Genetic Disruption of WASHC4 Drives Endo-Lysosomal Dysfunction and Cognitive-Movement Impairments in Mice and Humans

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Abstract

Mutation of the WASH complex subunit, SWIP, is implicated in human intellectual disability, but the cellular etiology of this association is unknown. We identify the neuronal WASH complex proteome, revealing a network of endosomal proteins. To uncover how dysfunction of endosomal SWIP leads to disease, we generate a mouse model of the human WASHC4$^{c.3056C>G}$ mutation. Quantitative spatial proteomics analysis of SWIP^{P1019R} mouse brain reveals that this mutation destabilizes the WASH complex and uncovers significant perturbations in both endosomal and lysosomal pathways. Cellular and histological analyses confirm that SWIP^{P1019R} results in endo-lysosomal disruption and uncover indicators of neurodegeneration. We find that SWIP^{P1019R} not only impacts cognition, but also causes significant progressive motor deficits in mice. Remarkably, a retrospective analysis of SWIPP1019R patients confirms motor deficits in humans. Combined, these findings support the model that WASH complex destabilization, resulting from SWIP^{P1019R}, drives cognitive and motor impairments via endo-lysosomal dysfunction in the brain.

Introduction

Neurons maintain precise control of their subcellular proteome using a sophisticated network of vesicular trafficking pathways that shuttle cargo throughout their elaborate processes. Endosomes function as a central hub in this vesicular relay system by coordinating protein sorting between multiple cellular compartments, including surface receptor endocytosis and recycling, as well as degradative shunting to the lysosome. How endosomal trafficking is modulated in neurons remains a vital area of research due to the unique degree of spatial segregation between organelles in neurons, and its strong implication in neurodevelopmental and neurodegenerative diseases.

In non-neuronal cells, an evolutionarily conserved complex, the Wiskott-Aldrich Syndrome protein and SCAR Homology (WASH) complex, coordinates endosomal trafficking (derivery 2010, linardopoulou 2007). WASH is composed of five core protein components: WASHC1 (aka WASH1), WASHC2 (aka FAM21), WASHC3 (aka CCDC53), WASHC4 (aka SWIP), and WASHC5 (aka Strumpellin) (encoded by genes Washc1-Washc5, respectively), which are broadly expressed in multiple organ systems (Alekhina et al., 2017; Kustermann et al., 2018; McNally et al., 2017; Simonetti and Cullen, 2019; Thul et al., 2017). The WASH complex plays a central role in non-neuronal endosomal trafficking by activating Arp2/3-dependent actin branching at the outer surface of endosomes to influence cargo sorting and vesicular scission (Gomez and Billadeau, 2009; Lee et al., 2016; Phillips-Krawczak et al., 2015; Piotrowski et al., 2013; Simonetti and Cullen, 2019). WASH also interacts with at least three main cargo adaptor complexes — the Retromer, Retriever, and COMMD/CCDC22/CCDC93 (CCC) complexes — all of which associate with distinct sorting nexins to select specific cargo and enable their trafficking to other cellular locations (Binda et al., 2019; Farfán et al., 2013; McNally et al., 2017; Phillips-Krawczak et al., 2015; Seaman and Freeman, 2014; Singla et al., 2019). Loss of the WASH complex in non-neuronal cells has detrimental effects on endosomal structure and function, as its loss results in aberrant endosomal tubule elongation and cargo mislocalization (Bartuzi et al., 2016; Derivery et al., 2009; Gomez et al., 2012; Gomez and Billadeau, 2009; Phillips-Krawczak et al., 2015; Piotrowski et al., 2013). However, whether the WASH complex performs an endosomal trafficking role in neurons remains an open question, as no studies have addressed neuronal WASH function to date.

Consistent with the association between the endosomal trafficking system and pathology, dominant missense mutations in WASHC5 (protein: Strumpellin) are associated with hereditary spastic paraplegia (SPG8) (De Bot et al., 2013; Valdmanis et al., 2007), and autosomal recessive point mutations in WASHC4 (protein: SWIP) and WASHC5 are associated with syndromic and non-syndromic intellectual disabilities (Assoum et al., 2020; Elliott et al., 2013; Ropers et al., 2011). In particu-

lar, an autosomal recessive mutation in WASHC4 (c.3056C>G; p.Pro1019Arg) was identified in a cohort of children with non-syndromic intellectual disability (Ropers et al., 2011). Cell lines derived from these patients exhibited decreased abundance of WASH proteins, leading the authors to hypothesize that the observed cognitive deficits in SWIPP1019R patients resulted from disruption of neuronal WASH signaling (Ropers et al., 2011). However, whether this mutation leads to perturbations in neuronal endosomal integrity, or how this might result in cellular changes associated with disease, are unknown.

Results

Identification of the *in vivo* WASH complex proteome

While multiple mutations within the WASH complex have been identified in humans (Assoum et al., 2020; Elliott et al., 2013; Ropers et al., 2011; Valdmanis et al., 2007), how these mutations lead to neurological dysfunction remains unknown **(Figure 1A)**. Given that previous work in non-neuronal cultured cells and non-mammalian organisms have established that the WASH complex functions in endosomal trafficking, we first aimed to determine whether this role was conserved in the mouse nervous system (Alekhina et al., 2017; Billadeau et al., 2010; Derivery et al., 2009; Gomez et al., 2012; Gomez and Billadeau, 2009). To discover the likely molecular functions of the neuronal WASH complex, we utilized an in vivo BioID (iBioID) paradigm developed in our laboratory to identify the WASH complex proteome from brain tissue (Uezu et al., 2016). BioID probes were generated by fusing a component of the WASH complex, WASH1 (gene: Washc1), with the promiscuous biotin ligase, BioID2 (WASH1-BioID2, Figure 1B), or by expressing BioID2 alone (negative control, solubleBioID2) under the neuron-specific, human Synapsin-1 promoter (Kim et al., 2016). We injected adenoviruses (AAV) expressing these constructs into the cortex of wild-type postnatal day zero (P0) mice (Figure 1B). Two weeks post-injection, we administered daily subcutaneous biotin for seven days to biotinylate in vivo substrates. The viruses displayed efficient expression and activity in brain tissue, as evidenced by colocalization of the WASH1-BioID2 viral epitope (HA) and biotinylated proteins (Streptavidin) (Figures 1C-F). For label-free quantitative high-mass accuracy LC-MS/MS analyses, whole brain samples were collected at P22, snap-frozen, and processed as previously described (Uezu et al., 2016). A total of 2,311 proteins were identified across all three experimental replicates, which were further analyzed for those with significant enrichment in WASH1-BioID2 samples over solubleBioID2 negative controls (Table S1).

The resulting neuronal WASH proteome included 174 proteins that were significantly enriched (Fold-change $\geq$ 3.0, Benjamini-Hochberg P-Adjust $<$ 0.1, Figure 1G). Of these proteins, we identified all five WASH complex components (Fig-

ure 1H), as well as 13 previously reported WASH complex interactors (Figure 1I) (McNally et al., 2017; Phillips-Krawczak et al., 2015; Simonetti and Cullen, 2019; Singla et al., 2019), which provided strong validity for our proteomic approach and analyses. Additional bioinformatic analyses of the neuronal WASH proteome identified a network of proteins implicated in vesicular trafficking, including 23 proteins enriched for endosomal functions (Figure 1J) and 24 proteins enriched for endocytic functions (Figure 1K). Among these endosomal and endocytic proteins were components of the recently identified endosomal sorting complexes, CCC (CCDC93 and COMMD9) and Retriever (VPS35L) (Phillips-Krawczak et al., 2015; Singla et al., 2019), as well as multiple sorting nexins important for recruitment of trafficking regulators to the endosome and cargo selection, such as SNX1-3, and SNX16 (Kvainickas et al., 2017; Maruzs et al., 2015; Simonetti et al., 2017). These data demonstrated that the WASH complex interacts with many of the same proteins in neurons as it does in yeast, amoebae, flies, and mammalian cell lines. Furthermore, there were 32 proteins enriched for cytoskeletal regulatory functions (Figure 1L), including actin-modulatory molecules such as the Arp2/3 complex subunit ARPC5, which is consistent with WASH's role in activating this complex to stimulate actin polymerization at endosomes for vesicular scission (Billadeau et al., 2010; Derivery et al., 2009). The WASH1-BioID2 isolated complex also contained 28 proteins known to localize to the excitatory post-synapse (Figure 1M). This included many core synaptic scaffolding proteins, such as SHANK2-3 and DLGAP2-4 (Chen et al., 2011; Mao et al., 2015; Monteiro and Feng, 2017; Wan et al., 2011), as well as modulators of synaptic receptors such as SYNGAP1 and SHISA6 (Barnett et al., 2006; Clement et al., 2012; Kim et al., 2003; Klaassen et al., 2016), which was consistent with the idea that vesicular trafficking plays an important part in synaptic function and regulation. Taken together, these results support a major endosomal trafficking role of the WASH complex in mouse brain.

SWIP^{P1019R} destabilizes the WASH complex

To determine how disruption of the WASH complex may lead to disease, we generated a mouse model of a human missense mutation found in children with intellectual disability, WASHC4c.3056c>g (protein: SWIPP1019R) (Ropers et al., 2011). Due to the sequence homology of human and mouse Washc4 genes, we were able to introduce the same point mutation in exon 29 of murine Washc4 using CRISPR (Derivery and Gautreau, 2010; Ropers et al., 2011). This C>G point mutation results in a Proline>Arginine substitution at position 1019 of SWIP's amino acid sequence (Figure 2A), a region thought to be critical for its binding to the WASH component, Strumpellin (Jia et al., 2010; Ropers et al., 2011). Western blot analysis of brain lysate from adult homozygous SWIPP1019R mutant mice (referred to from here on as MUT mice) displayed significantly decreased abundance of two WASH complex members, Strumpellin and WASH1 (Figure 2B).

These results phenocopied data from the human patients (Ropers et al., 2011) and suggested that the WASH complex is unstable in the presence of this SWIP point mutation in vivo. To test whether this mutation disrupted interactions between WASH complex subunits, we compared the ability of wild-type SWIP (WT) and SWIPP1019R (MUT) to co-immunoprecipitate with Strumpellin and WASH1 in HEK cells. Compared to WT, MUT SWIP co-immunoprecipitated significantly less Strumpellin and WASH1 (IP: 54.8suggesting that the SWIPP1019R mutation hinders WASH complex formation (Figure 2-figure supplement 1). Together these data support the notion that SWIPP1019R is a damaging mutation that not only impairs its function, but also results in significant reductions of the WASH complex as a whole.

Spatial proteomics analysis of SWIP^{P1019R} mutant mouse brain

Next, we aimed to understand the impact of the SWIPP1019R mutation on the subcellular organization of the mouse brain proteome. We performed spatial proteomics by following the protocol established by Geladaki et al., with modifications for homogenization of brain tissue (Geladaki et al., 2019; Hallett et al., 2008). We isolated seven subcellular fractions from brain tissue and quantified proteins in these samples using 16-plex TMT proteomics. Using this spatial proteomics dataset, we developed a data-driven clustering approach to classify proteins into subcellular compartments. This approach, which differs from the support vector machine learning algorithm employed by Geladaki et al. (2019), was motivated by the lack of a large corpus of brain-specific protein subcellular localization information, and the greater complexity of brain tissue compared to cultured cells. In addition to evaluating differential protein abundance between WT and SWIPP1019R MUT brain, we utilized this spatial proteomics dataset to analyze network-level changes in groups of covarying proteins to better understand WASH's function and explore the cellular mechanisms by which SWIPP1019R causes disease.

Brains from 10-month-old mice were gently homogenized to release intact organelles, followed by successive centrifugation steps to enrich subcellular compartments into different fractions based on their density (Figure 2C) (Geladaki et al., 2019). Seven WT and seven MUT fractions (each prepared from one brain, 14 samples total) were labeled with unique isobaric tandem-mass tags and concatenated. We also included two sample pooled quality controls (SPQCs), which allowed us to assess experimental variability and perform normalization between experiments. By performing this experiment in triplicate, deep coverage of the mouse brain proteome was obtained—across all 48 samples we quantified 86,551 peptides, corresponding to 7,488 proteins. After data pre-processing, normalization, and filtering we retained 5,897 reproducibly quantified proteins in the final dataset (Table S2).

We used generalized linear models (GLMs) to assess differential protein abundance for intra-fraction comparisons between WT and MUT genotypes, and for overall comparisons between WT and MUT groups, adjusted for baseline differences in subcellular fraction. In the first analysis, there were 85 proteins with significantly altered abundance in at least one of the 7 subcellular fractions (Benjamini-Hochberg P-Adjust < 0.1, Table S2 and Figure 2-figure supplement 2). Five proteins were differentially abundant between WT and MUT in all 7 fractions, including four WASH proteins and RAB21A—a known WASH interactor that functions in early endosomal trafficking (WASHC1, WASHC2, WASHC4, WASHC5, Figure 2E) (Del Olmo et al., 2019; Simpson et al., 2004). The abundance of the remaining WASH complex protein, WASHC3, was found to be very low and was not retained in the final dataset due to its sparse quantification. These data affirm that the SWIPP1019R mutation destabilizes the WASH complex. Next, to evaluate global differences between WT and MUT brain, we analyzed the average effect of genotype on protein abundance across all fractions. At this level, there were 687 differentially abundant proteins between WT and MUT brain (Bonferroni P-Adjust < 0.05) (Table S2). We then aimed to place these differentially abundant proteins into a more meaningful biological context using a systems-based approach.

For network-based analyses, we clustered the protein covariation network defined by pairwise correlations between all 5,897 proteins. Our data-driven, quality-based approach used Network Enhancement (Wang et al., 2018) to remove biological noise from the covariation network and employed the Leiden algorithm (Traag et al., 2019) to identify optimal partitions of the graph. We enforced module quality by permutation testing (Ritchie et al., 2016) to ensure that identified modules exhibited a non-random topology. Clustering of the protein covariation graph identified 255 modules of proteins that strongly covaried together (see Methods for complete description of clustering approach). To test for module-level differences between WT and MUT brain, we summarized modules for each biological replicate (a single subcellular fraction prepared from either a WT or MUT mouse) as the sum of their proteins, and extended our GLM framework to identify changes in module abundance (adjusted for fraction differences) between genotypes. 37 of the 255 modules exhibited significant differences in WT versus MUT brain (Bonferroni P-Adjust < 0.05; Table S3). Of note, the module containing the WASH complex, M19, was predicted to have endosomal function by annotation of protein function, and was enriched for proteins identified by WASH1-BioID2 (hypergeometric test P-Adjust < 0.05, bold node edges, Figure 2D). Similar to the WASH iBioID proteome (Figure 1), M19 contained components of the CCC (CCDC22, CCDC93, COMMD1-3, COMMD6-7, and COMMD9) and Retriever sorting complexes (VPS26C and VPS35L), but not the Retromer sorting complex, suggesting that in the brain, the WASH complex may not interact as closely with Retromer as it does in other cells (Figure 2D). Across all fractions, the abundance of M19 was significantly

lower in MUT brain compared to WT, providing evidence that the SWIPP1019R mutation reduces the stability of this protein subnetwork and impairs its function (Figure 2F-G).

In contrast to the decreased abundance of the WASH complex/endosome module, M19, we observed three modules (M2, M159, and M213) which were enriched for lysosomal protein components (Geladaki et al., 2019), and exhibited increased abundance in MUT brain (Figure 3). M159 (Figure 3B) contained the lysosomal protease Cathepsin A (CTSA), while M213 (Figure 3D) contained Cathepsin B (CTSB), as well as two key lysosomal hydrolases GLB1 and MAN2B2, and M2 (Figure 3C) contained two Cathepsins (CTSS and CTSL) and several lysosomal hydrolases (e.g. GNS, GLA, and MAN2B1) (Eng and Desnick, 1994; Mayor et al., 1993; Mok et al., 2003; Moon et al., 2016; Patel et al., 2018; Regier and Tifft, 1993; Rosenbaum et al., 2014). Notably, M2 also contained the lysosomal glycoprotein progranulin (GRN), which is integral to proper lysosome function and whose loss is widely linked with neurodegenerative pathologies (Baker et al., 2006; Pottier et al., 2016; Tanaka et al., 2017; Zhou et al., 2018). In addition, M2 contained the hydrolase IDS, whose loss causes a lysosomal storage disorder that can present with neurological symptoms (Hopwood et al., 1993; Schröder et al., 1994). The overall increase in abundance of modules M2, M159, and M213, and these key lysosomal proteins (Figure 3E-G), may therefore reflect an increase in flux through degradative lysosomal pathways in SWIPP1019R brain.

Furthermore, Module 2 (Figure 3C) included multiple membrane proteins and extracellular proteins, such as ITGA5 (an integrin shown to be upregulated and redistributed upon loss of WASH1), ATP13A2 (a cation transporter whose loss causes a Parkinsonian syndrome), and MMP17 (an extracellular metalloprotease), suggesting a link between these proteins and lysosomal enzymatic function (English et al., 2000; Ramirez et al., 2006; Zech et al., 2011). Increased abundance of these M2 proteins in MUT brain may indicate that WASH complex disruption alters their cellular localization. Taken together, these changes appear to reflect a pathological condition characterized by distorted lysosomal metabolism and altered cellular trafficking.

In addition to these endo-lysosomal changes, network alterations were evident for an endoplasmic reticulum (ER) module (M83), supporting a shift in the proteostasis of mutant neurons (Figure 2-figure supplement 3B). Notably, within the ER module, M83, there was increased abundance of chaperones (e.g. HSPA5, PDIA3, PDIA4, PDIA6, and DNAJC3) that are commonly engaged in presence of misfolded proteins (Bartels et al., 2019; Kim et al., 2020; Montibeller and de Belleroche, 2018; Synofzik et al., 2014; Wang et al., 2016). This elevation of ER stress modulators can be indicative of neurodegenerative states, in which the unfolded protein response (UPR) is activated to resolve misfolded species (Garcia-Huerta et al., 2016; Hetz and Saxena, 2017). These data demonstrate that loss of WASH

function not only alters endo-lysosomal trafficking, but also causes increased stress on cellular homeostasis. Finally, besides these endo-lysosomal and homeostatic changes, we also observed two synaptic modules (M35 and M248) that were reduced in MUT brain (Figure 2-figure supplement 3C-D). These included mostly excitatory post-synaptic proteins such as HOMER2 and DLG4 (also identified in WASH1-BioID, Figure 1), consistent with endosomal WASH influencing synaptic regulation. Decreased abundance of these modules indicates that loss of the WASH complex may result in failure of these proteins to be properly trafficked to the synapse.

SWIP mutant neurons display endo-lysosomal structural abnormalities

Combined, the proteomics data strongly suggested that endo-lysosomal pathways are altered in adult SWIPP1019R mutant mouse brain. Next, we analyzed whether structural changes in this system were evident in primary neurons. Cortical neurons from littermate WT and MUT P0 pups were cultured for 15 days in vitro (DIV15, Figure 4A), then fixed and stained for established markers of early endosomes (Early Endosome Antigen 1, EEA1; Figures 4B and 4C) and lysosomes (Cathepsin D, CathD; Figures 4D and 4E). Reconstructed three-dimensional volumes of EEA1 and Cathepsin D puncta revealed that MUT neurons display larger EEA1+ somatic puncta than WT neurons (Figures 4G and 4J), but no difference in the total number of EEA1+ puncta (Figure 4F). This finding is consistent with a loss-of-function mutation, as loss of WASH activity prevents cargo scission from endosomes and leads to cargo accumulation (Bartuzi et al., 2016; Gomez et al., 2012). Conversely, MUT neurons exhibited significantly less Cathepsin D+ puncta than WT neurons (Figure 4H), but the remaining puncta were significantly larger than those of WT neurons (Figures 4I and 4K). These data support the finding that the SWIPP1019R mutation results in both molecular and morphological abnormalities in the endo-lysosomal pathway.

SWIPP1019R mutant brains exhibit markers of abnormal endo-lysosomal structures and cell death in vivo. As there is strong evidence that dysfunctional endo-lysosomal trafficking and elevated ER stress are associated with neurodegenerative disorders, adolescent (P42) and adult (10 month-old, 10mo) WT and MUT brain tissue were analyzed for the presence of cleaved caspase-3, a marker of apoptotic pathway activation, in four brain regions (Boatright and Salvesen, 2003; Porter and Jänicke, 1999). Very little cleaved caspase-3 staining was present in WT and MUT mice at adolescence (Figures 5A, 5B, and Figure 5-figure supplement 1). However, at 10mo, the MUT motor cortices displayed significantly greater cleaved caspsase-3 staining compared to age-matched WT littermate controls (Figures 5D, 5E, and 5H). Furthermore, this difference appeared to be selective for the motor cortex, as we did not observe significant differences in cleaved

caspase-3 staining at either age for hippocampal, striatal, or cerebellar regions (Figure 5-figure supplement 1). These data suggested that neurons of the motor cortex were particularly susceptible to disruption of endo-lysosomal pathways downstream of SWIPP109R, perhaps because long-range corticospinal projections require high fidelity of trafficking pathways (Blackstone et al., 2011; Slosarek et al., 2018; Wang et al., 2014).

To further examine the morphology of primary motor cortex neurons at a subcellular resolution, samples from age-matched 7-month-old WT and MUT mice (7mo, 3 animals each) were imaged by transmission electron microscopy (TEM). Strikingly, we observed large electron-dense inclusions in the cell bodies of MUT neurons (arrows, Figure 5L; pseudo-colored region, 5N). These dense structures were associated electron-lucent lipid-like inclusions (asterisk, Figure 5N), and were visually consistent with lipofuscin accumulation at lysosomal residual bodies (Poët et al., 2006; Valdez et al., 2017; Yoshikawa et al., 2002). Lipofuscin is a by-product of lysosomal breakdown of lipids, proteins, and carbohydrates, which naturally accumulates over time in non-dividing cells such as neurons (Höhn and Grune, 2013; Moreno-García et al., 2018; Terman and Brunk, 1998). However, excessive lipofuscin accumulation is thought to be detrimental to cellular homeostasis by inhibiting lysosomal function and promoting oxidative stress, often leading to cell death (Brunk and Terman, 2002; Powell et al., 2005). As a result, elevated lipofuscin is considered a biomarker of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and Neuronal Ceroid Lipofuscinoses (Moreno-García et al., 2018). Therefore, the marked increase in lipofuscin area and number seen in MUT electron micrographs (Figures 5O and 5P, respectively) is consistent with the increased abundance of lysosomal pathways observed by proteomics, and likely reflects an increase in lysosomal breakdown of cellular material. Together these data indicate that SWIPP1019R results in pathological lysosomal function that could lead to neurodegeneration.

SWIP^{P1019R} mutant mice display persistent deficits in cued fear memory recall

To observe the functional consequences of the SWIPP1019R mutation, we next studied WT and MUT mouse behavior. Given that children with homozygous SWIPP1019R point mutations display intellectual disability (Ropers et al., 2011) and SWIPP1019R mutant mice exhibit endo-lysosomal disruptions implicated in neurodegenerative processes, behavior was assessed at two ages: adolescence (P40-50), and mid-late adulthood (5.5-6.5 mo). Interestingly, MUT mice performed equivalently to WT mice in episodic and working memory paradigms, including novel object recognition and Y-maze alternations (Figure 6-figure supplement 1). However, in a fear conditioning task, MUT mice displayed a significant deficit in cued fear memory (Figure 6). This task tests the ability of a mouse to asso-

ciate an aversive event (a mild electric footshock) with a paired tone (Figure 6A). Freezing behavior of mice during tone presentation is attributed to hippocampal or amygdala-based fear memory processes (Goosens and Maren, 2001; Maren and Holt, 2000; Vazdarjanova and McGaugh, 1998). Forty-eight hours after exposure to the paired tone and footshock, MUT mice showed a significant decrease in conditioned freezing to tone presentation compared to their WT littermates (Figures 6B and 6C). To ensure that this difference was not due to altered sensory capacities of MUT mice, we measured the startle response of mice to both electric foot shock and presented tones. In line with intact sensation, MUT mice responded comparably to WT mice in these tests (Figure 6-figure supplement 2). These data demonstrate that although MUT mice perceive footshock sensations and auditory cues, it is their memory of these paired events that is significantly impaired. Additionally, this deficit in fear response was evident at both adolescence and adulthood (top panels, and bottom panels, respectively, Figures 6B and 6C). These changes are consistent with the hypothesis that SWIPP109R is the cause of cognitive impairments in humans.

SWIP^{P1019R} mutant mice exhibit surprising motor deficits that are confirmed in human patients

Because SWIPP1019R results in endo-lysosomal pathology consistent with neurodegenerative disorders in the motor cortex, we next analyzed motor function of the mice over time. First, we tested the ability of WT and MUT mice to remain on a rotating rod for five minutes (Rotarod, Figures 7A-7C). At both adolescence and adulthood, MUT mice performed markedly worse than WT littermate controls (Fig 7C). Mouse performance was not significantly different across trials, which suggested that this difference in retention time was not due to progressive fatigue, but more likely due to an overall difference in motor control (Mann and Chesselet, 2015).

To study the animals' movement at a finer scale, the gait of WT and MUT mice was also analyzed using a TreadScan system containing a high-speed camera coupled to a transparent treadmill (Figure 7D) (Beare et al., 2009). Interestingly, while the gait parameters of mice were largely indistinguishable across genotypes at adolescence, a striking difference was seen when the same mice were aged to adulthood (Figures 7E-7G). In particular, MUT mice took slower (Figure 7E), longer strides (Figure 7F), stepping closer to the midline of their body (track width, Figure 7- figure supplement 1), and their gait symmetry was altered so that their strides were no longer perfectly out of phase (out of phase=0.5, Figure 7G). While these differences were most pronounced in the rear limbs (as depicted in Figure 7E-7G), the same trends were present in front limbs (Figure 7-figure supplement 1). These findings demonstrate that SWIPP1019R results in progressive motor function decline that was detectable by the rotarod task at adolescence, but which became

more prominent with age, as both gait and strength functions deteriorated.

These marked motor findings prompted us to re-evaluate the original reports of human SWIPP1019R patients (Ropers et al., 2011). While developmental delay or learning difficulties were the primary impetus for medical evaluation, all patients also exhibited motor symptoms (mean age = 10.4 years old, Figure 7H). The patients' movements were described as "clumsy" with notable fine motor difficulties, dysmetria, dysdiadochokinesia, and mild dysarthria on clinical exam (Figure 7H). Recent communication with the parents of these patients, who are now an average of 21 years old, revealed no notable symptom exacerbation. It is therefore possible that the SWIPP1019R mouse model either exhibits differences from human patients or may predict future disease progression for these individuals, given that we observed significant worsening at 5-6 months old in mice (which is thought to be equivalent to 30-35 years old in humans) (Dutta and Sengupta, 2016; Zhang et al., 2019).

Discussion

Methods and Materials