TAOK1 is associated with neurodevelopmental disorder and essential for neuronal maturation and cortical development

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Abstract

Thousand and one amino-acid kinase 1 (TAOK1) is a MAP3K protein kinase, regulating different mitogen-activated protein kinase pathways, thereby modulating a multitude of processes in the cell. Given the recent finding of TAOK1 involvement in neurodevelopmental disorders (NDDs), we investigated the role of TAOK1 in neuronal function and collected a cohort of 23 individuals with mostly de novo variants in TAOK1 to further define the associated NDD. Here, we provide evidence for an important role for TAOK1 in neuronal function, showing that altered TAOK1 expression levels in the embryonic mouse brain affect neural migration in vivo, as well as neuronal maturation in vitro. The molecular spectrum of the identified TAOK1 variants comprises largely truncating and nonsense variants, but also missense variants, for which we provide evidence that they can have a loss of function or dominant-negative effect on TAOK1, expanding the potential underlying causative mechanisms resulting in NDD. Taken together, our data indicate that TAOK1 activity needs to be properly controlled for normal neuronal function and that TAOK1 dysregulation leads to a neurodevelopmental disorder mainly comprising similar facial features, developmental delay/intellectual disability and/or variable learning or behavioral problems, muscular hypotonia, infant feeding difficulties, and growth problems.

KEYWORDS
cortical development, functional genomics, in utero electroporation, neurodevelopmental disorders, TAOK1

1 | INTRODUCTION

Next-generation sequencing has led to rapid advances in understanding the genetic background for many neurodevelopmental disorders (NDDs). Indeed, genome sequencing (GS) or exome sequencing (ES) is now often used as diagnostic tools for unexplained NDDs, and the list of novel, defined syndromes has been expanding rapidly (Deciphering Developmental Disorders Study, 2017; Lelieveld et al., 2016; Wright et al., 2018). Here, we describe the identification of TAOK1 variants in patients with NDD, as identified by ES, and define the associated core phenotype.

Thousand and one amino-acid protein kinases (TAOKs) are part of the Ste20p protein kinase family and include TAOK1 (also called PSK2 or MARKK), TAOK2 (also called PSK1), and TAOK3 (also called JNK [c-Jun NH2-terminal kinase]-inhibiting kinase). These kinases act upstream in the mitogen-activated protein kinase cascade, thereby regulating many cellular processes (Dan et al., 2001). Knockdown of Tao1 in Drosophila (which is the only representative of the TAOK family in Drosophila) is shown to affect brain volume at larval stages (Poon et al., 2016) and results in early lethality, a smaller ventral nerve cord, and a reduced number of neuromuscular junction endings, implying a critical role of the TAOK family in Drosophila neurodevelopment (Dulovic-Mahlow et al., 2019). In the mammalian TAOK family, TAOK2 plays an important role in neurodevelopment as Taok2 knockout mice show cognitive deficits as well as abnormal neural connectivity with shorter dendrites and fewer...
spines on neurons in the prefrontal cortex (Richter et al., 2018). TAOK2 has also been implicated in human neurodevelopment, as it is located at 16p11.2, a region associated with autism spectrum disorder (ASD) and schizophrenia (Pucilowska et al., 2015; L. A. Weiss et al., 2008). Indeed, patients with ASD harboring de novo variants in TAOK2 were identified recently, and these variants affect TAOK2 protein function, causing either loss-of-function (LoF) or a dominant-negative effect (Richter et al., 2018).

The role of TAOK1 in neuronal function has been less well documented. TAOK1 is a MAP3K protein kinase, which regulates the p38 as well as the JNK pathways either directly (Hutchison et al., 1998; Raman et al., 2007; Zihni et al., 2006) or indirectly through interleukin-17 (Z. Zhang et al., 2018). TAOK1 is highly homologous to TAOK2, consisting of a catalytic domain, a substrate-binding domain, a spacer, and a tail (Timm et al., 2003). The major difference between the two kinases arises from the distinct tail domains, where TAOK2 contains a microtubule-binding domain, which is absent in TAOK1 (Zihni et al., 2006). TAOK1 is involved in neurite outgrowth, axonal transport regulation, and in differentiating PC12 cells through the activation of PAR-1 (Timm et al., 2003). Additionally, human TAOK1 was shown to be involved in mitogenesis by being an important regulator of checkpoint control, indicating a developmental role (Draviam et al., 2007). Human TAOK1 is located on 17q11.2, close to NF1 (Zihni et al., 2006). An individual with developmental delay, dysmorphic features, microcephaly, and short stature, was reported to harbor a de novo microdeletion at chromosome 17q11.2, resulting in haploinsufficiency of seven candidate genes, including TAOK1 (Mahlow et al., 2019).

Here, we provide further evidence that TAOK1 is involved in mammalian brain development by manipulating TAOK1 expression during mouse brain development and describe the molecular and clinical data of 22 additional individuals carrying mutations affecting TAOK1.

## 2 MATERIAL AND METHODS

### 2.1 Patients

We collected the molecular and clinical features of 20 individuals with an intragenic TAOK1 variant and three patients with a chromosomal deletion, including TAOK1. Most patients were included as a result of a collaboration facilitated by GeneMatcher (Sorbera et al., 2015) in which multiple clinical and research groups independently identified individuals with intellectual disability/developmental delay (ID/DD) or other related phenotypes with rare variations in TAOK1. Furthermore, a Pubmed search has been executed to find an additional case in which a small chromosome deletion involving TAOK1 was thought to be causative for the patient’s phenotype (Xie et al., 2016).

Clinical analysis of these patients was performed during regular consultations focusing on medical history, physical examination, and observational analysis of behavioral features. In all patients, exome sequencing and variant filtering were performed, according to the routine procedures at each institute (Farwell et al., 2014; Farwell Hagman et al., 2016; Retterer et al., 2015; Twigg et al., 2015; K. Weiss et al., 2016).

Informed consent to publish data was obtained from all patients, either as part of the diagnostic workflow or as part of a research study. Informed consent to publish clinical photographs was also obtained if applicable. Informed consent matched the local ethical guidelines.

### 2.2 In silico modeling

For homology modeling, the protein sequence of TAOK1 (NP_065842.1) was submitted to the I-TASSER protein structure prediction server (Yang & Zhang, 2015). For structural representation, the model with the highest confidence (C-score) and topological similarity (Tm-score) was used. Structural representations were made with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

### 2.3 Constructs

The complementary DNA (cDNA) sequence from human TAOK1 WT (NM_020791.2) was obtained from a human brain cDNA library by polymerase chain reaction (PCR) (Phusion high fidelity; Thermo Fisher Scientific) using the following primers: Fw 5′-GGGGCCCATCCCTCAGGGCCCATACAATACAGACAGG-3′ and Rev 5′-TATTTAATATGAATCATATGAGTGGGCAAGTAGG-3′.

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Constructs were generated by PCR (Phusion high fidelity, Thermo Fisher Scientific) using the following primers: Fw 5′-TAOK1-Rev 5′-c.449G>T (p.Arg150Le). Fw 5′-CA

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The single-nucleotide point mutation was introduced by PCR (Phusion high fidelity, Thermo Fisher Scientific) using the following primers: Fw 5′-TTCTCATACTATATATATATATATTAAGAGG-3′ and Rev 5′-GATATTTCCTGCTTTGATATCTATATGAGTGGGCAAGTAGG-3′. The truncated TAOK1 was also generated by PCR (Phusion high fidelity, Thermo Fisher Scientific) using the following

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primers: TAOK1-c.2442T (p.Tyr815Ter), Fw 5'-GAATCCGGCGCCG
cCATCACCAGTAACAGGAGC-3' and Rev 5'-GAATCTTTAAT
TAAATAGCTTCAACAGCAGTTCC-3'. For all the in vivo and in
vitro experiments, the dual promoter expression vector without a gene
insert was used as control (control vector). Short hairpin RNA (shRNA)
constructs were obtained from the MISSION shRNA library for mouse
genomes of Sigma Life Sciences and The RNAi Consortium. For knock-
down of TAOK1 we used three different shRNA plasmids, each targeting
a different sequence: (1) GCCATTACAAAGTGGAAATAA, (2) CCATCT
CAACACTATTCCAGAA, and (3) GACTCGAAAGTTAGCCATCTT. The
control shRNA plasmid is the MISSION nontarget shRNA control vector:
CAACAGATGAAGAGACCAAA.

2.4 | Mice

For the neuronal cultures, FvB/NHsd females were crossed with
FvB/NHsd males (both ordered at 8- to 10-weeks-old from Envigo).
For the in utero electroporation, female FvB/NHsd (Envigo) were
crossed with male C57Bl6/J (ordered at 8- to 10-weeks-old from
Charles River). All mice were kept group-housed in IV cages
(Seasafe 1145T; Tecniplast) with bedding material (Lignocel BK 8/15
from Rettenmayer) on a 12-h light/dark cycle at 21°C ± 1°C,
humidity at 40%–70% and with food pellets (801727CRM(P) from
Special Dietary Service) and water available ad libitum. All animal
experiments were conducted in accordance with the European
Commission Council Directive 2010/63/EU (CCD approval
AVD101002017893).

2.5 | HEK-293T cell transfections

To test the expression levels of the TAOK1 constructs, we chose
HEK-293T cells, a cell line easy to transfect and culture. These cells
were not authenticated. HEK-293T cells were cultured in DMEM/
10% fetal calf serum/1% penicillin/streptomycin in six-well plates
and transfected when 60% confluent with the following DNA con-
structs: Control vector, TAOK1WT, TAOK1Arg150Del, TAOK1Leu167Arg,
TAOK1Met231Val, TAOK1Leu167Arg, or TAOK1Leu548Pro (3 μg per six-well
dish). Transfection of the plasmids was done using poly-
ethyleneimine according to the manufacturer's instructions (Sigma).
After 4–6 h of transfection, the medium was changed to reduce
toxicity. Transfected cells were then used for Western blot analysis.

2.6 | Western blot analysis

Two to three days after transfection, HEK-293T cells were harvested
and homogenized in lysis buffer (10 mM Tris-HCl 6.8, 2.5% sodium
dodecyl sulfate, 2 mM EDTA), containing protease inhibitor cocktail
(#P8340; Sigma), phosphatase inhibitor cocktail 2 (#P5726; Sigma),
and phosphatase inhibitor cocktail 3 (#P0044; Sigma). Protein
concentration was determined using the bicinchoninic acid protein assay
tdTomato) were taken from each coverslip for each experiment with at least two independent replications. For the analysis of the intensity of TAOK1 signal and neuronal morphology, the ImageJ software was used. For the intensity, the fluorescent signal of endogenous TAOK1 was measured in somas of transfected and five nontransfected surrounding neurons. The intensity of the transfected neuron was then divided over the nontransfected neurons. For neuronal morphology, the dendrites with their branches were measured using the NeuronJ plugin of ImageJ. Total neurite length was measured and analyzed and for the arborization, the number of branching of each primary neurite (=coming directly from the soma) was counted and analyzed. All values were normalized against the mean value for each parameter of the control (empty vector control). The analysis was done by an experimenter blinded for the transfection conditions.

2.9 | In-utero electroporation

The procedure was performed as described previously (Proietti Onori et al., 2018). In short, pregnant FvB/NhsD mice at E14.5 of gestation were anesthetized, and the uterus was exposed. The DNA construct (1.5–3 μg/μl) was diluted in fast green (0.05%) and injected in the lateral ventricle of the embryos while still in the uterus, using a glass pipette controlled by a Picospritzer® III device. To ensure the proper electroporation of the injected DNA constructs (1–2 μl) into the progenitor cells, five electrical square pulses of 45 V with a duration of 50 ms per pulse and 150-ms interpulse interval were delivered using tweezer-type electrodes connected to a pulse generator (ECM 830, BTX Harvard Apparatus). The electrodes were placed in such a way that the positive pole was targeting the developing somatosensory cortex. The following plasmids were injected: control vector, TAOK1WT, TAOK1Arg150Ile, TAOK1Leu167Arg, TAOK1Met231Val, TAOK1Leu315Phe, or TAOK1Leu549Pro or for knockdown experiments: a pool of the Taok1 shRNAs with an RFP plasmid (Addgene) or the control shRNA with an RFP plasmid. To make sure the effect on migration was specific for the knockdown of Taok1, the single shRNAs were also tested individually and resulted in similar migration deficits (data not shown). After birth, pups (M/F) were sacrificed at P1 or P7 for histochemical processing.

2.10 | Immunohistochemistry

Mice were deeply anesthetized with an overdose of nembutal and transcardially perfused with 4% paraformaldehyde (PFA). Brains were extracted and post-fixed in 4% PFA. Brains were then embedded in gelatin and cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB), frozen on dry ice, and sectioned using a freezing microtome (40–50-μm thick). Free-floating coronal sections were washed in PBS and blocked in PBS containing 10% normal horse serum (NHS) and 0.5% Triton X-100 for 1 h at ambient temperature. Afterward, slices were incubated with primary antibody RFP (#600401379, 1:2000; Rockland), diluted in PBS containing 2% NHS, 0.5% Triton X-100, at 4°C for 48–72 h. Slices were washed three times with PBS and the secondary antibody was added (Cy3 donkey-anti-rabbit, 1:400; Jackson ImmunoResearch) diluted in PBS containing 2% NHS, 0.5% Triton-X 100. Finally, the slices were counterstained with 4,6-diamidino-2-phenylindole solution (1:10,000; Invitrogen) before being mounted with Mowiol on the glass. Overview images of the coronal sections were acquired by tile scan imaging using an LSM700 confocal microscope (Zeiss) with a ×10 objective.

Neuronal migration analysis was performed using confocal images (×10 objective, 0.5 zoom, 1024 × 1024 pixels) obtained from two to three nonconsecutive sections from at least three successfully targeted animals per plasmid, as previously described (Küry et al., 2017; Proietti Onori et al., 2018; Reijnders et al., 2017). Briefly, images were rotated to correctly position the cortical layers, and the number of cells in different layers were counted using ImageJ (Analyze Particles option). Cortical areas from the pia to the ventricle were divided into 10 equally sized bins and the percentage of tdTomato-positive cells per bin was counted systematically.

2.11 | Statistical analysis

All data were assumed to be normally distributed. Statistical difference between the conditions for the in vitro and in vivo overexpression experiments was determined using one-way analysis of variance (ANOVA) followed by Dunnett’s posthoc test for multiple comparisons and for the knockdown in vivo experiments using the two-tailed unpaired t test (dual comparison). For the western blot analysis, a two-tailed unpaired t test was used (dual comparison). Neuronal migration was analyzed based on the proportion of electroporated cell targeted to the cortical plate at P1 (defined as the most proximal 40% of the dorsoventral distance between the pia and ventricle [first 4 of 10 equally spaced bins]) or to layer 2/3 of the somatosensory cortex, defined as the proximal 30% of the dorsoventral distance between the proximal 10% of the cortex (assumed to represent layer 1) and ventricle (bin 2–4 of equally spaced bins). On the basis of our previous neuronal morphology analyses (Proietti Onori et al., 2018), we performed two replicates of a minimum of 10 neurons per condition. For neuronal migration analyses, we used a minimum of three targeted pups per condition. For the western blot analysis, we used at least three replicates.

3 | RESULTS

3.1 | TAOK1 plays an important role in cortical development

In utero electroporation in mice or rats is a valuable technique to study the role of specific proteins in neuronal function as an impaired neuronal function can result in abnormal cortical migration, a key...
aspect of neurodevelopment (Saito & Nakatsuji, 2001; Tabata & Nakajima, 2001; Taniguchi et al., 2012). Hence, we used this technique to assess whether loss of TAOK1 would affect neuronal migration in vivo. First, we assessed the level of shRNA-mediated knockdown of mouse Taok1 in neurons, using primary hippocampal culture in vitro as the antibody for TAOK1 did not work well on slices. We found, on average 35% reduction in TAOK1 immunofluorescence intensity for both the single shRNAs and the pooled conditions (Figures S1A and S1B).

We performed the in utero electroporation in mouse embryos at embryonic day 14.5, a well-established time in murine fetal development when neural progenitor cells divide to give rise to immature neurons that will migrate to their final destination within the cortical plate, which will form the cerebral cortex layer 2/3. We found that shRNA-mediated knockdown of Taok1 (using a pool of three different shRNAs specifically targeting Taok1) at this time resulted in a clear migration deficit of the neurons coming from the transfected neural progenitor cells, with significantly fewer transfected cells reaching the cortical plate (CP) at postnatal day 1 (P1) compared to control (scramble shRNA) condition (70% vs. 90% respectively; $t_{[23]} = 4.21$, $p = .0003$, unpaired Student’s t test, Figure 1a,b).

This migration deficit persists at P7 when only 75% of the targeted neurons were present in layer 2 of 3 of the somatosensory cortex compared to 95% in control conditions ($t_{[15]} = 2.84$, $p = .0124$, unpaired Student’s t test, Figure 1c,d). Similar migration deficits were found when the shRNAs were tested separately, confirming their specificity (Figure S1C–E). Together these results indicate that TAOK1 plays a critical role in neuronal function and that reduction of TAOK1 levels results in impaired brain development.

3.2 | Phenotypic characterization of the variants found in TAOK1

Nineteen unrelated individuals with variants affecting TAOK1 were identified, which were heterozygous for a missense variant ($n = 5$), premature stop variant ($n = 7$), indel ($n = 4$), or canonical splice site variant ($n = 3$) (Tables 1 and S1). In addition, four individuals (individuals 18, 21, 22, and 23) carried a chromosomal microdeletion ranging in size from 807 bp to 2 Mb, of which patient 23 was previously published (Xie et al., 2016). The deletion found in individual 22 was inherited from a parent with a history of cognitive
| Gender and age | Chromosome position (GRCh37) | cDNA change (NM_020791.2) | Amino acid change | Inheritance | Heterozygous/Homozygous |
|----------------|--------------------------------|-----------------------------|-------------------|-------------|--------------------------|
| Patient 1      | Male, 5 years chr17:g.27861216del | c.2442del                  | p.Tyr815Ilefs*31  | De novo     | Heterozygous             |
| Patient 2      | Male, 9 years chr17:g.27818884dup | c.831+1dupG                | p.?               | De novo     | Heterozygous             |
| Patient 3      | Male, 8 years chr17:g.27837949T>C | c.1643 T>C                  | p.Leu548Pro       | De novo     | Heterozygous             |
| Patient 4      | Female, 14 years chr17:g.27822746dup | c.999+1dupG               | p.?               | De novo     | Heterozygous             |
| Patient 5      | Male, 14 years chr17:g.27844583C>T | c.1819 C>T                 | p.Gln607Ter       | De novo     | Heterozygous             |
| Patient 6      | Female, 2 years and 6 months chr17:g.27818877_27818878insCT | c.825_826insCT | p.Lys277Ter   | De novo     | Heterozygous             |
| Patient 7      | Male, 6 years chr17:g.27816684G>T | c.658G>T                   | p.Glu220Ter       | Maternal, familial NDD | Heterozygous             |
| Patient 8      | Male, 20 years chr17:g.27849514C>T | c.2125C>T                  | p.Arg709Ter       | Paternal    | Heterozygous             |
| Patient 9      | Male, 2 years chr17:g.27805365G>T | c.449G>T                   | p.Arg150Ile       | De novo     | Heterozygous             |
| Patient 10     | Male, 17 years chr17:g.27807436T>G | c.500T>G                   | p.Leu167Arg       | De novo     | Heterozygous             |
| Patient 11     | Male, 3 years chr17:g.27849472C>T | c.2083C>T                  | p.Arg695Ter       | De novo     | Heterozygous             |
| Patient 12     | Female, 3 years chr17:g.27805366G>C | c.449+1G>C              | p.?               | De novo     | Heterozygous             |
| Patient 13     | Female, 1 year and 11 months chr17:g.27805309dup | c.393dupT   | p.Thr132Terfs*19 | De novo     | Heterozygous             |
| Patient 14     | Male, 5 years chr17:g.27849493C>T | c.2104C>T                  | p.Arg702Ter       | Unknown     | Heterozygous             |
| Patient 15     | Male, 6 years chr17:g.27822689C>T | c.943C>T                  | p.Leu315Phe       | De novo     | Heterozygous             |
| Patient 16     | Male, 10 years chr17:g.27829690delA | c.1287delA | Lys78Valfs*42  | De novo     | Heterozygous             |
| Patient 17     | Female, 1 year and 8 months chr17:g.27802715_27802716del | c.232_233delAA | Lys78Valfs*20  | De novo     | Heterozygous             |
| Patient 18     | Male, 10 years chr17:g.27848999_27849799del | c.1909_2148+262del | p.? (exon 17 deletion) | De novo     | Heterozygous             |
| Patient 19     | Female, 21 years chr17:g.27816717A>G | c.691 A>G     | p.Met231Val     | Unknown     | Heterozygous             |
| Patient 20     | Female, 1 year and 2 months chr17:g.27844597C>T | c.1813C>T | p.Arg605Ter     | Unknown     | Heterozygous             |
| Patient 21     | Female, 7 years del17q11.2,27:08−29.08 Mb × 1 | 2 MB | N/A            | Heterozygous |                      |
| Patient 22     | Male, 6 years del chr17:27,670,438−27,934,287 × 1 | 264 kb | Maternally inherited (ID in parents) | Heterozygous |                      |
| Patient 23     | Female, 1 year and 9 months del chr17:27,064,286−28,761,847 × 1 | 1.69 MB | De novo       | N/A         |                      |

Note: p.? indicates that the effect on protein is unknown.
Abbreviations: cDNA, complementary DNA; ID, intellectual disability; NDD, neurodevelopmental disorder; N/A, not assessed.
impairment. For individuals 14, 19, and 20, no parental genotype data were available, so inheritance could not be determined. Two single nucleotide variants were inherited (individual 7 and 8). The variant found in individual 7 was inherited maternally. Although for the mother, no formal IQ was known, she was unable to follow conventional school. Moreover, there is a clear familial context of delayed development. The half-brother (maternal side) had an IQ of 72 and a grand-uncle (maternal side) was known with ID, so the TAOK1 variant could potentially further segregate in this family. The variant found in individual 8 was paternally inherited. Here, although the father has symptoms of autism, he was never formally diagnosed or assessed for neurodevelopmental issues. This variant predicts a premature stop and is present once out of 251344 alleles in gnomAD (https://gnomad.broadinstitute.org/). All other variants in TAOK1 identified in our cohort occurred de novo. There were additional de novo variants reported in our cohort in 11 different genes (Table S1; individuals 1, 7, 9, 10, 11, 17, 19, and 20). The de novo ZEB2 variant in patient 10 is considered to be likely pathogenic and probably contributing to the phenotype, although the patient does not have clinically suspected Mowat-Wilson syndrome.

We compiled the clinical data and compared that with the clinical data that have been reported previously (Dulovic-Mahlow et al., 2019) (Tables 2 and S1). This reveals that the collective features present in the majority of individuals that define the core clinical picture of the TAOK1-associated syndrome are: Varying degrees of ID/DD, neonatal feeding difficulties, overlapping facial features, behavior problems, hypotonia, and joint hypermobility. Similar facial features observed comprise frontal bossing, downslanting palpebral fissures, long philtrum, and bulbous nasal tip (Figure 2a). Of note, patient 11 presented with multisuture craniosynostosis, which may have been related to prenatal ventriculomegaly and macrocephaly, causing the fetal head to be stuck in the pelvis. Some patients were too young to establish a formal diagnosis of ID, and for three individuals (patients 5, 8, and 15) no ID was present. Therefore, the cognitive phenotype might be very mild and could explain the presence of two vertical transmissions (patients 7 and 8) and presence in gnomAD of the variant in individual 8.

Microdeletions in the 17q11.2 region often encompass the NF1 gene (Kehrer-Sawatzki et al., 2017). The microdeletions presented here, however, are proximally located to this more common deleted region. Previously, a novel de novo deletion at 17q11.2 adjacent to the NF1 gene was reported in an individual with developmental delay, short stature, postnatal microcephaly, underweight, and dysmorphic features, including flat facial profile, dolichocephaly, hypertelorism, short philtrum, flat nasal bridge, and posteriorly rotated and low set ears. Chromosomal microarray analysis revealed a 1.69 Mb de novo deletion at 17q11.2 adjacent to NF1 gene, which involved 43 RefSeq genes. We included this information in Table 1 (individual 23). The authors compared in their study the proband to three other cases with overlapping small deletions from databases (Xie et al., 2016). On the basis of their findings, they hypothesized that TAOK1 might be involved in the developmental delay and microcephaly in their patient. However, the information we collected in our study from the individuals with TAOK1 variants and the two individuals with overlapping microdeletions indicate that

| Parameter                  | Frequency | Previous | Total (%) |
|----------------------------|-----------|----------|-----------|
| **Delivery**               |           |          |           |
| C-section                  | 5/20      | N/R      | 5/20 (25) |
| **Growth**                 |           |          |           |
| Small stature (height for age <2.0 SD) | 4/20   | N/R      | 4/20 (20) |
| Overweight (weight for height >2.0 SD) | 6/20   | N/R      | 6/20 (30) |
| Macrocephaly (>2.0 SD)     | 7/18      | 3/8      | 10/26 (38) |
| **Neurodevelopmental**     |           |          |           |
| Global developmental delay | 18/20     | 6/8      | 24/28 (86) |
| Intellectual disability    | 14/20     | 4/8      | 18/28 (64) |
| Behavior problems          | 12/20     | 2/8b     | 14/28 (50) |
| Hypotonia                  | 10/20     | 6/8      | 16/28 (57) |
| **Gastrointestinal**       |           |          |           |
| Neonatal feeding difficulties | 9/18   | N/R      | 9/18 (50) |
| **Musculoskeletal**        |           |          |           |
| Joint hypermobility        | 6/20      | 2/8      | 8/28 (29) |
| Recurrent ear/airway infections | 6/18 | N/R      | 6/18 (33) |

Abbreviations: N/R, not reported; TAOK1, thousand and one amino-acid kinase 1.

*Dulovic-Mahlow et al. (2019).

bAttention deficit hyperactivity disorder (ADHD) and short attention span.
microcephaly is not likely caused by loss of TAOK1, as the patients concerned have either occipitofrontal circumference in the normal range or above.

TAOK1 consists of a kinase domain in the N-terminal region (amino acids 34–295), a substrate-binding domain (SBD, amino acids 296–431), a spacer, and a tail, both containing a coiled-coil domain (CC). There is a high overall amino acid sequence conservation (~80% identity) between the human TAOK1 and TAOK1 from different vertebrate classes (Figure S2), with the least conserved part being the C-terminal region. Of the missense variants found in our cohort, two were located in the kinase domain, one in the SBD, and one in the first CC domain, all of which involved strictly conserved residues (Figure S2). Of the premature stop and indel variants, three were located in the kinase domain, one in the SBD, two in the CC, and three in the spacer (Figure 2b, top), indicating no real hotspot for variants within the protein. The same applies to the variants found in the previous study (Dulovic-Mahlow et al., 2019) (Figure 2b, top, variants indicated in gray).

3.3 | Functional assessment of TAOK1 variants

Consistent with the finding that reduced TAOK1 levels affect neuronal function, the majority of the identified variants are predicted to cause a premature stop, indicating haploinsufficiency as the underlying molecular mechanism for the NDD, as was also shown previously (Dulovic-Mahlow et al., 2019). However, for the missense variants, the effect on protein function is less predictable. All missense variants are localized in highly conserved residues, with the identical amino acid sequence comparing human, mouse, rat, frog,
chicken, and zebrafish (Figure S2). In silico prediction of the variants using different prediction tools revealed that depending on the prediction tool used, the variants are damaging, likely damaging, or benign, hence, of unknown significance. Besides the variable outcomes obtained by these tools, they also do not address whether a missense variant causes an LoF or a gain of function (GoF). Therefore, we tested the effect of the TAOK1 missense variants on protein function. One of the variants, TAOK1Leu548Pro, is predicted to be located in an alpha-helix structure in the protein (Figure 2c) and proline is in general considered to perturb the alpha-helix; hence, this variant could affect protein stability. To test the stability of the protein in the presence of different variants, HEK-293T cells were transfected with different constructs and the protein levels were assessed. Surprisingly, Western blot analysis revealed significantly reduced protein expression for two of the TAOK1 variants, predicted to be located in a linker region in the kinase domain of the protein (TAOK1Arg150Ile and TAOK1Leu167Arg), whereas the TAOK1Leu548Pro variant instead showed significantly higher protein level compared to TAOK1WT, indicating that this variant does not cause intrinsic instability of the protein. The variants TAOK1Met231Val and TAOK1Leu315Phe did not alter the expression levels compared to TAOK1WT (Figure 3a, one-way ANOVA, F[5,66]=14.35, p < .0001; TAOK1WT vs. TAOK1Arg150Ile, p = .0029; TAOK1WT vs. TAOK1Leu167Arg, p = .0019; TAOK1WT vs. TAOK1Met231Val, p = .13; TAOK1WT vs. TAOK1Leu315Phe, p = .32; TAOK1WT vs. TAOK1Leu548Pro, p = .0029, Dunnett’s multiple comparison test).

Having established the effect of the variants at the protein level, we continued testing whether the variants behaved differently from TAOK1WT in vitro and in vivo assays to assess their pathogenicity. For the in vitro assay, mouse primary hippocampal neurons were

**FIGURE 3** Differential effect of TAOK1 variants on protein expression and neuronal development in vitro. (a) Western blot analysis revealing altered expression levels of some TAOK1 variants compared to TAOK1WT when overexpressed in HEK-293T cells. The number of samples is indicated between brackets for each condition. (b) Representative confocal images of hippocampal neurons transfected with empty vector (EV), TAOK1WT, or TAOK1 variants, showing clear overexpression of the TAOK1 protein upon staining for TAOK1 for each TAOK1 condition. (c) Total neurite length and arborization normalized to control. Data are presented as mean ± SEM. The number of independently analyzed culture wells/cells is indicated between brackets for each condition. TAOK1, thousand and one amino-acid kinase 1; WT, wild-type. *Compared to empty vector condition and #compared to TAOK1WT condition. **p < .01, ***p < .001, and ###p < .001.
Transfection of primary hippocampal neurons was performed after growing the neurons for 3 days in vitro (DIV) when the neurons start to form secondary and tertiary branches. Overexpression of TAOK<sup>WT</sup> affected neuronal maturation by significantly reduced neurite length and arborization compared to the empty vector (EV)-transfected neurons (Figure 3b,c) (Neurite length: one-way ANOVA, F[6,185] = 18.39, p < .0001; empty vector vs. TAOK<sup>WT</sup>, p < .0001, Dunnett’s multiple comparison test; Arborization: one-way ANOVA, F[6,185] = 38.93, p < .0001; empty vector vs. TAOK<sup>WT</sup>, p < .0001, Dunnett’s multiple comparison test). TAOK<sup>1<sub>Arg150Ile</sub></sup>, TAOK<sup>1<sub>Met231Val</sub></sup>, and TAOK<sup>1<sub>Leu315phe</sub></sup> behaved similarly to TAOK<sup>1WT</sup> upon overexpression (Neurite length: TAOK<sup>1<sub>Arg150Ile</sub></sup> vs. TAOK<sup>WT</sup>, p = .99; TAOK<sup>1<sub>Met231Val</sub></sup> vs. TAOK<sup>WT</sup>, p = .99; TAOK<sup>1<sub>Leu315phe</sub></sup> vs. TAOK<sup>WT</sup>, p = .17, Dunnett’s multiple comparison test; Arborization: TAOK<sup>1<sub>Arg150Ile</sub></sup> vs. TAOK<sup>WT</sup>, p = .10; TAOK<sup>1<sub>Met231Val</sub></sup> vs. TAOK<sup>WT</sup>, p = .97; TAOK<sup>1<sub>Leu315phe</sub></sup> vs. TAOK<sup>WT</sup>, p = .91, Dunnett’s multiple comparison test). TAOK<sup>1<sub>Leu167Arg</sub></sup> and TAOK<sup>1<sub>Leu548Pro</sub></sup> showed a milder effect on neuronal development. Neuronal arborization was still significantly decreased upon overexpression of the TAOK<sup>1<sub>Leu167Arg</sub></sup> and TAOK<sup>1<sub>Leu548Pro</sub></sup> variants compared to empty vector, but the effect was not as strong as wild-type TAOK1 overexpression as the arborization was significantly higher in TAOK<sup>1<sub>Leu167Arg</sub></sup> and TAOK<sup>1<sub>Leu548Pro</sub></sup> compared to TAOK<sup>1WT</sup> (empty vector vs. TAOK<sup>1<sub>Leu167Arg</sub></sup>, p < .0001; empty vector vs. TAOK<sup>1<sub>Leu548Pro</sub></sup>, p = .0002; TAOK<sup>1<sub>Leu167Arg</sub></sup> vs. TAOK<sup>WT</sup>, p = .0002; TAOK<sup>1<sub>Leu548Pro</sub></sup> vs. TAOK<sup>WT</sup>, p < .0001, Dunnett’s multiple comparison test). The effect on neurite length was in fact for the TAOK<sup>1<sub>Leu548Pro</sub></sup> variant opposite of TAOK<sup>WT</sup> overexpression, showing a significantly increased neurite length compared to empty vector and compared to TAOK<sup>WT</sup> (empty vector vs. TAOK<sup>1<sub>Leu548Pro</sub></sup>, p = .0009; TAOK<sup>1<sub>Leu548Pro</sub></sup> vs. TAOK<sup>WT</sup>, p < .0001, Dunnett’s multiple comparison test). The TAOK<sup>1<sub>Leu167Arg</sub></sup> variant only partially restored the effect of TAOK<sup>1WT</sup> on neurite length, revealing a trend, though not significant toward increased neurite length compared to TAOK<sup>1WT</sup>, and showing no significant difference compared to empty vector (empty vector vs. TAOK<sup>1<sub>Leu167Arg</sub></sup>, p = .49; TAOK<sup>1<sub>Leu167Arg</sub></sup> vs. TAOK<sup>WT</sup>, p = .083, Dunnett’s multiple comparison test).

Finally, knowing that the in vivo neuronal migration assay is sensitive to reduced TAOK1 function, we evaluated the effect of overexpression of the different TAOK1 variants compared to overexpression of TAOK<sup>WT</sup> using again the in utero electroporation technique. Similar to the knockdown of Taok1, though less severe, overexpression of TAOK<sup>1WT</sup> resulted in a migration deficit when assessed at P1, with only 70% of the cells reaching the CP compared to 95% in the empty vector condition (Figure 4) (one-way ANOVA, F[7,67] = 28.66, p < .0001; empty vector vs. TAOK1<sup>1WT</sup>, p = .0011, Dunnett’s multiple comparison test).

**FIGURE 4** Differential effect of thousand and one amino-acid kinase 1 (TAOK1) variants on neuronal migration in vivo. (a) Representative images from postnatal Day 1 brain, showing the transfected cells (tdTomato+) from the subventricular zone (SVZ; indicated by the arrow) to the cortical plate (CP; indicated by the arrowheads). (b) Cumulative distribution of the transfected neurons at P1 from the CP to the intermediate zone (IZ). (c) Percentage of neurons reaching the superficial layers of the cortex (sum of bins 1–4). Data are presented as mean ± SEM. Number of images analyzed is indicated for each condition. *Compared to empty vector condition and †compared to TAOK1<sup>WT</sup> condition. *p < .05, **p < .01, ***p < .001, ##p < .01, and ###p < .001
Dunnett’s multiple comparison test), indicating that the expression level of TAOK1 needs to be well regulated during early neurodevelopment. Interestingly, whereas overexpression of TAOK1Leu167Arg and TAOK1Leu315Phe revealed a stronger migration deficit compared to TAOK1WT, TAOK1Leu548Pro showed normal migration compared to the empty vector condition and a strong trend toward improved migration compared to TAOK1WT (Figure 4, TAOK1Leu167Arg vs. TAOK1WT p < .0005; TAOK1Leu315Phe vs. TAOK1WT p < .0001; empty vector vs. TAOK1Leu548Pro: p = .57; TAOK1WT vs. TAOK1Leu548Pro: p = .19, Dunnett’s multiple comparison test). Overexpression of TAOK1Arg150Ile and TAOK1Met231Val revealed similar migration deficits as seen upon overexpression of TAOK1WT (empty vector vs. TAOK1Arg150Ile, p = .016; empty vector vs. TAOK1Met231Val, p < .0001; TAOK1Arg150Ile vs. TAOK1WT, p = .9; TAOK1Met231Val vs. TAOK1WT, p = .53, Dunnett’s multiple comparison test), indicating a mild LoF effect.

To study the effect of truncating variants, we generated a truncated TAOK1, ending at c.2442 (p.Tyr815Ter; TAOK1trunc), and evaluated the effect on migration. Overexpression of TAOK1trunc resulted in an improved migration pattern when compared to empty vector and TAOK1WT, showing no significant difference compared to each of these conditions (empty vector vs. TAOK1trunc: p = .14; TAOK1WT vs. TAOK1trunc: p = .6, Dunnett’s multiple comparison test), indicating a mild LoF effect.

Taken together, these results show that overexpression of TAOK1WT is damaging for neurons both in vitro and in vivo, and that whereas some variants in TAOK1 behave as LoF variants, others affect the protein in different ways (see Table 3 for a summary of the effects found in the different functional assays), suggesting that TAOK1-related disorder can be caused by distinct pathophysiological mechanisms.

4 | DISCUSSION

With the increased genetic diagnostic yield in cohorts of patients with NDDs, novel genes are found to play an important role in neurodevelopment. Yet, determining the function of these genes and the encoded proteins often lags behind. Of the human TAOK family, so far only for TAOK2, a role in neurodevelopment was shown (Richter et al., 2018). Recently, eight individuals with NDD carrying de novo variants in TAOK1 were described (Dulovic-Mahlov et al., 2019), but the functional consequences of the TAOK1 missense variants on neuronal function were not assessed. Here, we provide compelling evidence that TAOK1 is important for neuronal function: We show that changing the expression level of TAOK1 (either increasing or decreasing it) in a subset of neural progenitor cells disrupts neural migration, suggesting that the expression level of TAOK1 needs to be regulated for proper neuronal function. In addition, we show that overexpression of TAOK1 affects neuronal maturation in vitro. Moreover, we further delineate the clinical spectrum as well as the molecular mechanisms associated with TAOK1 mutations: (1) The majority of 22 novel individuals carrying different variants in, or deletion of, TAOK1 we identified, had a neurodevelopmental disorder characterized by similar facial features, ID/DD and/or variable learning or behavioral problems, muscular hypotonia, infant feeding difficulties, and growth problems and (2) we show that missense and truncating variants affect TAOK1 protein functioning in different ways, thereby showing that besides haploinsufficiency, also other molecular mechanisms are at play. Unfortunately, patient material was not available, refraining us from testing whether frameshift and nonsense variants result in a truncated protein or whether these mutations induce nonsense-mediated messenger RNA decay.

The eight individuals with de novo variants in TAOK1 that were recently described share common features of global developmental delay, muscular hypotonia, and in the four of eight cases that could be assessed, a formal diagnosis of ID (Dulovic-Mahlov et al., 2019). Here, we describe 20 additional individuals with TAOK1 intragenic variants allowing us to further define the TAOK1-associated disorder. We noted that the global developmental delay might be very mild and that behavior problems and/or overlapping facial features might be the main reason for referral for genetic testing. Other significant features are growth abnormalities (small stature, overweight, and macrocephaly), neonatal feeding difficulties, joint hypermobility, and recurrent ear and airway infections.

Using in vivo and in vitro assays, we have shown that TAOK1 plays an important role in neural development. In our in vitro assay, overexpression of TAOK1 reduces neurite length and arborization in

### TABLE 3: Overview of functional assessment of TAOK1 missense variants

| Protein expression compared to WT TAOK1 | Neuronal morphology compared to WT TAOK1 | Neuronal migration compared to WT TAOK1 | Inferred pathophysiological mechanism |
|----------------------------------------|----------------------------------------|----------------------------------------|---------------------------------------|
| Empty vector                           | Increased complexity                   | Increased                               | LoF                                    |
| p.Arg150Ile                            | Reduced                                | Similar                                 | Loss of Function                       |
| p.Leu167Arg                            | Reduced                                | Increased complexity                    | Dominant acting                       |
| p.Met231Val                            | Unaffected                             | Similar                                 | None                                   |
| p.Leu315Phe                            | Unaffected                             | Severe impaired                         | Dominant acting                       |
| p.Leu548Pro                            | Increased                              | Increased complexity                    | Loss of Function                       |

Abbreviations: TAOK1, thousand and one amino-acid kinase 1; WT, wild-type.
Moreover, the regulation of ParTao1 knockdown in mammals have three representative genes (Mahlow et al., 2019; King et al., 2011; Poon et al., 2016). It is hypothesized that this is the result of dysregulation of the Par1/Tau pathway, which affects the microtubule dynamics in developing neurons (King & Heberlein, 2011). However, as earlier findings, showing that in differentiating PC12 cells, TAOK1 is required for neurite outgrowth as the absence of TAOK1 severely reduces the number of differentiating neurons with neurites (Timm et al., 2003). However, it should be noted that we induce the overexpression of TAOK1 from DIV3, which is after the initiation of neurite outgrowth (Dotti et al., 1988). Additionally, we are using a different neuronal population (primary hippocampal neurons) and do not induce neuronal differentiation. Finally, overexpression might affect different pathways compared to knockdown.

Of the TAOK1 variants tested, TAOK1Arg150Ile and TAOK1Leu167Arg both located in the kinase domain of the protein, showed reduced protein expression in HEK293T cells, which could indicate an LoF mutation. In the functional assays, we observed no difference between the TAOK1Arg150Ile and TAOK1WT, which could indicate that the function of TAOK1Arg150Ile is reduced but potentially can be compensated for by overexpression. TAOK1Leu167Arg does behave differently compared to TAOK1WT in the functional assays, showing a partial rescue of the neuronal morphology phenotype, but a much more severe migration deficit compared to TAOK1WT. These results could suggest that the TAOK1Leu167Arg mutation acts in a dominant acting manner. The TAOK1Met231Val variant is also located in the kinase domain and behaves in all the assays similar to TAOK1WT. Therefore, based on our functional data no conclusions can be drawn with respect to the pathogenicity of this variant. Additionally, for this patient (patient 21), the inheritance is unknown and the phenotype is not specific. It, therefore, remains unclear whether this variant is the (major) cause for the NDD phenotype seen in this individual.

TAOK1Leu315Pho is located in the substrate-binding domain and does not affect expression levels nor neuronal morphology. Interestingly, this variant does severely affect the in vivo migration of neurons when overexpressed, therefore, likely having a dominant-negative effect on TAOK1 function. The TAOK1Leu548Pro variant yielded increased protein expression levels, improved neuronal morphology in vitro as well as improved neuronal migration in vivo compared to TAOK1WT, indicating an LoF effect on TAOK1. Together these results reveal that dysregulation of TAOK1 in several ways might result in a neurodevelopmental disorder.

The precise TAOK1 downstream signaling pathways responsible for the neurodevelopmental deficits remain to be elucidated, as literature on the role of TAOK1 in the brain is limited. Most data come from studies in Drosophila melanogaster, which has a single TAOK ortholog, called Tao. Tao1 in Drosophila plays a role in axon guidance, and its deletion leads to malformations of the fly brain (Dulovic-Mahlow et al., 2019; King et al., 2011; Poon et al., 2016). It is hypothesized that this is the result of dysregulation of the Par1/Tau pathway, which affects the microtubule dynamics in developing neurons (King & Heberlein, 2011). However, as Drosophila has only one representative gene (Tao1) for the TAOK family, whereas humans have three TAOK genes, it is difficult to translate findings of Tao1 knockdown in Drosophila to the role of TAOK1 in mammals. Moreover, the regulation of Par-1 by TAOK1 appears to be opposite when comparing the Drosophila with mammalian neurons. In Drosophila, Par-1 is thought to be negatively regulated by Tao1 (Wang et al., 2007), whereas in mammalian neurons (PC12 cells differentiated with NGF), TAOK1 was shown to stimulate PAR-1, which leads to phosphorylation of Tau, followed by microtubule dynamics required for neurite outgrowth (Biernat et al., 2002; Timm et al., 2003). Thus, future studies will have to unravel the precise mechanism through which TAOK1 causes NDD.

Taken together, our data show that TAOK1 plays an important role in mammalian neuronal maturation and that dysregulation of TAOK1 either through haploinsufficiency, dominant-negative effects, or even other mechanisms, causes a neurodevelopmental disorder characterized by similar facial features, ID/DD, and/or variable learning or behavioral problems, muscular hypotonia, infant feeding difficulties, and growth problems.

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CONFLICT OF INTERESTS

Mari Rossi is employed by and receives a salary from Ambry Genetics, one of whose commercially available tests is exome sequencing. Amanda Noyes, Katherine G. Langley, Alice Brooks, Jennifer Humberson, Maria J. Guillen Sacoto, Jane Juusola, Kristin G. Monaghan, and Sumit Punj are employees of GeneDx, Inc.

AUTHOR CONTRIBUTIONS

Geeske M. van Woerden, Ype Elgersma, and Tijtske Kleefstra designed the study. Karen W. Gripp identified a patient and initiated the collaboration through Genematcher. Melanie Bos and Tijtske Kleefstra performed clinical cohort analyses. Geeske M. van Woerden, Rossella Avagliano Trezza, Charlotte de Konink, Fatima Rehman, Saskia Brulleman, Róisín McCormack, and Gwynna de Geus performed functional experiments and analysis. Ben Distel performed the in silico modeling. GeneDx: Amanda Noyes and Katherine G. Langley, performed case identification and data collection; Aida Telegrafi, Amy Blevins, Jessica Hoffman, Maria J. Guillen Sacoto, Jane Juusola, Kristin G. Monaghan, and Sumit Punj are employees of GeneDx, Inc.
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DATA AVAILABILITY STATEMENT
All data are available upon request. Accession number used for...
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