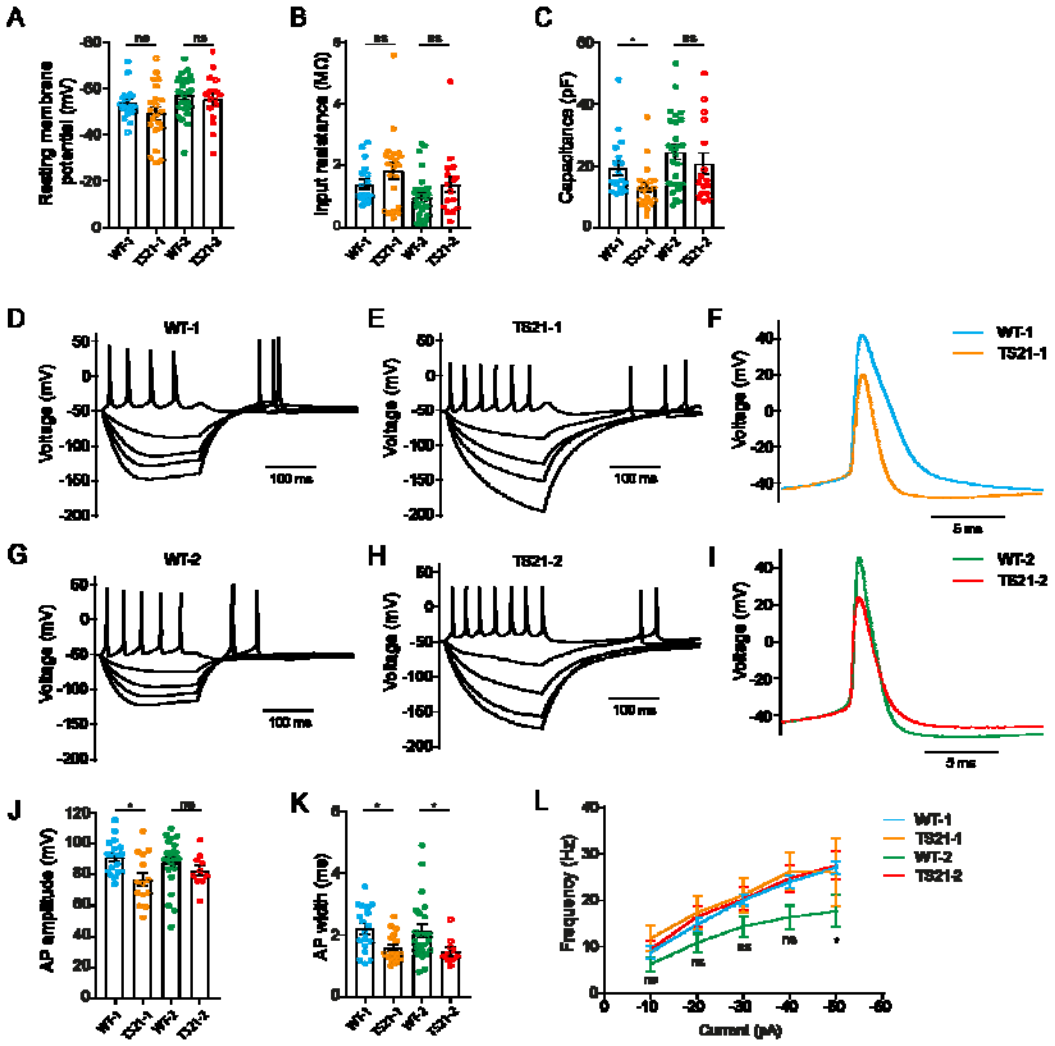
S3



Reduced activity in trisomic TS21 neuronal networks at day 50

A: Average power spectra of TS21 and WT control neurons. B: averaged neuronal power spectra of traces shown in A. Note the decreased activity in both trisomic TS21 lines compared to WT, control neurons. Values show the mean integral of the power spectra over 0.1 and 1 Hz (WT-1 vs. WT-2: ns, WT-1 vs. TS21-1: **, WT-1 vs. TS21-2: ***, WT-2 vs. TS21-1: ***, WT-2 vs. TS21-2: ***, TS21-1 vs. TS21-2, ns, Tukey's multiple comparisons test, *P <0.05).

S4

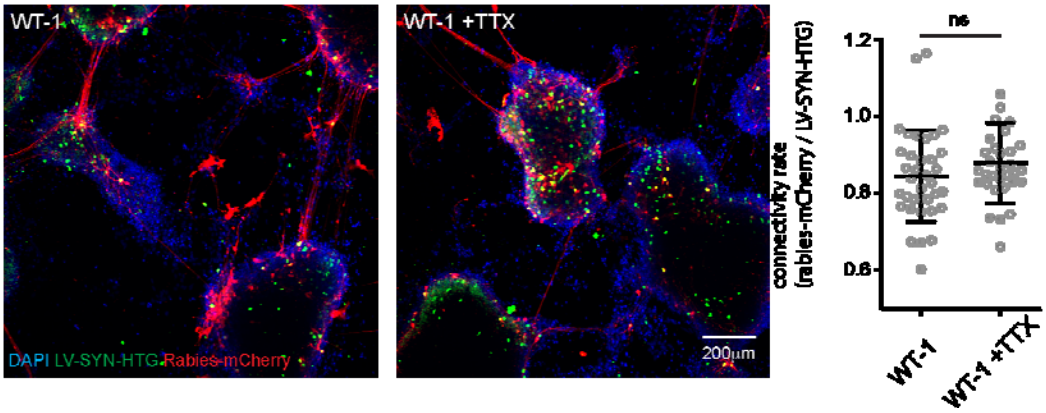


***Ex vivo* electrophysiological characterisation of TS21 and WT neurons**

A: Resting membrane potential. Unpaired two-tailed t-test; ns, not significant. B: Input resistance. Mann-Whitney U-test; ns, not significant. C: Capacitance. Mann-Whitney U-test, **P <0.01; ns, not significant. (D-E, G-H) Representative current clamp recordings of membrane potentials after current injection in WT-1 (D), TS21-1 (E), WT-2 (G) and TS21-2 (H) neurons. F: Detail of an action potential in WT-1 and TS21-1 neurons. I: Detail of an action potential in WT-2 and TS21-2 neurons. J: Action potential amplitude. Mann-Whitney U-test, **P <0.01; ns, not significant. K:

Action potential width. Mann-Whitney U-test, *P <0.05. L: Frequency of action potentials in response to positive current injection. Error bars represent SEM.

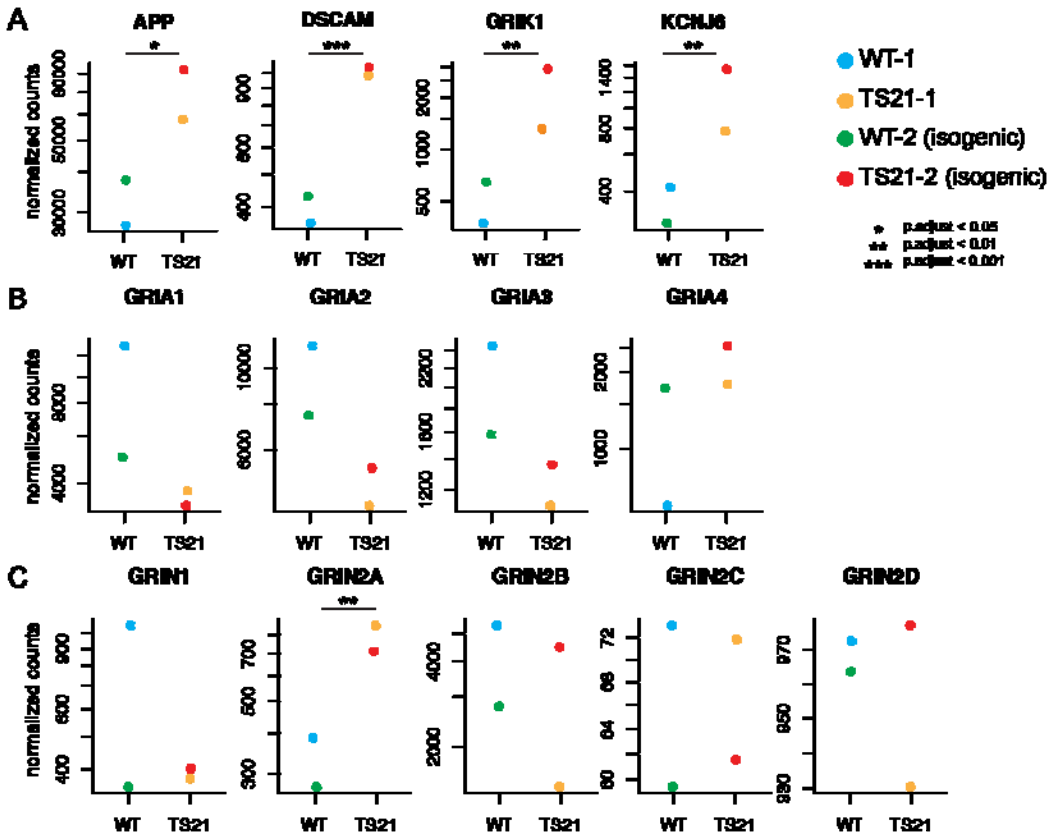
S5



Pseudotyped rabies spread is independent of neuronal activity

Representative images of mock and TTX treated WT-1 neurons co-infected with a lentivirus expressing TVA and nuclear GFP (TVA-NLS-GFP) and a pseudotyped mCherry expressing rabies virus (Rabies-mCherry) (left). Quantification of connectivity (right). Note no difference in connectivity levels in TTX treated neuronal cultures.

S6

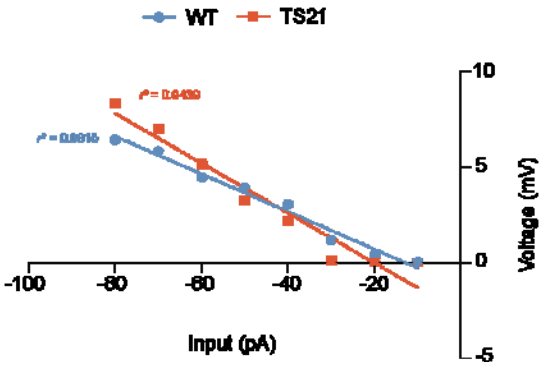


Gene expression levels of significantly up or down-regulated genes in TS21 neurons at day 50

A: genes located on chromosome 21 are significantly up-regulated in TS21 neurons.

B: Expression of AMPA receptor subunits, B: Expression of NMDA receptor subunits.

S7



IV curves after hyperpolarizing current injection in TS21 and control neurons ex *vivo*

Positive correlation between hyperpolarization (input) and current (voltage) in WT (r2 = 0.9815) and TS21 (r2 = 0.9439) neurons ex *vivo*.

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