Early maturation and hyperexcitability is a shared phenotype of cortical neurons derived from different ASD-causing mutations

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Abstract: Autism Spectrum Disorder (ASD) is characterized mainly by social and sensory-motor abnormal and repetitive behavior patterns. Over 1000 genetic variants were reported to be highly penetrant and causative of ASD. Many of these mutations cause comorbidities such as epilepsy and intellectual disabilities (ID). In this study, we measured cortical neurons derived from induced pluripotent stem cells (iPSCs) of patients with four mutations in the genes GRIN2B, SHANK3, UBTF, as well as chromosomal duplication in the 7q11.23 region and compared them to neurons derived from a first degree relative without the mutation. Using a whole-cell patch-clamp, we observed that the mutant cortical neurons demonstrated hyperexcitability and early maturation compared to control lines. These changes were characterized by increased sodium currents, increased amplitude and rate of excitatory postsynaptic currents (EPSCs), and more evoked action potentials in response to current stimulation in early-stage cell development (3-5 weeks post differentiation). These changes that appeared in all the different mutant lines, together with previously reported data, indicate that an early maturation and hyperexcitability may be a convergent phenotype of ASD cortical neurons.

INTRODUCTION

Autism Spectrum Disorder (ASD) was first defined by Leo Kanner in 1943 and named "early infantile autism" as an independent disorder from the psychotic disorder of schizophrenia, describing 11 children with social, biological, and emotional abnormalities such as the inability to relate to others or objects in a traditional way, an anxious and obsessive desire for consistency, early eating difficulties and hearing problems[1]. Recently, more symptoms entered the category of autistic-like behaviors such as social and sensory-motor abnormal behaviors, attention deficit hyperactivity disorder (ADHD), poorly integrated verbal and non-verbal communication, abnormalities in eye contact, hyper or hypo-reactivity to sensory input, repetitive body movements, and more[2,3].

On the genomic aspect, there has been more attention to of many variants in which chromosomal subregions are deleted or duplicated in an inherited and de novo manner as well[2]. Various genes and mutations have been reported to be associated with ASD, such as *AVPR1a*, *CHD8*, *DISC1*, *7Dup11.23*, *DYX1C1*, *GRIN2B*, *ITGB3*, *NLGN1*, *NLGN3*, *NRXN1*, *PARK2*, *PTB2*, *RELN*, *RPL10*, *SHANK3*, *SLC6A4*, *SLC1A4*, *UBTF*, *UB3A* [2–8]. On the neurobiological aspect, no specific brain area nor system has been confirmed to be entirely associated with the disorder, but an overall brain impairment has been shown starting from childhood[3]. The areas in the brain that are thought to be affected include cortical as well as non-cortical regions: the prefrontal cortex (PFC), Brodmann's areas 9, 21, and 22, orbitofrontal cortex (OFC) fusiform gyrus, fronto-insular

cortex, cingulate cortex, hippocampus, amygdala, cerebellum, and brainstem[8]. Our study focuses on the following four genetic mutations:

Dup7

7q11.23 Duplication Syndrome, briefly known as ***Dup7***, is caused by a duplication of 1.5-1.8 Mb in section q11.23 of chromosome 7[9], also known as the Williams-Beuren syndrome critical region (WBSCR) and is inherited in an autosomal dominant manner[10]. Extra copies of the genes are located in the critical region and include the Elastin gene (ELN)- encoding elastin, a structural protein and a critical component of elastic fibers[11], and the General Transcription Factor IIi gene (GTF2I)- encoding general transcription factor IIi, an inducible multifunctional transcription factor and a regulator of agonist-induced calcium entry to the cytoplasm[12]. These are thought to contribute significantly to the Syndrome's symptoms[13,14], such as the increased risk for aortic dilatation and separation anxiety[10].

Common symptoms of *Dup7* patients include anxiety disorders (primarily social anxiety), selective mutism, ASD, ADHD, intellectual disability, seizures, severe speech delays and hypotonia[15,16]; Facial features including a broad forehead, a short philtrum, a thin upper lip and a facial asymmetry have been reported as well[13]. *Dup7* shares some overlapping and contrasting phenotypes with the 7q11.23 deletion syndrome, commonly known as the Williams-Beuren Syndrome (WS); most notably, social anxiety is common in 7q11.23 duplication patients, while WS patients tend to have a social personality with no social anxiety[17]. Beyond that, magnetic resonance imaging (MRI) showed various abnormalities such as cerebral atrophy, ventriculomegaly, increased cortical thickness, alterations in white matter's volume and cerebellar hypoplasia and an increased cortical extra-axial space[16].

SHANK3

The SHANK protein family, also known as ProSAP, including *SHANK1*, *SHANK2* and *SHANK3*, is a family of scaffold proteins first identified using guanylate kinase-associated protein (GKAP) – a PSD-95-binding protein and a major component of the postsynaptic density (PSD), as bait.[18,19] These proteins contain five key sites for protein-protein interaction: ankyrin repeats, Src homology 3 (SH3) domain, a vast proline-rich region, a C-terminal sterile α-motif (SAM) domain, and a PSD-95/discs large/zonula occludens-1 (PDZ) domain mediating its interaction with a variety of proteins as GKAP which moderates its binding to the NMDA and AMPA receptors[18,20,21].

The cognate gene, ***SHANK3*** gene, is located at the terminal long arm of chromosome 22, coding for master scaffolding protein found in the body's tissues and notably in the brain. It plays a critical role in the postsynaptic density of glutamatergic synapses and synaptic functions[18,22]. Mutations in this gene have been linked to deficits in synaptic function and plasticity, accompanied by lower reciprocal social interactions[21,23–26], ASD and other neurodevelopmental symptoms[24,27]. The individuals affected by the 22q13 deletion syndrome[27,28], also known as Phelan-McDermid syndrome (PMS), suffer from developmental delays, a low muscular tone, a weakened perception of pain, delayed speech, seizures, ASD and autistic-like behaviors with a prevalence of 84%[29–31]. The patients also exhibit cortical alterations[32].

GRIN2B

Glutamate Ionotropic Receptor NMDA type Subunit 2B (***GRIN2B,*** *GLUN2B*, *NMDAR2B*, or *NR2B*) is a gene located on the 'p' arm of chromosome 12 (12p13.1 is its exact location)[33,34]. This gene is one member of a family of genes encoding various proteins that together form the

NMDA receptor[35] – glutamate-gated ion channel, which allows positively charged particles to flow through cells in the brain, activating neurons to send signals to each other[36,37].
Mutations in this gene lead to a production of a nonfunctional GluN2B protein or completely prevent the production of GluN2B proteins from one copy of the gene in each cell; A shortage or dysfunction of this protein may cause an extreme reduction of the number of the functional NMDA receptors[36,38], causing neurodevelopmental disorders[39]. This disorder is associated with West syndrome, characterized by intellectual disability, delayed development of speech and motor skills, focal seizures, weak muscle tone (hypotonia), movement disorders, schizophrenia and behavioral problems common in ASD[33,39–43]. In addition, MRI scans revealed a consistent malformation of cortical development (MCD) consistent with that of tubulinopathies, in addition to hypoplastic corpus callosum of varying degrees, enlarged and mildly dysplastic basal ganglia, hippocampal dysplasia as well as enlarged tecta and generalized cerebral volume loss[44]. GRIN2B-related disorders seem to have the highest prevalence among all GRIN-related disorders with 5.91 **predicted incidence per 100,000 births**[45]; This was previously reported to make up ~ 0.22% of the total neurodevelopmental disorders[44].

UBTF

The upstream Binding Transcription Factor (***UBTF)*** is a gene that codes for Upstream Binding Factor (UBF) – a protein that acts as a transcription factor in RNA polymerase I (Pol I). UBF is critical for ribosomal RNA transcripts (rRNA) and synthesis from ribosomal DNA (rDNA) in the nucleolus[46]; A loss of UBF induces nuclear disruptions, including inhibition of cell proliferation, rapid and synchronous apoptosis as well as cell death[47]. Moreover, mutations in the *UBTF* gene lead to a production of an increased amount of ribosomal RNA transcript (rRNA), inducing depletion of RNA binding proteins, altered disposal machinery, and ribosome biogenesis[48]. These disruptions lead to pediatric neurodevelopmental regression starting at young onset (2.5 to 3 years). The degeneration starts with cognitive-motor deficits, reported in humans as well as knockout mice[7]. This regression is accompanied by dystonia, parkinsonism, severe intellectual disability, feeding difficulties, autistic-like behaviors, a slow loss of motor, cognitive and speech capabilities, as well as severe epilepsy [48–52]. Recent MRI studies showed gradual widespread brain atrophy in both supratentorial and infratentorial areas affecting both grey and white matter in patients with similar neuroregression patterns[48,50]. Furthermore, increased pre-rRNA and 18S rRNA expression was reported in addition to nucleolar abnormalities quantified by quantitative real-time polymerase chain reaction (qRT-PCR), increased number of DNA breaks, defective cell cycle progression by comet assay, and apoptosis by TUNEL assay[48].

RESULTS

Dup7 cortical neurons display increased sodium and potassium currents, increased synaptic activity and hyperexcitability early in the differentiation.

We performed whole-cell patch clamp experiments five weeks (day 34) after the start of the differentiation of 16 Dup7-mutant neurons and 13 control neurons derived from a first-degree relative of the same gender. In a voltage clamp mode, EPSC recordings were performed by holding the cell at -60mV. We observed an increase in the rate of EPSCs of the mutant neurons compared to the controls (0.13 $\pm$ 0.08 Hz in Dup7-mutant neurons and 0.07 $\pm$ 0.07 Hz in the control neurons, p=0.03), as shown in Fig. 1a-c. Fig. 1a-b presents representative traces, while Fig. 1c represents the average over all the recordings. Additionally, a significant increase in the

mean amplitude of the EPSCs was observed. The Dup7-mutant neurons had a larger amplitude compared to the control neurons (12.72 ± 2.86 pA for the mutant neurons and 7.12 + 5.09 pA for the control neurons (p=0.002, (Fig. 1d.)). The cumulative distribution of the EPSC amplitudes for Dup7-mutant neurons is slightly right-shifted compared to control neurons indicating larger amplitudes of EPSCs (Fig. 1e).

Next, we recorded in voltage clamp mode the sodium and potassium currents. We observed a significantly larger normalized sodium current in the Dup7-mutant neurons compared to the control neurons (F (1,38) = 9.43, p=0.004). Representative traces of the recordings are shown in Fig. 1f (control) and 1g (mutant). The average sodium currents are presented in Fig. 1h. Additionally, we observed increased slow and fast potassium currents (normalized by the capacitance) in the Dup7-mutant neurons compared to controls (F (1,14) = 5.61; p=0.03 for the slow potassium currents and F (1,14) = 8.36; p=0.01 for the fast potassium currents) over the 10-80 mV range (Fig. 1h-j). We next measured the number of evoked action potentials in a current clamp mode as a measure of the neuronal excitability. We observed a hyperexcitability pattern for the Dup7-mutant neurons compared to control neurons. The total number of evoked potentials (see Methods) for the Dup7-mutant neurons was 47.63 ± 49.61; for the control neurons, it was 28.5 ± 69.48 (p=0.006). A representative example is presented in Fig. 1k (control) and 1l (Dup7), and the average over all recordings is shown in Fig. 1m. Spike shape analysis (see Methods) is presented in Table S1. Fig. 1n. is an example ICC image for neuronal markers MAP2 and the cortical marker TBR1.

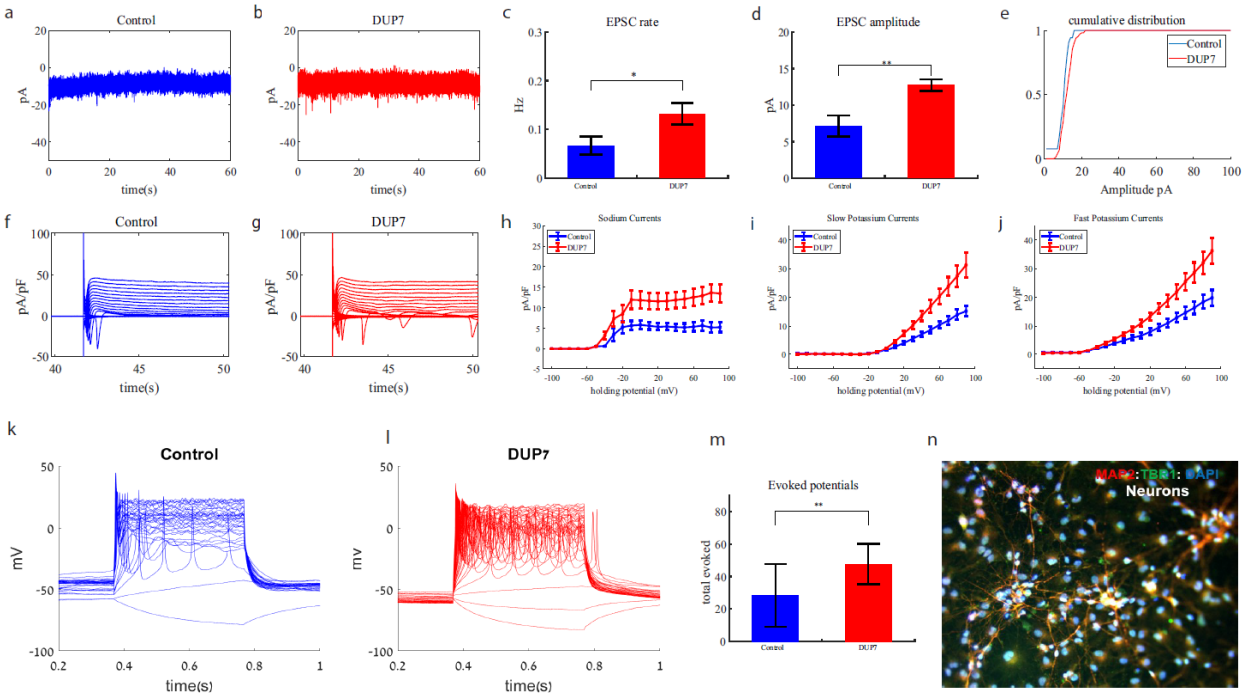

Fig. 1. Young (5 weeks post differentiation) Dup7-mutant neurons are hyperexcitable compared to control neurons.

a A representative trace of (EPSCsmeasured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a dup7-mutant neuron at five weeks post-differentiation. **c** The mean rate of synaptic events was higher in the dup7-mutant neurons compared to control neurons (p=0.029). **d** The average amplitude of EPSCs was increased in the dup7-mutant neurons (p =0.002). **e** The cumulative distribution of the amplitude of EPSCs is slightly right-shifted in the dup7-mutant neurons, indicating an increase in the amplitudes. **f**

A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in control neurons. **g** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in dup7-mutant neurons. **h** The average sodium currents in dup7-mutant neurons is increased compared to control neurons (p=0.004). **i** The average slow potassium currents in dup7-mutant neurons is increased compared to control neurons (p=0.03). **j** The average fast potassium currents is increased in dup7-mutant compared to control neurons (p=0.01). **k** A representative recording of evoked action potentials in a current-clamp mode of a control neuron. **l** A representative recording of evoked action potentials in a current-clamp mode of a dup7-mutant neuron. **m** The total number of evoked action potentials is larger in dup7-mutant neurons compared to control neurons (p=0.006). **n** A representative image of neurons that were immunostained for DAPI, MAP2 and TBR1.

GRIN2B cortical neurons display increased sodium and potassium currents and hyperexcitability early in the differentiation.

We performed whole-cell patch clamp experiments three weeks (day 19) after the start of the differentiation of 12 GRIN2B-mutant neurons and five weeks (day 27) of 7 control neurons. In a voltage clamp mode, EPSC recordings were performed by holding the cell at -60mV. We observed an increase in the rate of EPSCs of the mutant neurons compared to the controls (0.39$\pm$ 0.27 Hz in GRIN2B-mutant neurons and 0.17 $\pm$ 0.2 Hz in the control neurons, p=0.03) as shown in Fig. 2a-c. Fig. 2a-b presents representative traces, while Fig. 2c. represents the average over all the recordings. No significant difference in the mean amplitude of the EPSCs was observed (Fig. 2d.). The cumulative distribution of the EPSC amplitudes was similar between the control and GRIN2B-mutant neurons (Fig. 2e.).

Next, we recorded in voltage clamp mode the sodium and potassium currents. We observed a significantly larger normalized sodium current in the GRIN2B-mutant neurons compared to the control neurons ($F(1,38) = 14.1$, p=0.0006). Representative traces of the recordings are shown in Fig. 2f. (control) and 2g. (mutant). The average sodium currents are presented in Fig. 2h. Additionally, we observed larger slow and fast potassium currents (normalized by the capacitance) in the GRIN2B-mutant neurons compared to controls ($F(1,16) = 4.6$, p=0.04 for the slow potassium currents and $F(1,16) = 5.39$, p = 0.03 for the fast potassium currents) over the 0-80 mV range (Fig. 2h-j).

We next measured the number of evoked action potentials in current clamp mode as a measure of the neuronal excitability. We observed a hyperexcitability pattern for the GRIN2B-mutant neurons compared to control neurons. The total number of evoked potentials (see Methods) in the GRIN2B-mutant neurons was 38.86 $\pm$ 10.17, and in the control neurons, it was 22.37 $\pm$ 19.5 (p=0.003). A representative example is presented in Fig. 2k (control) and 2l (GRIN2B), and the average over all recordings is presented in Fig. 2m. Furthermore, we observed a significant increase in GRIN2B-mutant neurons' spike amplitude compared to control neurons (41.2 $\pm$ 12.5 mV in GRIN2B-mutant neurons and 20.5 $\pm$ 14.01 mV in control neurons, p =0.008); further spike shape analysis is presented in Table S1. Examples of ICC images are shown in Fig. 2n. (typical NPC markers PAX6 and NESTIN) and 2o. (neuronal markers MAP2, the cortical marker TBR1).

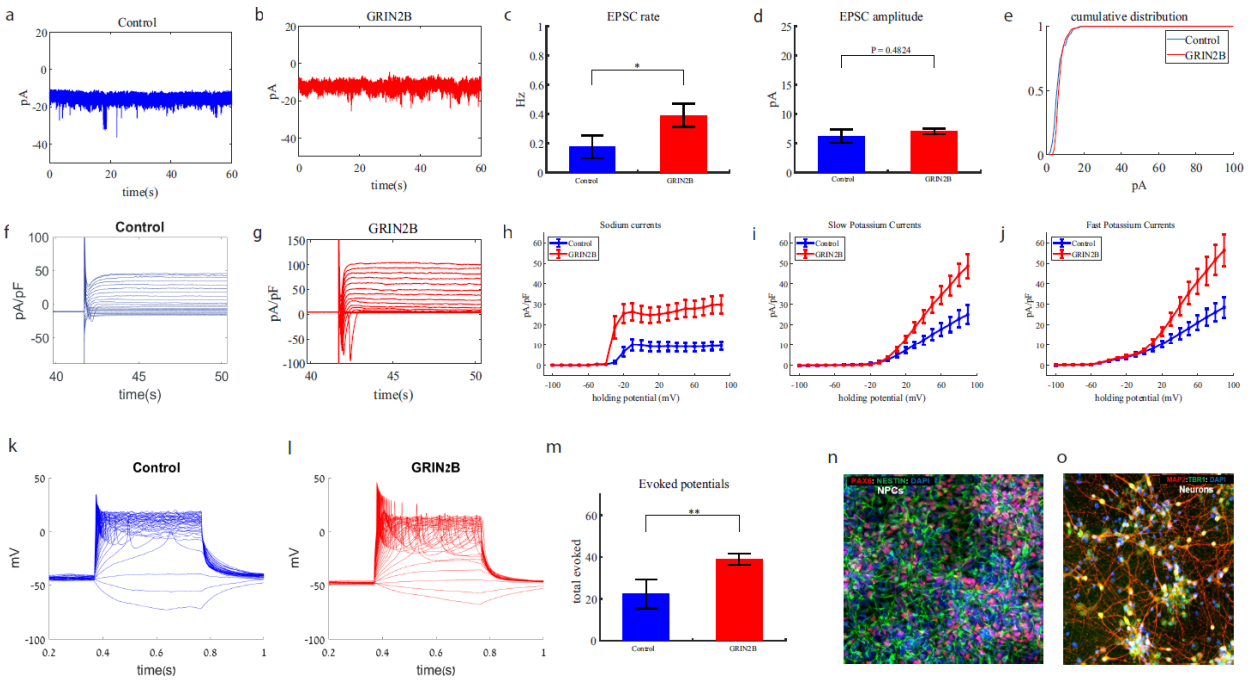

Fig. 2. Young (3 weeks post differentiation) GRIN2B-mutant neurons are hyperexcitable compared to control neurons (5 weeks post differentiation).

a A representative trace of EPSCs measured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a GRIN2B-mutant neuron at three weeks post-differentiation. **c** The mean rate of synaptic events was higher in the GRIN2B-mutant neurons (p=0.03). **d** The mean amplitude of EPSCs was increased but not significantly different in the GRIN2B-mutant neurons. **e** The cumulative distribution of the amplitude of the EPSCs of GRIN2B-mutant and control neurons looks similar. **F** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in control neurons. **g** A Representative trace of sodium and potassium currents recorded in voltage-clamp in GRIN2B-mutant neurons. **h** The average sodium currents in GRIN2B-mutant neurons is severely increased compared to control neurons (p=0.006). **I** The average slow potassium currents in GRIN2B-mutant neurons is increased compared to control neurons (p=0.04). **j** The average fast potassium currents in GRIN2B-mutant neurons is increased compared to control neurons (p=0.03). **k** A representative recording of evoked action potentials in a current-clamp mode of a control neuron. **l** A representative recording of evoked action potentials in a current-clamp mode of a GRIN2B-mutant neuron. **m** The total number of evoked action potentials is larger in the GRIN2B-mutant neurons compared to control neurons (p=0.003). **n, o** A representative images of **n** NPCs and **o** neurons that were immunostained for DAPI, MAP2, TBR1, PAX6 and NESTIN.

SHANK3 cortical neurons display increased sodium and slow potassium currents, a drastic increase in synaptic activity and hyperexcitability early in the differentiation.

We performed whole-cell patch clamp experiments five weeks (days 29-32) after the start of the differentiation of 21 SHANK3-mutant and 17 control neurons. In a voltage clamp mode, EPSC recordings were performed by holding the cell at -60mV. We observed a significant increase in the rate of EPSCs of the mutant neurons compared to the controls (0.28 $\pm$ 0.36 Hz in SHANK3-mutant neurons and 0.08 $\pm$ 0.06 Hz in the control neurons, p=0.003) as shown in Fig. 3a-c. Fig. 3a-b presents representative traces, while Fig. 3c represents the average over all the recordings. Additionally, a drastic increase in the mean amplitude of the EPSCs was observed. The SHANK3-mutant neurons had a larger amplitude compared to the control neurons (10.145 $\pm$ 3.26 pA for the mutant neurons and 5.29 + 1.65 pA for the control neurons, p=1.15e^{-5}, (Fig. 3d)).

The cumulative distribution of the EPSC amplitudes for SHANK3-mutant neurons is right shifted compared to control neurons indicating larger amplitudes of EPSCs (Fig. 3e).

Next, we recorded in voltage clamp mode the sodium and potassium currents. We observed a significantly larger normalized sodium current in the SHANK3-mutant neurons compared to the control neurons F (1,36) = 4.51, p=0.04. Representative traces of the recordings are shown in Fig. 3f (control) and 3g (mutant). The average sodium currents are presented in Fig. 3h. Additionally, we observed increased slow, but not fast, potassium currents (normalized by the capacitance) in the SHANK3-mutant neurons compared to controls, (F (1,10) = 5.68; p=0.03) over the 40-90 mV range (Fig. 3h-j).

We next measured the number of evoked action potentials in current clamp mode as a measure of the neuronal excitability. We observed a hyperexcitability pattern for the SHANK3-mutant neurons compared to control neurons. The total number of evoked potentials (see Method) for the SHANK3-mutant neurons was 31.32 $\pm$ 39.8; for the control neurons, it was 18.4 $\pm$ 22.96 (p=0.03). A representative example is presented in Fig. 3k. (control) and 3l. (SHANK3) and the average over all recordings are presented in Fig. 3m. Furthermore, we observed a more depolarized threshold (higher) in the SHANK3-mutant neurons compared to control neurons (30.4 $\pm$ 5.6 mV in the SHANK3-mutant neurons and 25.5 $\pm$ 4.2 mV in control neurons, p =0.02); further spike shape analysis is presented in Table S1. Examples of ICC images are shown in Fig. 3n. (typical NPC markers PAX6 and NESTIN) and 3o. (neuronal markers MAP2, the cortical marker TBR1).

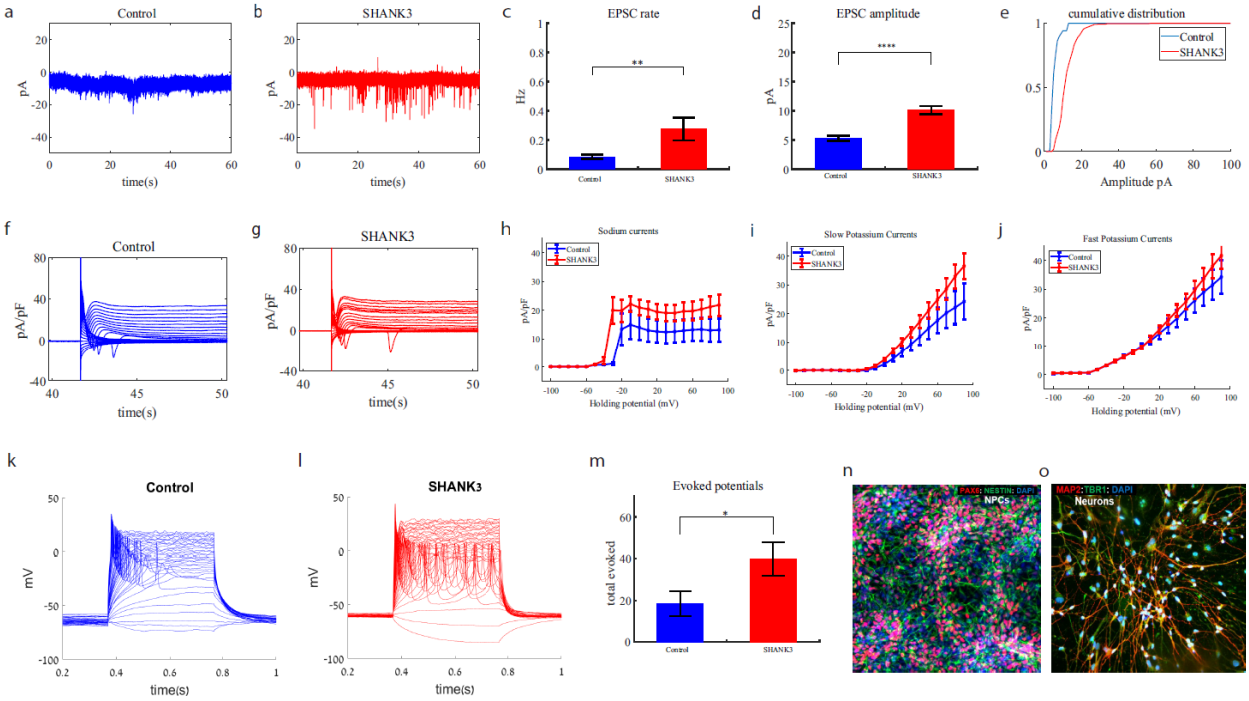

Fig. 3. Young (5 weeks post differentiation) SHANK3-mutant neurons are hyperexcitable compared to control neurons.

a A representative trace of excitatory postsynaptic currents (EPSCs) that were measured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a SHANK3-mutant neuron at five weeks post-differentiation. **c** The mean rate of synaptic events was higher in the SHANK3-mutant neurons (p=0.003). **d** The average amplitude of EPSCs was increased in the SHANK3-mutant neurons (p=1.15e^{-5}). **e** The cumulative distribution of the amplitude of EPSCs of SHANK3-mutant is right-shifted, indicating an increase in the amplitudes. **f** A Representative trace of sodium and potassium currents recorded in voltage-clamp in control

neurons. **g** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in SHANK3-mutant neurons. **h** The average sodium currents in SHANK3-mutant neurons is increased compared to control neurons (p=0.04). **I** The average slow potassium currents in SHANK3-mutant neurons is increased compared to control neurons (p=0.03). **j** The average fast potassium currents look similar and not significantly different in SHANK3-mutant compared to control neurons. **k** A representative recording of evoked action potentials in a current-clamp mode of control neurons. **l** A representative recording of evoked action potentials in a current-clamp mode of SHANK3-mutant neurons. **m** The total number of evoked action potentials is larger in SHANK3-mutant neurons compared to control neurons (p=0.03). **n, o** A representative images of NPCs (**n**) and neurons (**o**) that were immunostained for DAPI, MAP2, TBR1, PAX6 and NESTIN.

UBTF cortical neurons display increased sodium currents, an increase in synaptic amplitude, an increase in spontaneous activity and hyperexcitability early in the differentiation.

We performed whole-cell patch clamp experiments five weeks (day 32) after the start of the differentiation of 22 UBTF-mutant neurons and six weeks (days 37-38) after the beginning of the differentiation of 18 control neurons. In a voltage clamp mode, EPSC recordings were performed by holding the cell at -60mV. Fig. 4a-b presents representative traces, while Fig. 4c. represents the average EPSC amplitude over all the recordings. The EPSC rate of the UBTF-mutant neurons is slightly similar to the control neurons (Fig. 4c). We observed an increase in the mean amplitude of the EPSCs. The UBTF-mutant neurons had a larger amplitude compared to control neurons it was7.81$\pm$ 1.16 pA for the mutant neurons and 6.55 + 2.39 pA for control neurons, p=0.02 (Fig. 4d.). The cumulative distribution of the EPSC amplitudes for UBTF-mutant neurons is slightly right-shifted compared to control neurons indicating larger amplitudes of EPSCs (Fig. 4e.).

Next, we recorded in voltage clamp mode the sodium and potassium currents. We observed a significantly larger normalized sodium current in the UBTF-mutant neurons compared to the control neurons F (1,28) = 5.58, p=0.03 over the --50-90-mV range. Representative traces of the recordings are shown in Fig. 4f. (control) and 4g. (mutant). The average sodium currents is presented in Fig. 4h. An ANOVA test indicated no significant differences in the slow and fast potassium currents (Fig. 4h-j.).

We next measured the number of evoked action potentials (see Methods). for the UBTF-mutant neurons, it was 62.95 $\pm$ 31.56 and for the control neurons, it was 31.46$\pm$ 22.3 ($p=5.56e^{-4}$). A representative example is presented in Fig. 4k. (control) and 4l. (UBTF) and the average over all recordings are presented in Fig. 4m. Furthermore, we observed a significant increase in UBTF-mutant neurons' spike amplitude compared to control neurons (50$\pm$ 17.05 mV in the UBTF-mutant neurons and 36.04 $\pm$ 18.01 mV in control neurons, p =0.03). Besides, we observed a narrower spike in the UBTF-mutant neurons compared to the control neurons (3.3 $\pm$ 1.5 ms in the UBTF-mutant neurons and 12.5 $\pm$17.8 ms in the control neurons, p = 0.003); further spike shape analysis is presented in Table S1. The spontaneous neuronal activity (spontaneous action potentials) was measured in a holding potential of -45mV. We observed a significant increase in the spontaneous activity rate (p= 0.013) and amplitudes (p= 0.012) in the UBTF-mutant neurons compared to control neurons (Fig. S1a-b.). Examples of ICC images are shown in Fig. 4n. (typical NPC markers PAX6 and NESTIN) and 4o. (neuronal markers MAP2, the cortical marker TBR1).

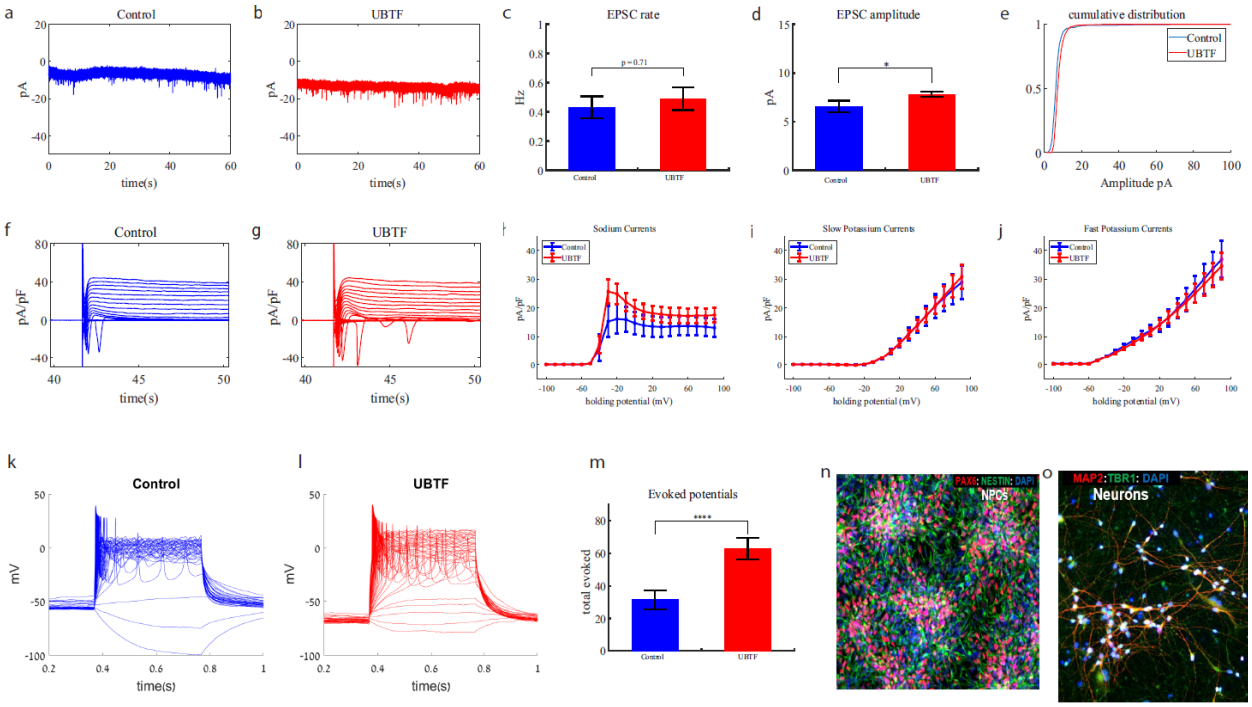

Fig. 4. Young (5 weeks post differentiation) UBTF-mutant neurons are hyperexcitable compared to control neurons (6 weeks post differentiation).

a A representative trace of excitatory postsynaptic currents (EPSCs) that were measured in control cortical neurons at six weeks post-differentiation. **b** A representative trace of EPSCs measured in a UBTF-mutant neuron at five weeks post-differentiation. **c** The mean rate of synaptic events was not significantly different in UBTF-mutant neurons compared to control neurons. **d** The average amplitude of EPSCs was increased in the UBTF-mutant neurons ($p = 0.02$). **e** The cumulative distribution of the amplitude of EPSCs of UBTF-mutant neurons is slightly right-shifted, indicating an increase in the amplitudes. **f** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in control neurons. **g** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in UBTF-mutant neurons. **h** The average sodium currents in UBTF-mutant neurons is increased compared to control neurons ($p<0.05$). **I** The average slow potassium currents look similar in the UBTF-mutant and control neurons. **j** The average fast potassium currents look identical in the UBTF-mutant and control neurons. **k** A representative recording of evoked action potentials in the current-clamp mode of control neurons. **l** A representative recording of evoked action potentials in a current-clamp mode of UBTF-mutant neurons. **m** The total number of evoked action potentials is higher in the UBTF-mutant neurons compared to control neurons ($p=5.56e^{-4}$). **n, o** A representative images of NPCs (**n**) and neurons (**o**) that were immunostained for DAPI, MAP2, TBR1, PAX6 and NESTIN.

DISCUSSION

In this study, iPSC technology was used in order to investigate the physiological features of cortical neurons derived from human patients with different ASD-related mutations: *Dup7*, *GRIN2B*, *SHANK3*, and *UBTF*. For that purpose, we differentiated patient-derived iPSCs into cortical neurons[53] since cortical alterations and malformations within the brain were identified in these mentioned mutations[16,32,44,50].

A broad range of mutations has been associated with ASD, sharing autistic-like behaviors such as difficulties in social communication and interaction and restricted or repetitive behaviors or interests. The broad spectrum of mutations is characterized by affecting different genes and pathways; For *Dup7* mutation, for example, a rare genetic syndrome caused by a micro-duplication in section q11.23 of chromosome 7, alterations in the ELN gene such as inherited and *de novo* deletions were reported, coding for the extracellular matrix protein, elastin, associated with connective-tissue malformations as reported in human patients[14]. *SHANK3* is a gene located at the terminal long arm of chromosome 22, coding for a master scaffolding protein found in the body's tissues, and importantly in the brain, and has a critical role in the postsynaptic density of glutamatergic synapses and synaptic

functions in rat and human brains[18,22]. The GRIN2B mutation results in a production of a nonfunctional GluN2B protein. A shortage or a dysfunction of this protein may cause an extreme reduction in the number of the functional NMDA receptors[38] causing neuronal impairments in both mice and human models[54,55]. *UBTF* is a gene coding for UBF, a transcription factor in RNA Pol I, critical for rRNA transcripts synthesis from rDNA in the nucleolus[46]. Loss of UBF induces nuclear disruptions, including inhibition of cell proliferation, rapid and synchronous apoptosis, and cell death in mice models[47].

Although these mutations are functionally very different from one another, they cause similar symptoms in the patients. Interestingly, we observed a hyperexcitability pattern in all these mentioned ASD-related mutations in an early-stage cell development (3-5 weeks post differentiation) that we measured by electrophysiological recordings. This hyperexcitability involved different aspects; In terms of sodium-potassium currents and activity, a consistent increase in sodium currents was observed within the four patient-derived neurons, which could, in turn, increase the excitability of the neurons by decreasing the action potential threshold[56]. These alterations of sodium currents can lead *to abnormal neuronal activity*, a phenomenon that also occurs in epilepsy[57]. It is interesting to note that all these four mutations have a strong association with epilepsy, and many of the patients also suffer from epilepsy[16,29,44,52]. Alteration in sodium currents had been previously reported in sodium channels associated mutations, such as *SCN1A*, *SCN2A* and *SCN3A*[58]. We also observed an increase in the EPSC rate and amplitude, indicating pre and postsynaptic changes that occur in the mutant neurons compared to the controls neurons. Similar findings were reported in mice models of autism [59–63]. Furthermore, more evoked action potentials were observed in response to current stimulation in the mutant-derived neurons. All these changes can indicate that the ASD mutant neurons develop faster, and at this early stage, when the control neurons are still very immature, they are already spiking and connecting with other neurons.

Several previous genetic studies showed a rise in cortical activity by documenting excitation-to-inhibition ratio system alterations[64–66], suggesting that periodic seizures and sensory hyperreactivity in ASD are caused by cortical hyperexcitability[67]. Previously, we reported a similar early time point hyperexcitability pattern in another ASD-related mutation - the A350V IQSEC2mutation[66]. In that study, we followed the IQSEC2-mutant neurons that started more active and more connected (5 weeks post differentiation) as they became hypoexcitable with reduced synaptic connections later on in the differentiation process. A reduction in synaptic connections was reported in a long line of ASD-related studies using mice and human models[68–71]. We speculate that there may be a connection between this early hyperexcitability and the later synaptic deficits, as perhaps this early hyperexcitability is neurotoxic to the cell at such an early stage of development.

Since, especially with mice studies, it is much harder to measure this early developmental stage, perhaps this is a stage that precedes the synaptic degradation in many other ASD mutations. More evidence to this early maturation was presented in a study with ASD patients with macrocephaly where the neurons derived from the patients were more arborized early in the development, and gene expression profiles also suggested an earlier maturation[72]. More evidence for functional hyperactivity in epilepsy and ASD-related mutations in human models were reported; briefly, in an engineered iPSC-derived neuron with the homozygous P924L mutation (one of many epilepsy-associated Slack mutations) displayed increased K_{Na} currents and more evoked action potentials in both single neurons and a connected neuronal network[73].

Our Findings present a shared phenotype of early maturation and hyperexcitability in four ASD-related mutations using patient-derived cortical neurons, indicating that there may be a common neurophysiological phenotype in ASD-related variants, sharing similar behavioral phenotypes but a different genotype. iPSC-derived neurons were previously used as a research tool for investigating physiological and cellular alterations characterizing various disorders including autism[66,72,74] and epilepsy[73,75,76]. Here we concentrated on the early developmental physiological alterations in 4 different ASD and epilepsy-related genes. The enhanced maturation and excitability in such young neurons may be deleterious to the cells and may later result in synaptic degeneration as was previously described in neurons derived from ASD and epilepsy patients[66,71–76].

MATERIALS AND METHODS

After obtaining institutional committee approval and written informed consent from all participants or their respective legal guardians, iPSCs were generated from the peripheral blood mononuclear cells (PBMCs) of one male child carrying *Dup7*, one female child carrying *GRIN2B*, one female child carrying *SHANK3*[77] and one female child carrying *UBTF* mutations and a corresponding number of controls sharing the same genetic backgrounds (first-degree relatives from the same gender, except for GRIN2B), Table S2 presents a description of the patients' cohort.

PBMC isolation

Participants had undergone a regular blood test (except for the SHANK3 cohort and GRIN2B-mutant line, in which iPSCs were generated from skin biopsies); blood was collected into heparin-coated tubes, diluted with phosphate-

buffered saline (PBS) and centrifuged for 30 minutes at 1800□×g at 23□C. The mononuclear cells (MCs) were collected by pipetting the buffy coat – the cell layer between the gel barrier and the plasma, into a sterile 15 ml conical centrifuge tube, adding PBS to reach a volume of 10 ml. This was followed by centrifuging (300 xg at room temperature for 15 min), resuspended using fetal bovine serum (FBS, F7524 Merck) 10% DMSO to freeze a total of ~$2x10^6$ cells per cryovial in 1ml volume were aliquoted, frozen and kept in liquid nitrogen.

Reprograming of PBMCs into iPSCs

PBMCs were grown in a 24-well plate and seeded in fresh StemPro™ SFM medium (10639011, Thermo Fisher Scientific). Sendai viral particle factors from CytoTune-iPS Sendai Reprogramming Kit (A16518, Life Technologies, Carlsbad, CA, USA) were added based on the manufacturer's recommendations[78], followed by centrifuging the cells (1,160□×g, at 35°C, for 30 minutes) and seeded on a 24-well plate and an overnight incubation in 5% CO_2 at 37°C. The next day, the cells were transferred into a low attachment coated 12-well plate. Later, they were plated in six-well Matrigel-coated plates for three days and fed with a complete PBMC medium: StemPro™-34 supplement and2mM of L-Glutamine, followed by two days of gradually transitioning into iPSC medium (DMEM/F-12, GlutaMAX, KnockOut™ Serum Replacement, 10mM MEM Non-Essential Amino Acids Solution and 55mM β-mercaptoethanol). This was followed by daily replacing spent medium with fresh mTeSR for 20 days. When iPSC colonies were large enough, manual picking of the colonies was done, and colonies were transferred onto six well-Matrigel-coated plates. Immunocytochemistry of TRA-1-60, SOX2 and OCT4 antibodies confirmed pluripotency.

Generating neuronal cultures

Cortical neurons were plated based on a previously described protocol[53]. In brief, iPSCs were grown to ~80% confluency, embryonic bodies (EBs) were formed by mechanical dissociation using dispase for 20 minutes and plated onto low-adherence plates in mTeSR medium with ROCK inhibitor for one day, followed by one day of mTeSR medium only. On the following ten days, cells were fed with EB media: (DMEM/F12 with Glutamax(1:100), B27 with Retinoic Acid (RA) (1:100), N2 supplement(1:100) and 0.1uM LDN), followed by plating onto polyornithine/laminin (Sigma)-coated dishes in DMEM/F12 (Invitrogen) plus N2, B27 and laminin for the following seven days. The rosettes were selected based on their morphology and were manually picked, dissociated with Accutase (Chemicon) and plated onto poly-l-ornithine/laminin-coated plates and fed with complete NPC medium (DMEM/F12 with Glutamax (1:100), B27 supplement with RA (1:100), N2 supplement (1:100), laminin (1 mg/ml) and 20ng/ml FGF2) for the following 15-20 days (based on their confluency)... NPCs were then differentiated into cortical neurons by feeding with the differentiation medium that contained: DMEM/F12, N2, B27, Glutamax, ascorbic acid (200 nM), cyclic AMP (cAMP; 500 mg/ml), laminin (1 mg/ml), BDNF (20 ng/ml), GDNF (20 ng/ml) for ten days. Between days 11-14, the cells were dissociated again and then fed with Brainphys medium with B27 supplement (1:100), N2 supplement (1:100), ascorbic acid (200 nM), cyclic AMP (500 mg/ml), BDNF (20 ng/ml), GDNF (20 ng/ml) and laminin (1 mg/ml).

Electrophysiology

Whole-cell patch-clamp recordings have been performed on neurons derived from patients carrying the *Dup7*, *GRIN2B*, *SHANK3* and *UBTF* mutations, as well as neurons derived from healthy controls based on previously described[79] with some modifications[80], three to five weeks post differentiation. Culture coverslips were placed inside a recording chamber filled with HEPES-based artificial cerebrospinal fluid (ACSF) containing (in mM): 10 HEPES,139 NaCl, 4 KCl, 2 $CaCl_2$, 10 D-glucose, and 1$MgCl_2$(pH 7.5, osmolarity adjusted to 310 mOsm) at room temperature. The recording micropipettes (tip resistance of 9-12 MΩ) were filled with an internal solution containing (in mM): 130 K-gluconate, 6 KCl, 4 NaCl, 10 Na-HEPES, 0.2 K-EGTA, 0.3 GTP, 2 Mg-ATP, 0.2 cAMP, 10 D-glucose, 0.15% biocytin and 0.06% rhodamine (pH 7.5, osmolarity adjusted to 290-300 mOsm). All measurements were done at room temperature using Clampex v11.1 with a sampling rate of 20 kHz.

Analysis of electrophysiological recordings

Analysis was performed based on previously described[80] analysis modified as follows:

Synaptic currents analysis. The mean and standard error (SE) of the excitatory postsynaptic currents (EPSCs) amplitudes for each active cell were calculated. The cumulative distribution of EPSCs amplitude was calculated for each group. For each cell, the rate of the events was calculated by dividing the number of events by the time period of the recording (non-active cells were included and had an event rate=0). The mean of all cells' rates and the standard error of the frequencies were computed for the control and mutant groups. Non-parametric statistical tests (Wilcoxon signed rank test) were performed for comparisons.

Sodium, fast, and slow potassium currents. Neurons were held in voltage clamp mode at -60 mV, and voltage steps of 400 ms were performed in the -100 to 90 mV range. Currents were typically normalized by the cells' capacitance; The sodium current was computed by subtracting the sodium current after stabilization from the lowest value of the inward sodium current. The fast potassium currents were measured by the maximum outward currents that appeared within a few milliseconds after a depolarization step. The slow potassium currents were measured after the 400 ms depolarization phase. A one-way ANOVA test was performed for the statistical analysis.

Evoked action potentials (APs). Neurons were held in current clamp mode at -60 mV with a constant holding current. Following this, current injections were given in 3-pA steps throughout 400 ms, starting 12 pA below the steady-hold current. A total of 38 depolarization steps were given. The total evoked action potential was the total number of action potentials that were counted in the 38 depolarization steps. Non-parametric statistical tests (Wilcoxon signed rank test) were performed for comparisons.

Action potential shape analysis. The first evoked action potential generated with the lowest amount of injected current was used for spike shape analysis. The spike threshold was defined as the membrane potential at which the slope of the depolarizing membrane potential increased dramatically, resulting in an action potential (the second derivative of the voltage versus time as the initial maximum). The spike height was calculated as the difference between the highest membrane potential during a spike and the threshold. The spike rise time is the time it takes the spike to reach the maximum. The spike width was calculated as the time it took the membrane potential to reach half of the spike amplitude in the rising part.

Immunocytochemistry (ICC)

Cells were fixed in 4% paraformaldehyde for 15 minutes, followed by three washes of DPBS, blocked and permeabilized in PBS containing 0.1%-0.2% Triton X-100 and 10% horse serum. The coverslips were incubated with primary antibodies; for NPCs: rabbit anti-PAX6 (1:250) and mouse-anti NESTIN (1:2000); for Neurons: chicken-anti MAP2 (1:500) and rabbit anti-TBR1 (1:250) in the blocking solution overnight at 4□C. On the next day, they were washed in DPBS and incubated with DAPI and corresponding secondary antibodies (1:1000) for neurons and (1:250) for NPCs for 60 minutes at room temperature. Then the coverslips were washed three times, mounted on glass slides using Fluromount-G (mounting medium) and dried overnight while being protected from light. Fluorescence signals were detected using a Leica THUNDER imager.

List of Supplementary Materials

Tables S1 and S2.

Fig S1.

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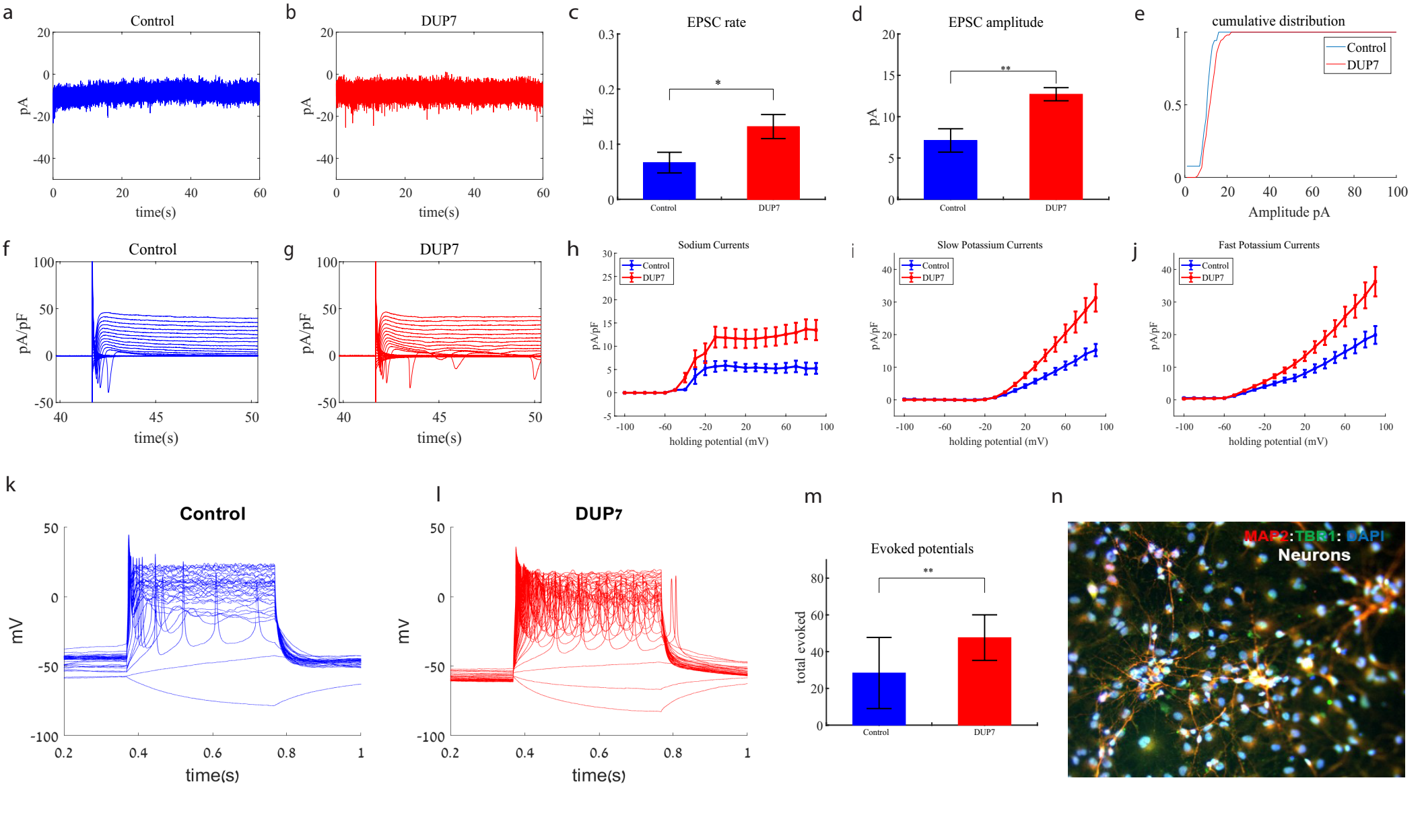
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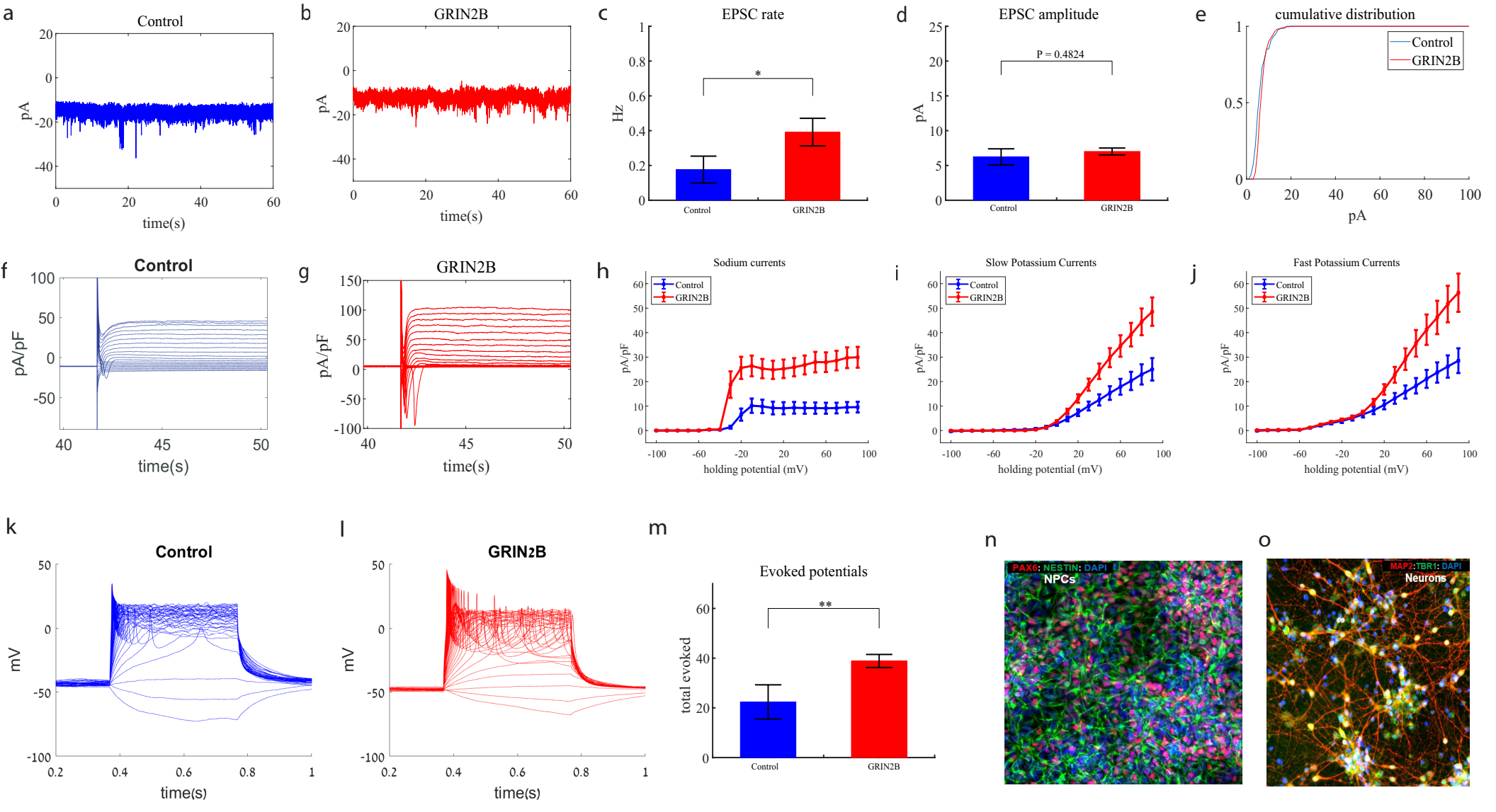
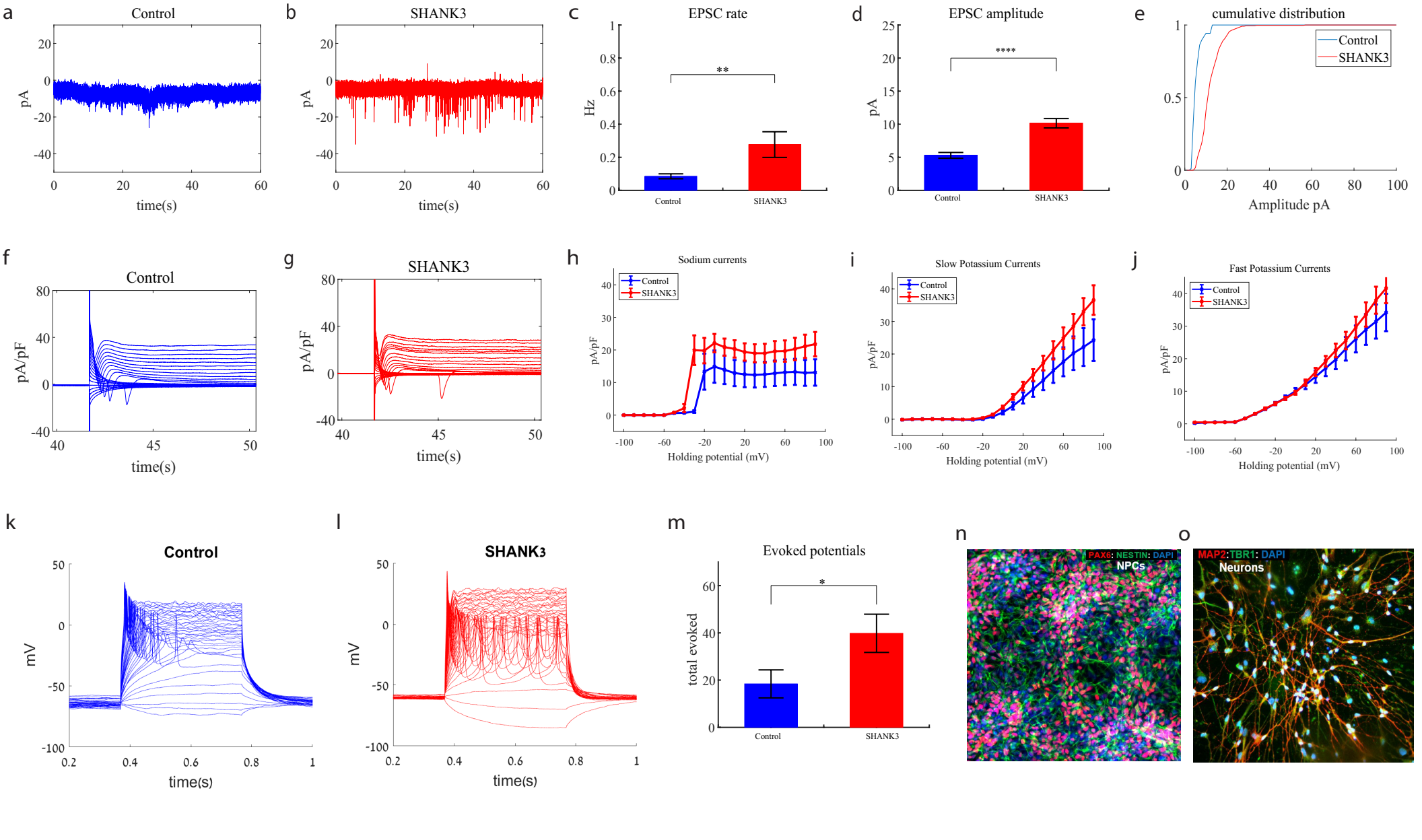
Evoked potentials
**
total evoked
Control
DUP7
n
MAP2:TBR1: DAPI
Neurons


a
Control
pA
time(s)
b
GRIN2B
pA
time(s)
c
EPSC rate
Hz
*
Control
GRIN2B
d
EPSC amplitude
pA
P = 0.4824
Control
GRIN2B
e
cumulative distribution
Control
GRIN2B
pA
f
Control
pA/pF
time(s)
g
GRIN2B
pA/pF
time(s)
h
Sodium currents
Control
GRIN2B
pA/pF
holding potential (mV)
i
Slow Potassium Currents
Control
GRIN2B
pA/pF
holding potential (mV)
j
Fast Potassium Currents
Control
GRIN2B
pA/pF
holding potential (mV)
k
Control
mV
time(s)
l
GRIN2B
mV
time(s)
m
Evoked potentials
**
total evoked
Control
GRIN2B
n
PAX6: NESTIN: DAPI
NPCs
o
MAP2:TBR1: DAPI
Neurons


a
Control
pA
time(s)
b
SHANK3
pA
time(s)
c
EPSC rate
Hz
**
Control
SHANK3
d
EPSC amplitude
pA
****
Control
SHANK3
e
cumulative distribution
Control
SHANK3
Amplitude pA
f
Control
pA/pF
time(s)
g
SHANK3
pA/pF
time(s)
h
Sodium currents
Control
SHANK3
pA/pF
Holding potential (mV)
i
Slow Potassium Currents
Control
SHANK3
pA/pF
Holding potential (mV)
j
Fast Potassium Currents
Control
SHANK3
pA/pF
Holding potential (mV)
k
Control
mV
time(s)
l
SHANK3
mV
time(s)
m
Evoked potentials
total evoked
*
Control
SHANK3
n
PAX6: NESTIN: DAPI
NPCs
o
MAP2: TBR1: DAPI
Neurons

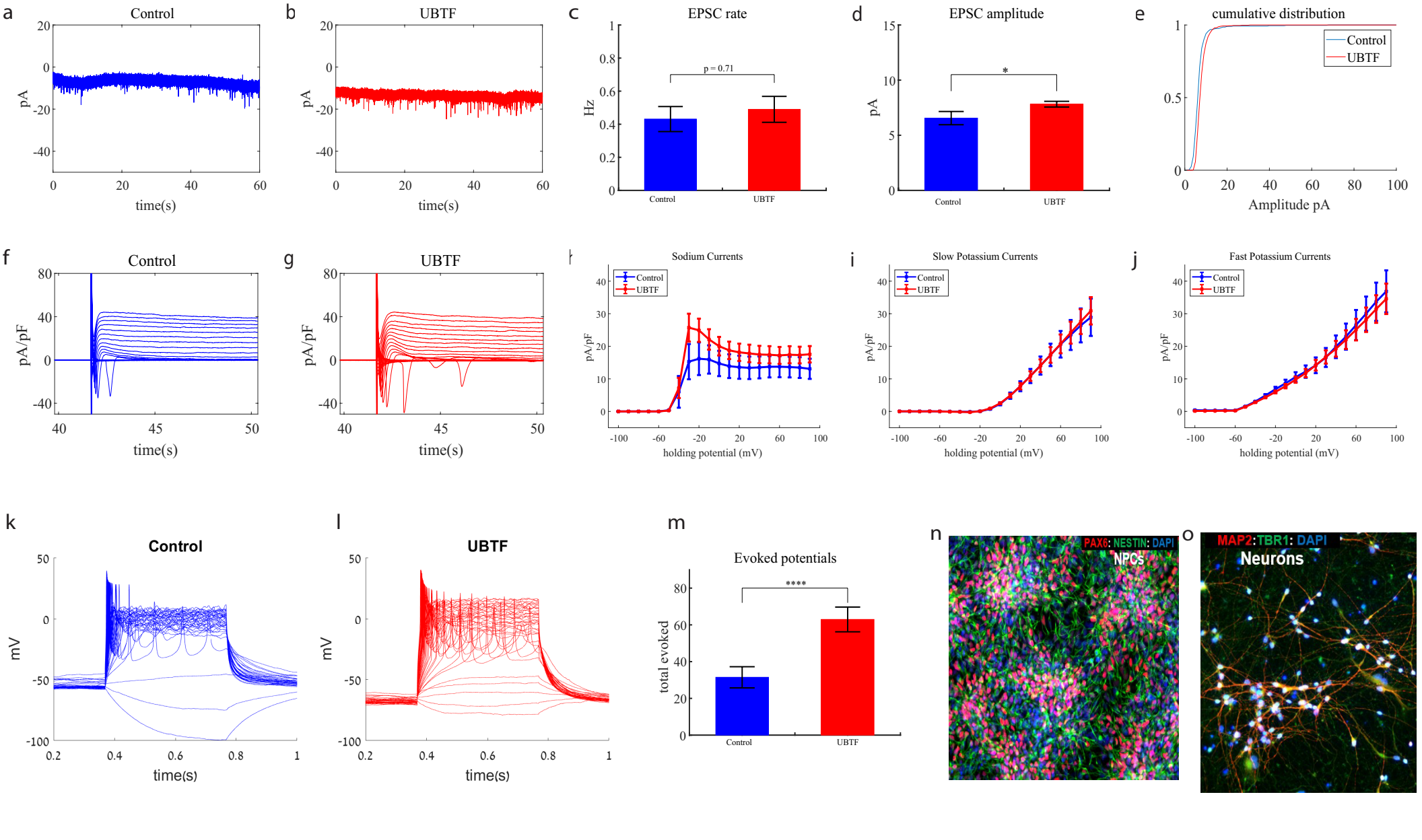

a
Control
pA
time(s)
b
UBTF
pA
time(s)
c
EPSC rate
p = 0.71
Hz
Control
UBTF
d
EPSC amplitude
*
pA
Control
UBTF
e
cumulative distribution
Control
UBTF
Amplitude pA
f
Control
pA/pF
time(s)
g
UBTF
pA/pF
time(s)
h
Sodium Currents
Control
UBTF
pA/pF
holding potential (mV)
i
Slow Potassium Currents
Control
UBTF
pA/pF
holding potential (mV)
j
Fast Potassium Currents
Control
UBTF
pA/pF
holding potential (mV)
k
Control
mV
time(s)
l
UBTF
mV
time(s)
m
Evoked potentials
****
total evoked
Control
UBTF
n
PAX6: NESTIN: DAPI
NPCs
o
MAP2:TBR1: DAPI
Neurons

Fig. 1. Young (5 weeks post differentiation) Dup7-mutant neurons are hyperexcitable compared to control neurons.

a A representative trace of (EPSCsmeasured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a dup7-mutant neuron at five weeks post-differentiation. **c** The mean rate of synaptic events was higher in the dup7-mutant neurons compared to control neurons (p=0.029). **d** The average amplitude of EPSCs was increased in the dup7-mutant neurons (p =0.002). **e** The cumulative distribution of the amplitude of EPSCs is slightly right-shifted in the dup7-mutant neurons, indicating an increase in the amplitudes. **f** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in control neurons. **g** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in dup7-mutant neurons. **h** The average sodium currents in dup7-mutant neurons is increased compared to control neurons (p=0.004). **i** The average slow potassium currents in dup7-mutant neurons is increased compared to control neurons (p=0.03). **j** The average fast potassium currents is increased in dup7-mutant compared to control neurons (p=0.01). **k** A representative recording of evoked action potentials in a current-clamp mode of a control neuron. **l** A representative recording of evoked action potentials in a current-clamp mode of a dup7-mutant neuron. **m** The total number of evoked action potentials is larger in dup7-mutant neurons compared to control neurons (p=0.006). **n** A representative image of neurons that were immunostained for DAPI, MAP2 and TBR1.

Fig. 2. Young (3 weeks post differentiation) GRIN2B-mutant neurons are hyperexcitable compared to control neurons (5 weeks post differentiation).

a A representative trace of EPSCs measured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a GRIN2B-mutant neuron at three weeks post-differentiation. **c** The mean rate of synaptic events was higher in the GRIN2B-mutant neurons (p=0.03). **d** The mean amplitude of EPSCs was increased but not significantly different in the GRIN2B-mutant neurons. **e** The cumulative distribution of the amplitude of the EPSCs of GRIN2B-mutant and control neurons looks similar. **F** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in control neurons. **g** A Representative trace of sodium and potassium currents recorded in voltage-clamp in GRIN2B-mutant neurons. **h** The average sodium currents in GRIN2B-mutant neurons is severely increased compared to control neurons (p=0.006). **I** The average slow potassium currents in GRIN2B-mutant neurons is increased compared to control neurons (p=0.04). **j** The average fast potassium currents in GRIN2B-mutant neurons is increased compared to control neurons (p=0.03). **k** A representative recording of evoked action potentials in a current-clamp mode of a control neuron. **l** A representative recording of evoked action potentials in a current-clamp mode of a GRIN2B-mutant neuron. **m** The total number of evoked action potentials is larger in the GRIN2B-mutant neurons compared to control neurons (p=0.003). **n, o** A representative images of **n** NPCs and **o** neurons that were immunostained for DAPI, MAP2, TBR1, PAX6 and NESTIN.

Fig. 3. Young (5 weeks post differentiation) SHANK3-mutant neurons are hyperexcitable compared to control neurons.

a A representative trace of excitatory postsynaptic currents (EPSCs) that were measured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a SHANK3-mutant neuron at five weeks post-differentiation. **c** The mean rate of synaptic events was higher in the SHANK3-mutant neurons (p=0.003). **d** The average amplitude of EPSCs was increased in the SHANK3-mutant neurons ($p=1.15e^{-5}$). **e** The cumulative distribution of the amplitude of EPSCs of SHANK3-mutant is right-shifted, indicating an increase in the amplitudes. **f** A Representative trace of sodium and potassium currents recorded in voltage-clamp in control neurons. **g** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in SHANK3-mutant neurons. **h** The average sodium currents in SHANK3-mutant neurons is increased compared to control neurons (p=0.04). **I** The average slow potassium currents in SHANK3-mutant neurons is increased compared to control neurons (p=0.03). **j** The average fast potassium currents look similar and not significantly different in SHANK3-mutant compared to control neurons. **k** A representative recording of evoked action potentials in a current-clamp mode of control neurons. **l** A representative recording of evoked action potentials in a current-clamp mode of SHANK3-mutant neurons. **m** The total number of evoked action potentials is larger in SHANK3-mutant neurons compared to control neurons (p=0.03). **n, o** A representative images of NPCs (**n**) and neurons (**o**) that were immunostained for DAPI, MAP2, TBR1, PAX6 and NESTIN.

Fig. 4. Young (5 weeks post differentiation) UBTF-mutant neurons are hyperexcitable compared to control neurons (6 weeks post differentiation).

a A representative trace of excitatory postsynaptic currents (EPSCs) that were measured in control cortical neurons at six weeks post-differentiation. **b** A representative trace of EPSCs measured in a UBTF-mutant neuron at five weeks post-differentiation. **c** The mean rate of synaptic events was not significantly different in UBTF-mutant neurons compared to control neurons. **d** The average amplitude of EPSCs was increased in the UBTF-mutant neurons ($p = 0.02$). **e** The cumulative distribution of the amplitude of EPSCs of UBTF-mutant neurons is slightly right-shifted, indicating an increase in the amplitudes. **f** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in control neurons. **g** A Representative trace of sodium and potassium currents recorded in a voltage-clamp mode in UBTF-mutant neurons. **h** The average sodium currents in UBTF-mutant neurons is increased compared to control neurons ($p<0.05$). **I** The average slow potassium currents look similar in the UBTF-mutant and control neurons. **j** The average fast potassium currents look identical in the UBTF-mutant and control neurons. **k** A representative recording of evoked action potentials in the current-clamp mode of control neurons. **l** A representative recording of evoked action potentials in a current-clamp mode of UBTF-mutant neurons. **m** The total number of evoked action potentials is higher in the UBTF-mutant neurons compared to control neurons ($p=5.56e^{-4}$). **n, o** A representative images of NPCs (**n**) and neurons (**o**) that were immunostained for DAPI, MAP2, TBR1, PAX6 and NESTIN.