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

Yara Hussein[1,*], Utkarsh Tripathi[1,*], Ashwani Choudhary[1], Ritu Nayak[1], David Peles[1], Idan Rosh[1],Tatiana Rabinski[2], Jose Djamus[1], Gad Vatine[2], Ronen Spiegel[3], Tali Garin-Shkolnik[3], Shani Stern[1,#]

[1]Sagol Department of Neurobiology, Faculty of Natural Sciences, University of Haifa; Haifa, Israel.
[2]The Department of Physiology and Cell Biology, Faculty of Health Sciences and RMSC, Ben-Gurion University of the Negev; Beer Sheva, Israel.
[3]Center for Rare Diseases, Emek Medical Center; Afula, Israel.
*Equally contributed
#Correspondence: Shani Stern, sstern@univ.haifa.ac.il

Abstract: Autism Spectrum Disorder (ASD) is characterized mainly by social and sensory-motor abnormal and repetitive behavior patterns. Over hundreds of genes and thousands of 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 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 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[4], and there seem to be a most definitive leaders: ADNP, CHD8, and SHANK3[5].

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[6]. 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[7], also known as the Williams-Beuren syndrome critical region (WBSCR) and is inherited in an autosomal dominant manner[8]. 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[9], 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[10]. These are thought to contribute significantly to the Syndrome's symptoms[11,12].

Common symptoms of *Dup7* patients include anxiety disorders, selective mutism, ASD, ADHD, intellectual disability, seizures, severe speech delays, and hypotonia[13,14] as well as Facial features including a broad forehead, a thin upper lip, and facial asymmetry[11]. Beyond that, magnetic resonance imaging (MRI) showed various abnormalities such as cerebral atrophy, increased cortical thickness, alterations in white matter's volume and cerebellar hypoplasia, and an increased cortical extra-axial space[14].

SHANK3

The SHANK protein family, also known as ProSAP, including *SHANK 1-3*, 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.[15,16] 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[15,17,18].

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[15,19]. Mutations in this gene have been linked to deficits in synaptic function and plasticity, accompanied by lower reciprocal social interactions[18,20–24], ASD, and other neurodevelopmental symptoms[21,25]. The individuals affected by the 22q13 deletion syndrome[25,26], also known as Phelan-McDermid syndrome (PMS), suffer from developmental delays, a low muscular tone, a weakened perception of pain, delayed speech, seizures ans ASD[27–29]. The patients also exhibit cortical alterations[30].

GRIN2B

Glutamate Ionotropic Receptor NMDA type Subunit 2B (***GRIN2B***) is a gene located on the 'p' arm of chromosome 12 (12p13.1 is its exact location)[31,32]. This gene is one member of a family of genes encoding various proteins that together form the NMDA receptor[33] – glutamate-gated ion channel, which allows positively charged particles to flow through cells in the brain, activating neurons to send signals to each other[34,35].

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[34,36,37], causing neurodevelopmental disorders[38]. This disorder is associated with West syndrome, characterized by intellectual disability, delayed development of speech and motor skills, focal seizures, weak muscle tone (hypotonia), pediatric encephalopathy, movement disorders, schizophrenia, and behavioral delays common in ASD[31,38–43]. In addition, MRI scans revealed a consistent malformation of cortical development (MCD) consistent with that of tubulinopathies, hypoplastic corpus callosum of varying degrees, enlarged and mildly dysplastic basal ganglia, hippocampal dysplasia as well as 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[49]. 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 [48,50–53]. 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[48,51]. 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].

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* (7q11.23 dup), one female child carrying *GRIN2B* (c.2065 G->T), one female child carrying *SHANK3*[54] (C.3679insG) and one female child carrying *UBTF* (E210K) mutations and a corresponding number of controls sharing the same genetic backgrounds (first-degree relatives from the same gender, except for GRIN2B), Table S1 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 PBMCs were extracted. Additional information can be found in the supplementary.

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[55] and iPSCs were generated. Additional information can be found in the supplementary.

Generating neuronal cultures

Cortical neurons were plated based on a previously described protocol[56]. Additional information can be found in the supplementary.

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[57] with some modifications[58], three to five weeks post differentiation. Additional information can be found in the supplementary.

Analysis of electrophysiological recordings

Analysis was performed based on previously described[58] analysis. Additional information can be found in the supplementary.

Immunocytochemistry (ICC)

Cells were fixed in 4% paraformaldehyde, blocked, and permeabilized in PBS containing 0.1%-0.2% Triton X-100 and 10% horse serum. The coverslips were incubated with primary antibodies overnight and incubated with DAPI and corresponding secondary antibodies on the next day, Then the coverslips were mounted. Fluorescence signals were detected using a Leica THUNDER imager. Additional information can be found in the supplementary.

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 $\pm$ 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 $\pm$ 49.61; for the control neurons, it was 28.5 $\pm$ 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 S2. Fig. 1n. is an example ICC image for neuronal markers MAP2 and the cortical marker 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 S2. Examples of ICC images are shown in Fig. 2n. (typical NPC markers PAX6 and NESTIN) and 2o. (neuronal markers MAP2, the cortical marker TBR1).

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, p0.003) 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 S2. Examples of ICC images are shown in Fig. 3n. (typical NPC markers PAX6 and NESTIN) and 3o. (neuronal markers MAP2, the cortical marker TBR1).

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 S2. 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).

DISCUSSION

In this study, iPSC technology was used 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[56] since cortical alterations and malformations within the brain were identified in these mentioned mutations[14,30,44,51].

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. Various genes affecting the cytoskeletal[59–63] and microtubule[64] dynamics are altered in ASD-associated genes, disturbing a critical stage in axons and dendrites development[65]; 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[12]. *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[15,19]. 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[36] causing neuronal impairments in both mice and human models[66,67]. *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[68]. These alterations of sodium currents can lead to abnormal neuronal activity, a phenomenon that also occurs in epilepsy[69]. 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[14,27,44,53]..

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 [71–75]. 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[76–78], suggesting that periodic seizures and sensory hyperreactivity in ASD are caused by cortical hyperexcitability[79,80]. Previously, we reported a similar early time point hyperexcitability pattern in another ASD-related mutation - the A350V IQSEC2mutation[78]. 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. An additional hyperexcitability pattern in 15q11-q13 Duplication Syndrome, a different ASD-associated mutation, was reported[81]. A reduction in synaptic connections was reported in a long line of ASD-related and ID-related studies using mice and human models[82–85], suggesting a potential shared accelerated aging mechanisms in ASD-associated mutations[63,64,86–88].

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[89]. More evidence for functional hyperactivity in epilepsy and ASD-related mutations in human models were reported; briefly, 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[90].

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[78,89,91,92] and epilepsy[90,93,94] and other neurodegenerative disorders such as Parkinson's[95]. 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[78,85,89–91,93,94].

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Competing interests
The authors declare that they have no conflict of interest.

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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).

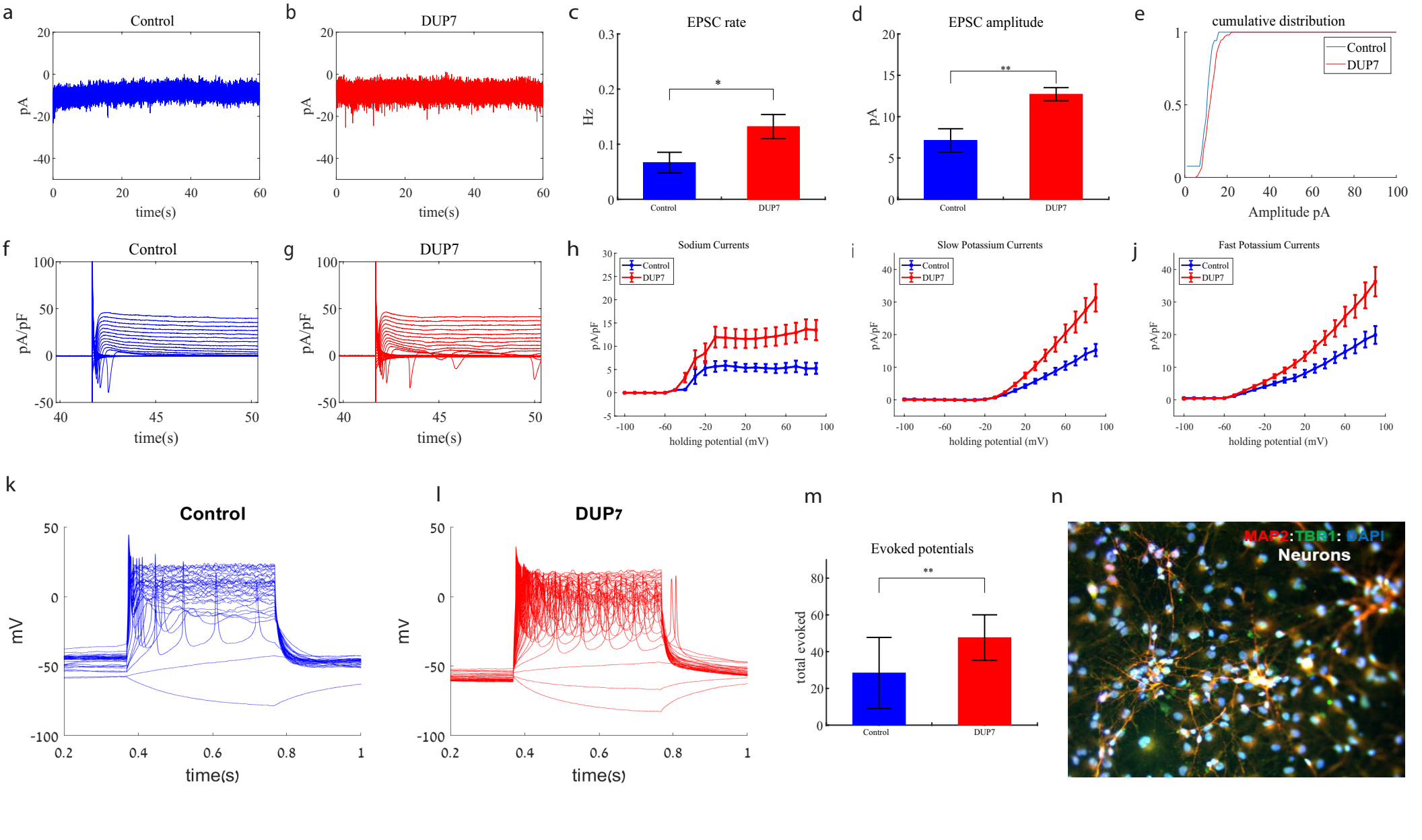
a A representative trace of EPSCs measured in control cortical neurons at five weeks post-differentiation. **b** A representative trace of EPSCs measured in a GRIN2B-mutant neuron at three weeks post-differentiation. **c** The mean rate of synaptic events was higher in the GRIN2B-mutant neurons (p=0.03). **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.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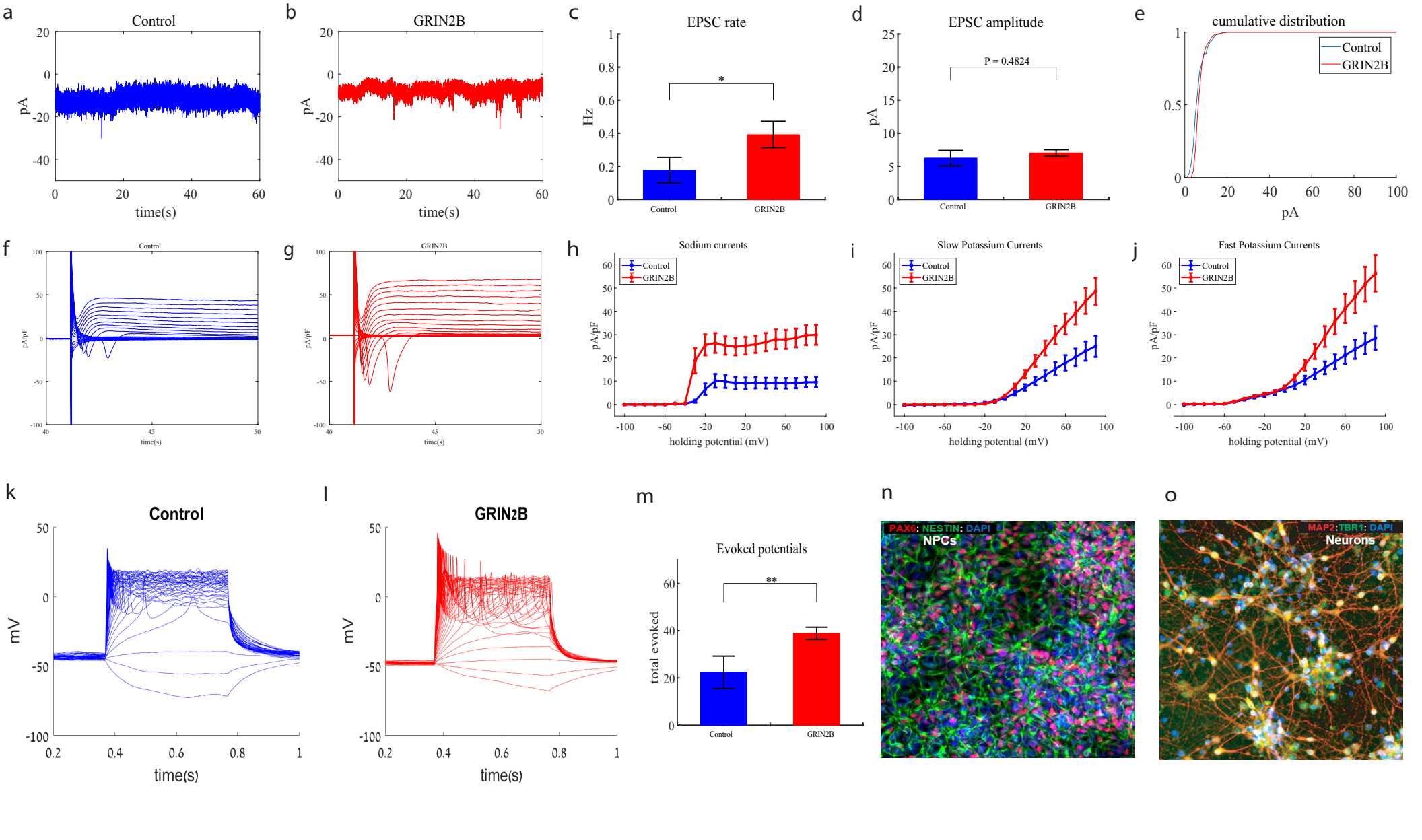
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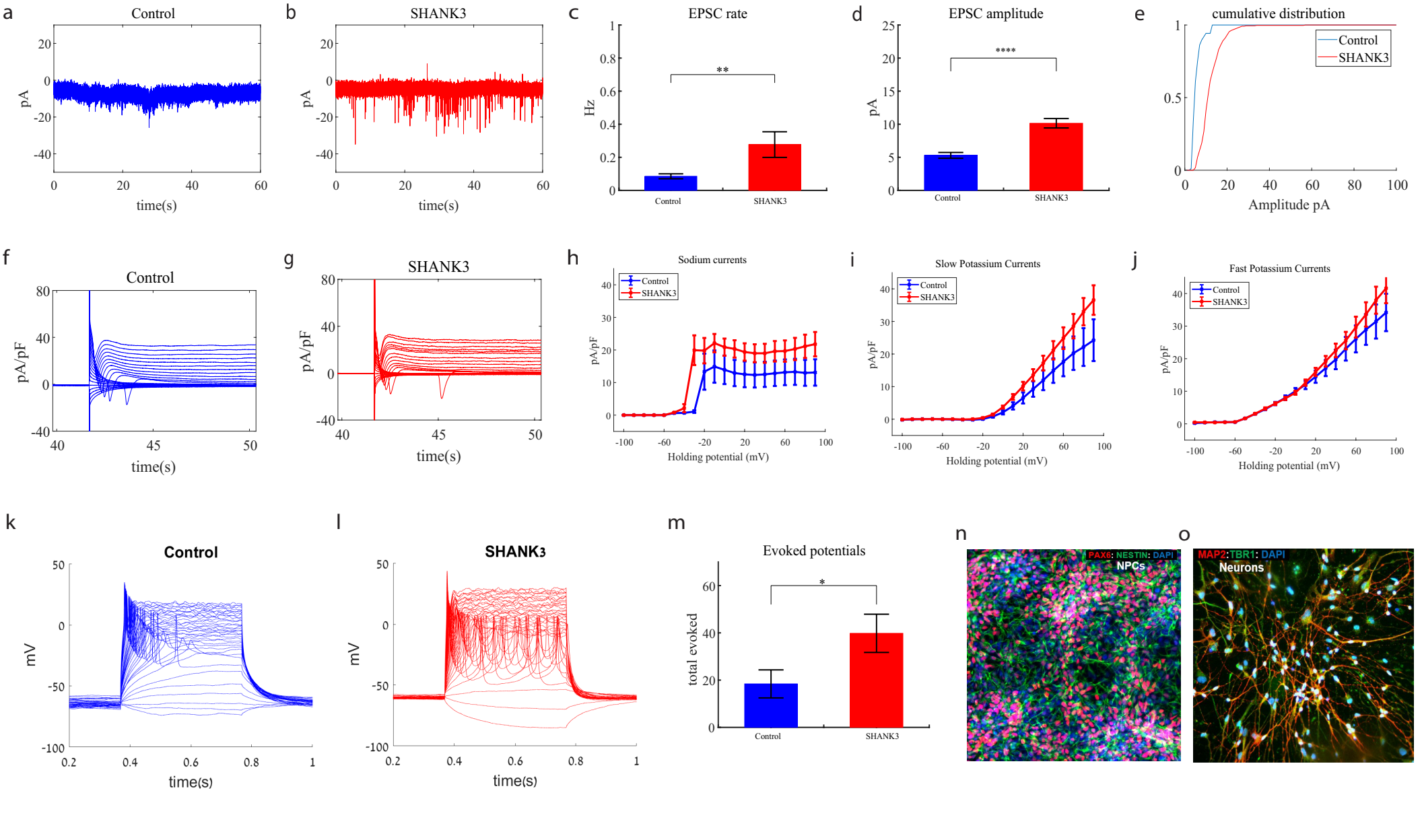
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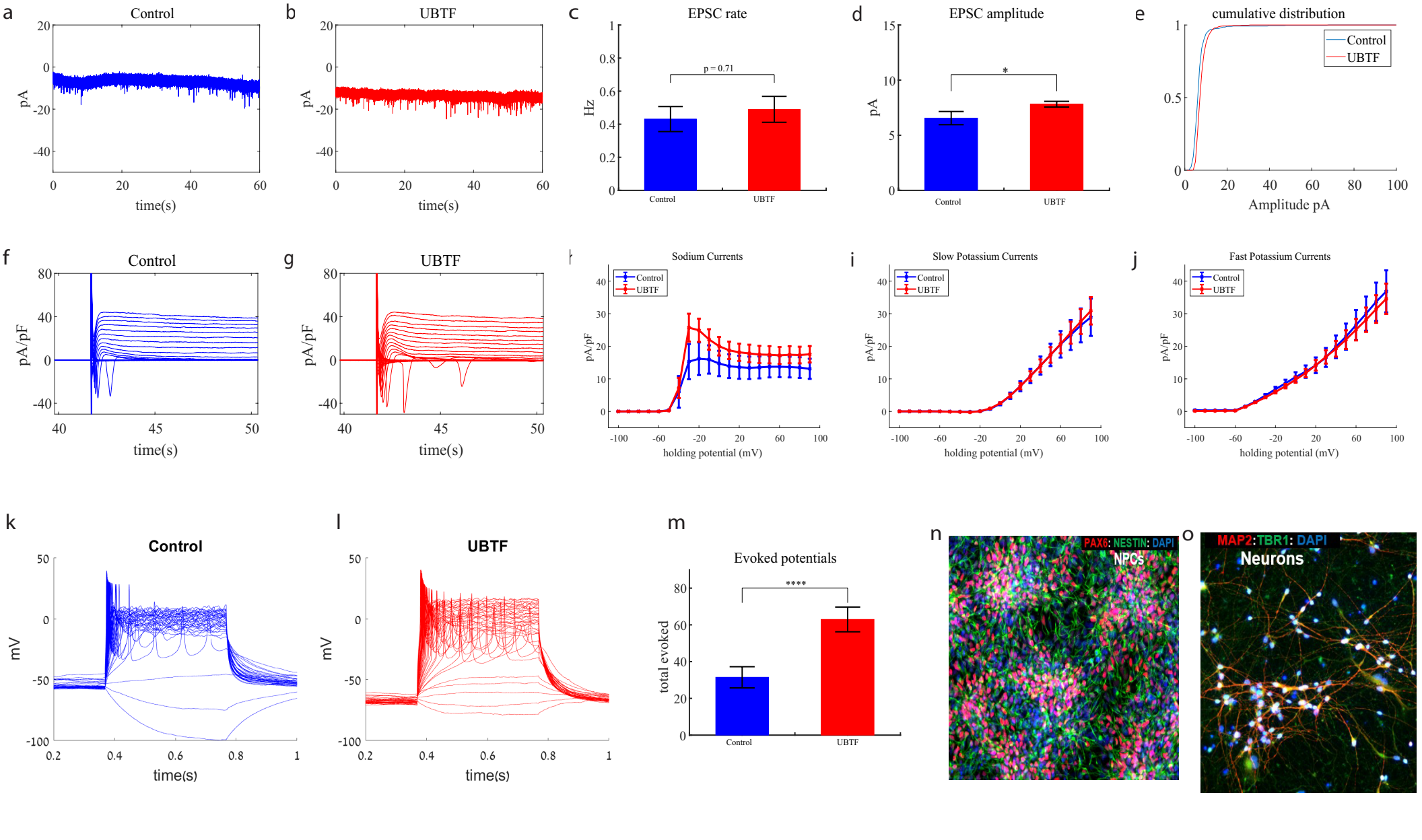
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