GLOBAL JOURNAL OF PEDIATRICS

Global Journal of Pediatrics

ISSN: 2832-4013

Three Siblings with Aarskog Scott Syndrome Together with Mental Retardation and Giant Megacolon

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Article Type: Review Article

Compiled date: February 26, 2024

Volume: 2

Issue: 1

Journal Name: Global Journal of Pediatrics

Publisher: Infact Publications LLC

Journal Short Name: Glob J Pedia

Article ID: INF1000278

Keywords: *ZFHX3*; Neuron; Constipation; Next generation sequencing

Cite this article: Kawashima H, Nishimata S, Shinji S, Morishima Y, Tsutsumi N, Kashiwagi K, et al. Three siblings with aarskog scott syndrome together with mental retardation and giant megacolon. Glob J Pedia. 2024;2(1):1–11.

Abstract

We found a new candidate disease-causing gene in 3 siblings who have Aarskog-Scott syndrome together with mental retardation and megacolon. Using exome analysis, *ZFHX3*, which has been reported to be associated with Hirschsprung disease, was identified as a candidate causative gene for their disease (mutations c.G756A:p.A2521T and c.G4428A:p.M1476I). In silico analysis suggested that A2521T was a functionally damaging mutation. Mice with a frame shift mutation in *ZFHX3* were fetal lethal. Mice with the A2530T (which corresponds to human A2521T) knockout mutation in *ZFHX3* were viable and did not have any obvious neurological abnormalities.

Introduction

Aarskog Scott Syndrome (AAS) is an inherited disease showing either X-linked recessive or autosomal-dominant inheritance (OMIM #305400) [1], and is known to be caused by abnormalities in the FGD1 gene [2]. However, mutations in the FGD1 gene have been found in only 18.3% of AAS patients, and other causative genes have remained unknown to date [3]. The disease is accompanied with skeletal dysplasia, urinary tract anomalies, telecanthus, external ear malformation, maxillary hypoplasia, and abnormalities of the sigmoid colon [4,5]. We present 3 siblings with AAS, who have chronic constipation with megacolon and psychomotor developmental retardation. The patients were found to have *ZFHX3* gene variants. *ZFHX3* is also called as Zinc finger homeobox protein 3 and encodes a transcription factor with multiple homeodomains and zinc finger motifs, and regulates myogenic and neuronal differentiation. However, as the patients also had mental retardation and giant megacolon, our data indicates that *ZFHX3* is not an independent candidate causative gene for AAS, and the possibility of clinical complications should be taken into consideration.

Case Presentation

The sister (middle of 4 siblings; (Figure 1) complained of intractable constipation and abdominal tension, and was admitted to our department at the age of 8 years. She had no particular past medical history except for psychomotor developmental delay. Problems with her bowel movements had been recognized since she was about 3-year-old. Analysis of her rectal biopsy revealed no pathological findings indicative of Hirschsprung disease. At the same time, Ehlers-Danlos syndrome was also suspected (ED score: 4 points), but electron microscopy analysis of her skin biopsy showed no obvious findings. She was hence treated with laxatives, such as oxidized Mg and laxoberon, but she only had

bowel movements once every 3 days to 5 days. At 13 years of age, she was admitted again for disimpaction. Her height was 134 cm (--3.8 SD), and her weight was 24 kg (--2.9 SD). Her fingers were slender, and webbing was observed between the fingers. She had hyperextension of the joints and facial anomalies (constant open mouth, thin and prominent jaw and nose, and raised ears). An enema, disimpaction, and an abdominal CT scan were performed. The CT displayed extreme megacolon that had displaced the bladder (Figure 2). Brain CT and MRI displayed no abnormalities. Her constipation improved after the treatment. After discharge, her condition was well controlled by laxatives. Her elder and younger brothers both had mental retardation IQ of the younger brother was 43 by WSIC-III. Both brothers had shown a tendency of constipation from 1-year-old. The brothers also visited our department because of their severe constipation and abdominal fullness, and they were hospitalized for examination at 12 years of age. The elder brother underwent a rectal mucosal biopsy, but Acetyl Cholinesterase (AChE) staining revealed that there was no proliferation of nerve fibers. Treatment with laxatives, such as oxidized Mg and laxoberon® was started, and they have since then experienced daily bowel movements. The younger brother also developed obesity and liver dysfunction at the age of 11 years, in addition to constipation. His liver biopsy showed chronic hepatitis and fatty liver, and he was diagnosed as having nonalcoholic steatohepatitis. Histologically, the rectal mucosa and superficial submucosal tissue showed mild chronic inflammatory cell infiltration within the mucosa. There was no proliferation of nerve fibers, which were positive for AChE staining. The biopsy samples showed no characteristics indicative of Hirschsprung disease. Brain MRI displayed mild atrophy without any vascular anomalies. All characteristics were shown in (Table 1).

Methods

Genomic Studies

Exome sequencing on blood samples from the 3 siblings was performed using Ion Proton sequencer. The effects of the detected variations on protein structure and function were mostly analyzed by SIFT and PolyPhen2. Alphafold2 was also used to predict the structure of the normal type and mutant type [6]. The output files were visualized using UCSF Chimera [7]. To understand how to evaluate the effects of mutations by AI, Alphamissense software was also used [8]. According to the results of exome sequencing and the damaging effects of the vairants, *ZFHX3* A2530T (which corresponds to human A2521T) knockout mice were created, and their neurological behavior was analyzed. C57BL/6N mice were purchased from CLEA Japan. The mutant mice generated by the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats / CRISPR associated proteins) system were maintained on the C57/BL6N background at Riken BioResource Research Center (BRC). We applied a standard procedure of the CRISPR/Cas9 system to introduce the pathogenic *ZFHX3* variant into the mouse genome.

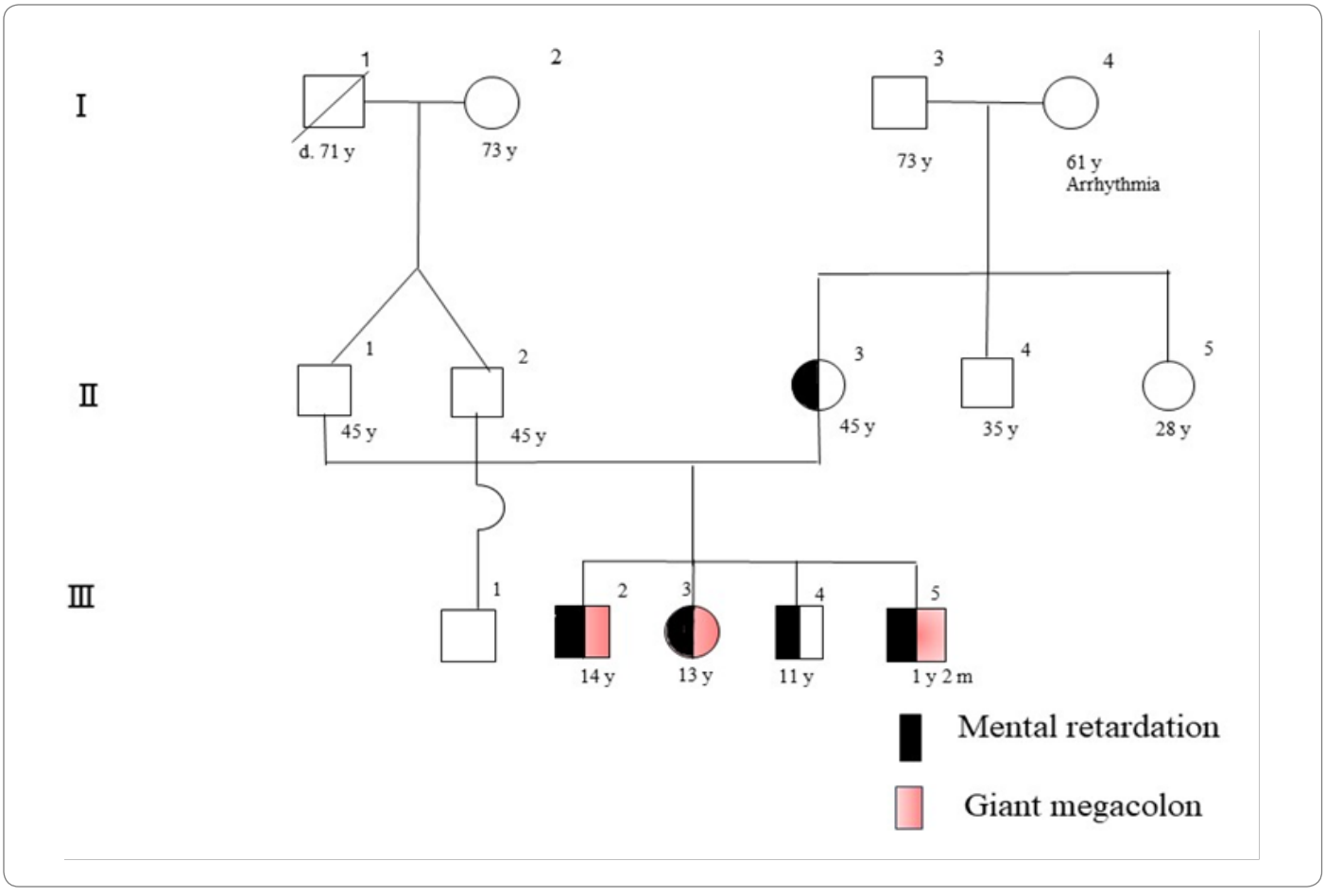


Figure 1: Pedigree of the patients.

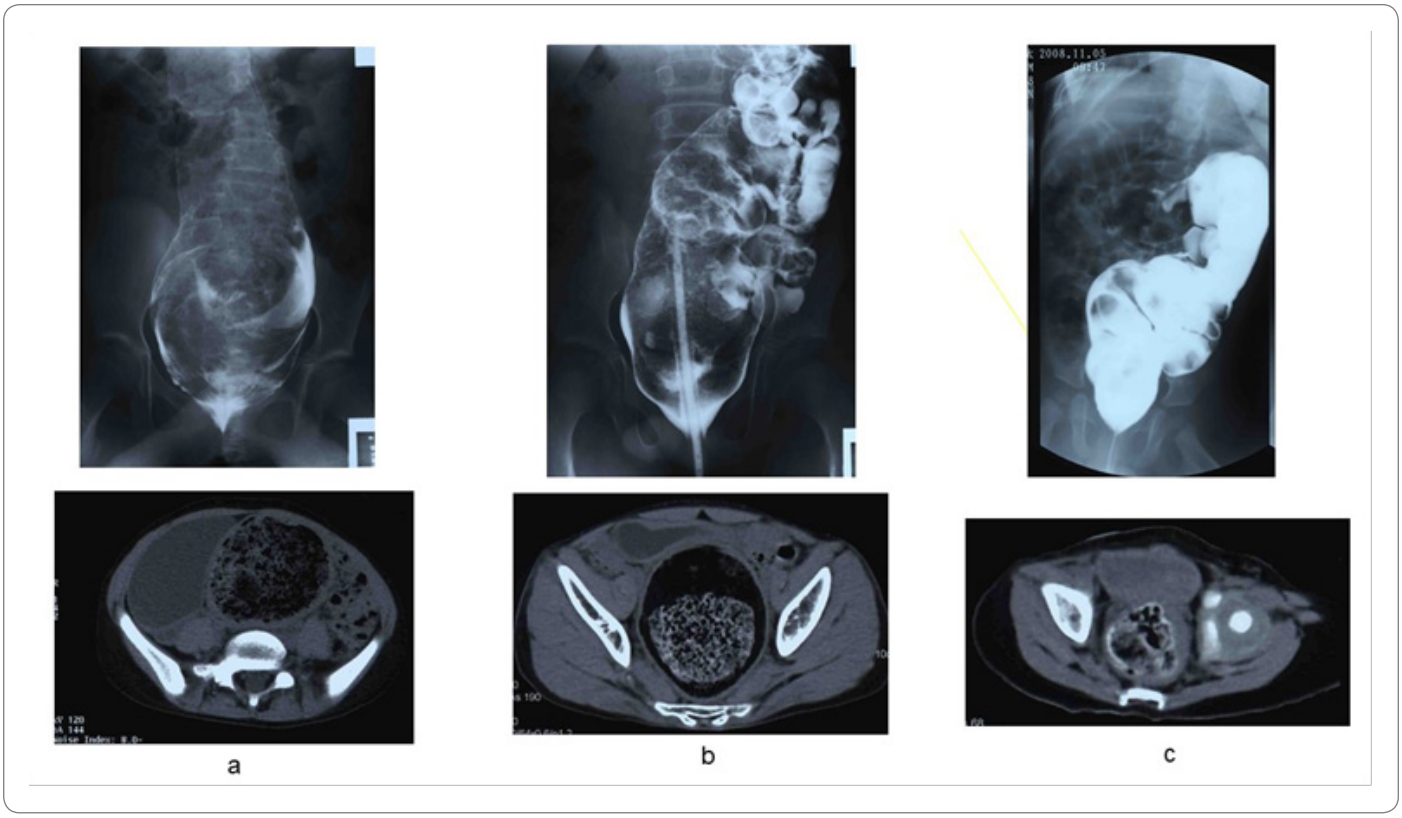


Figure 2: Pedigree of the patients.

Table 1: Profile of patients and typical cases of Aarskog-Scott syndrome.

	Patient 1	Patient 2	Patient 3	Aarskog-Scott syndrome
Inheritance pattern	ZFHX3 c.G756A:p.A2521T			XR or AD
	ZFHX3 c.G4428A:p.M1476I			Fdg1 genetic abnormality
Family history	Eldest daughter	Eldest son	Third son	
Gender	Male	Female	Male	
Mental retardation	+	+	+	+
Low stature	+			+
Colon	Megacolon	Megacolon	Megacolon	Sigma elongatum
Facial anomaly	Thin and prominent chin	Thin and prominent chin	Hypertelorism	Inverted triangular facehypertelorism
	Nasal tip	Nasal tip	Saddle nose	Upward nostrils
	Raised ears		Downward of the corners of the mouth	Widephiltrum
				Low set low etc.
Interdigital webbing	+	-	-	+
Finger and toe	Elongated fingers	Elongated fingers	Normal	Thick and short fingers
Others			Non-alcoholicsteatohepatitis	Collar-shaped scrotum
			Obesity	

The crRNA was designed to target the sequence 5'-CACGTCGACCCCTCAACAGCTGG-3' in the mouse *ZFHX3* locus. The gRNA was prepared by mixing equimolar amounts of crRNA and tracrRNA (Integrated DNA Technologies, Iowa 52241, USA). The gRNA duplex was mixed with 200 ng/μL of the Cas9 protein (Integrated DNA Technologies) and 200 ng/μL of a single stranded oligonucleotide donor in HEPES-buffered saline, and this was then introduced into fertilized eggs using CUY21EDIT II electroporator (Bex, Japan). After electroporation, eggs were cultured in KSOM medium (Arc Resource, Japan) for 1 day, and then transferred into the oviducts of recipient female mice. The sequence of the 120-nt single stranded oligonucleotide donor was 5′-CCTCGCAGCTCTCCCATCTGCCCCTCAAGCCCCTCCACACGTCGACCCCTCAACAGCTGaCAAATACCTCCTCAGCTAATCCCCTACCAGTGTGCAAGCTGGCGTGGGGTTCCCAT-3′. Phenotype data of mutant mice were collected and analyzed by the comprehensive phenotyping platform of Japan Mouse Clinic at Riken BRC (https://ja.brc.riken.jp/lab/jmc/mouse_clinic/en/index.html). All animal

experiments in this study were performed in accordance with the guidelines approved by the Animal Care and Use Committee of Riken BRC.

Results

Exome sequencing of the 3 siblings identified 2 variants of *ZFHX3* (c.G756A:p.A2521T and c.G4428A:p.M1476I) as candidates responsible for their diseases, as a compound-heterozygous model, as shown in (Table 2). The former mutation (A2521T) was shown to be damaging by SIFT and PolyPhen2. We analyzed the results of 100 residues, including the variants, to clarify the 3D structure of the variant proteins (Figure 3). However, it was difficult to evaluate the effects of these variants by Alphafold2, which predicts the structure of the normal and mutant. Alphamissense (AI) rated A2521T and M1476I as benign. The resultant mice with a frameshift in *ZFHX3* were fetal lethal. Mice with knockout did not show lethality, and their neurological and psychological characteristics were analyzed. These mice showed no significant difference with wild-type mice in all of the various neurological tests (light/dark transition test, open-field test, Crawley social interaction test, home-cage activity test, Y-maze, fear conditioning test, and pre-pulse inhibition test), as shown in (Figure 4A–4I).

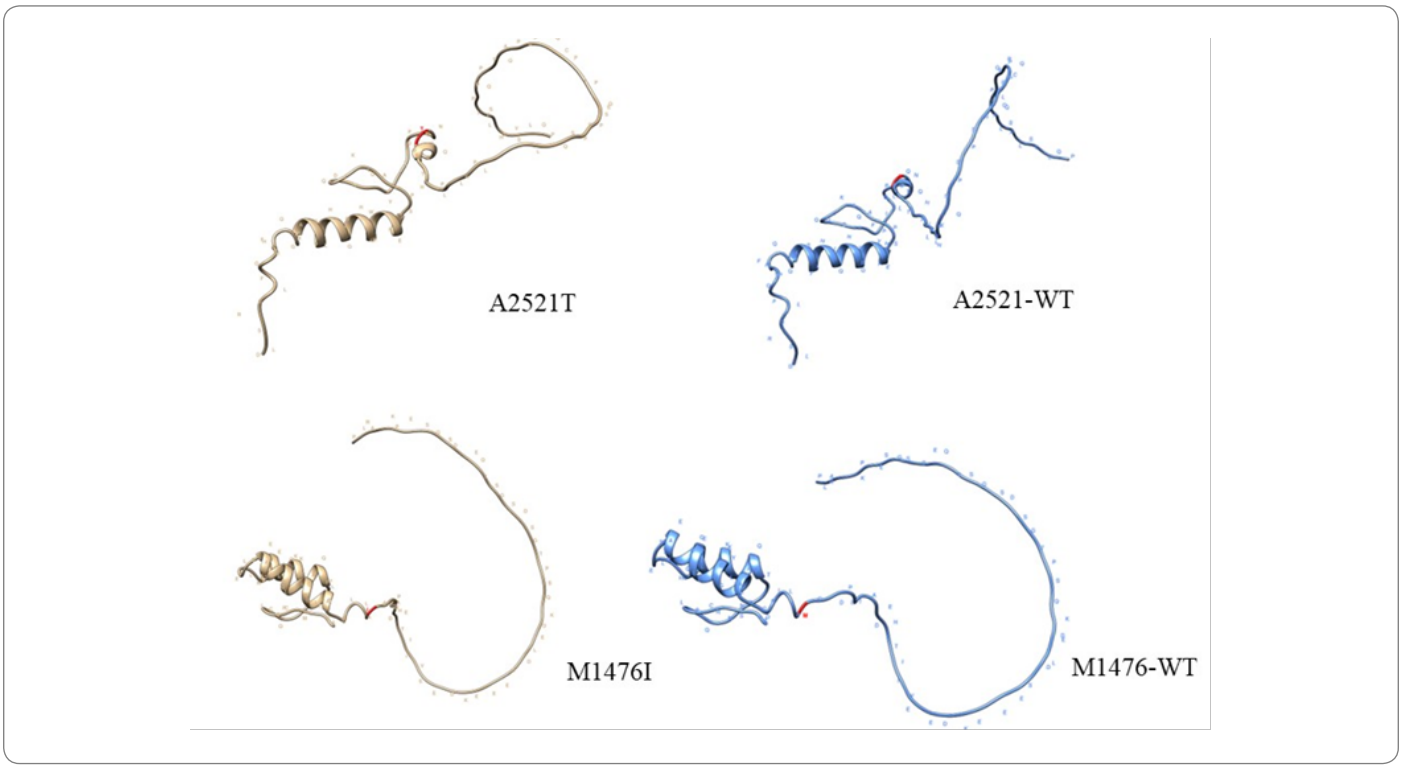


Figure 3: Predicted structure of normal and mutant *ZFHX3* by Alphafold2. The output files were visualized using UCSF Chimera.

Table 2: Annotation of exome in all families.

	Homozygous model		Compound hetero model
Exon, splice site in family	32,210		32,210
-Segmental duplication	29,268		29,268
-Synonymous, unknown	15,584		15,584
Positive in 3 affected patients	2,194		1892
Homozygous in 1 unaffected sibling	166	Heterozygous or none in the mother and father	964
Heterozygous in the mother and father	9	Heterozygous or none in 1 unaffected sibling	930
In house except neurological diseases	0	AF < 0.01(HGVD, 1000g, gnomAD)	126
		-In house except neurological diseases	53
		Compound hetero	2

Gene	Function	Exonic function	AA change	SIFT	PolyPhen2	Phenotype	InterVar
ZFHX3	Exonic	Nonsynonymous SNV	NM_006885:exon9:c.G7561A:p.A2521T	Tolerated 0.129	Damaged 0.995	Atrial fibrillation {Prostate cancer, susceptibility to, somatic}	Uncertain significance
ZFHX3	Exonic	Nonsynonymous SNV	NM_006885:exon9:c.G4428A:p.M1476I	Tolerated 0.396	Probably damagigng 0.573	Atrial fibrillation {Prostate cancer, susceptibility to, somatic}	Likely benign

Figure 4: Neurological and psychological analyses of ZFHX3 knockout mice.
No significant differences in neurological and psychological test results were observed between ZFHX3 knockout and wild-type mice.

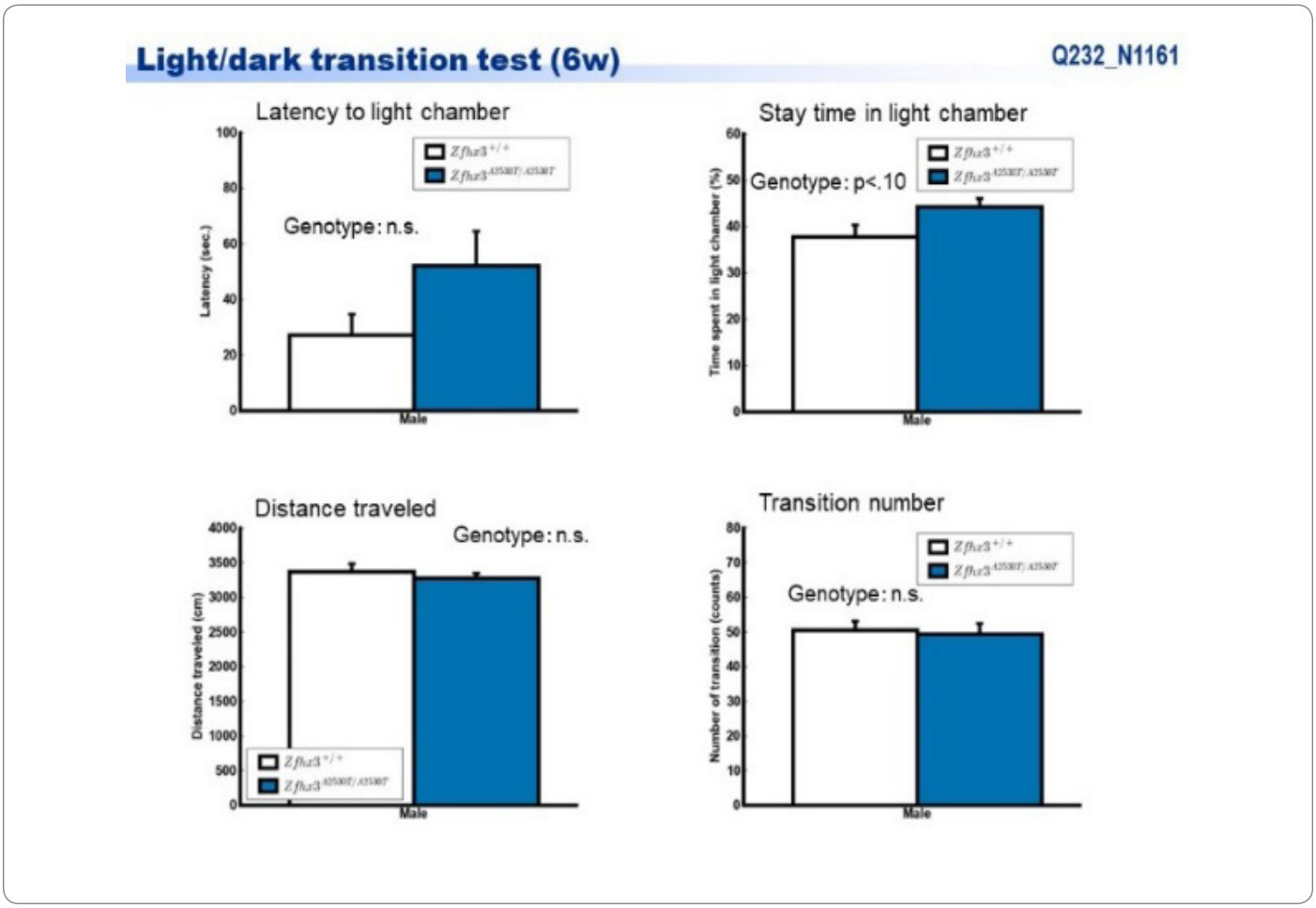
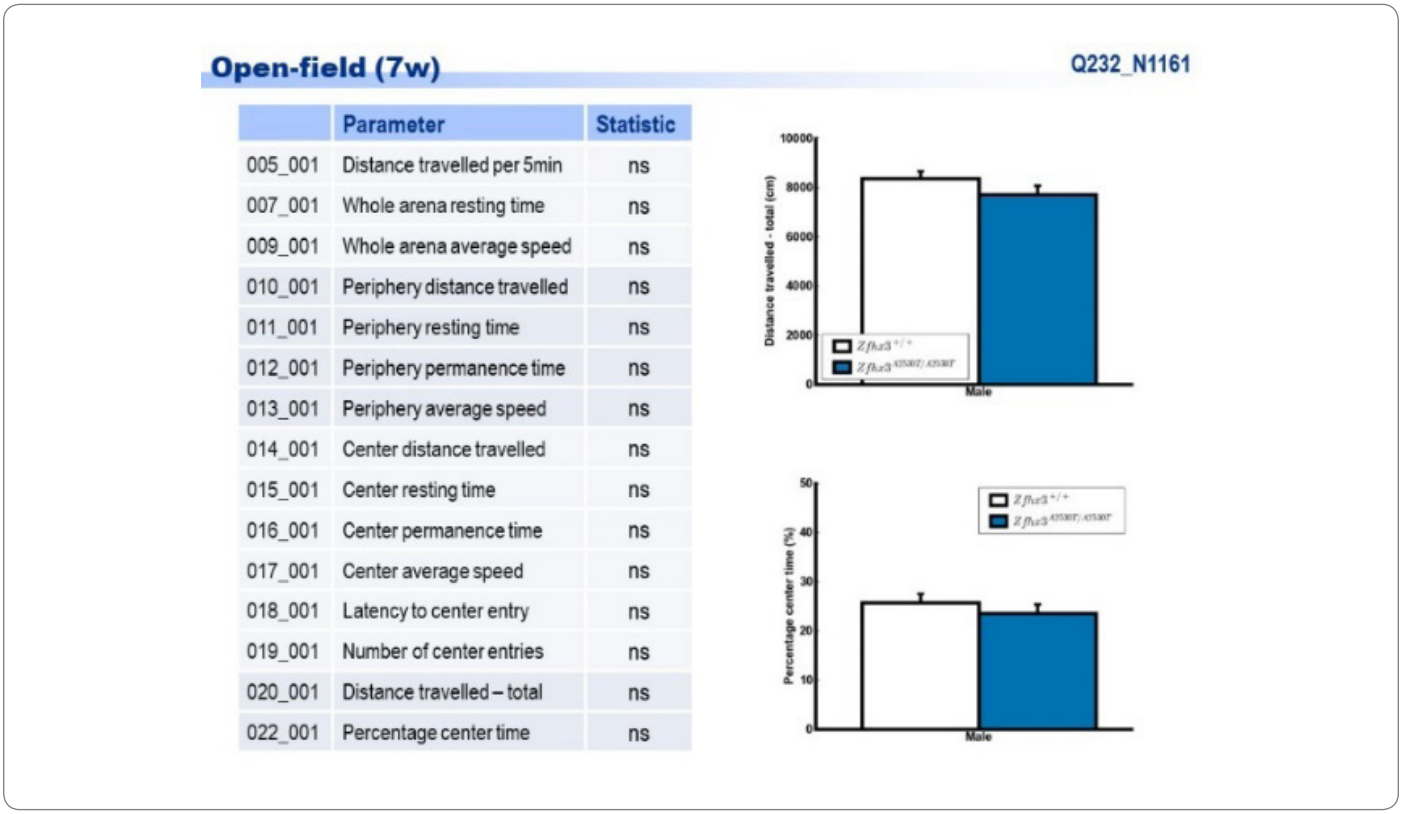


Figure 4A: Light/dark transition test (6W).



	Parameter	Statistic
005_001	Distance travelled per 5min	ns
007_001	Whole arena resting time	ns
009_001	Whole arena average speed	ns
010_001	Periphery distance travelled	ns
011_001	Periphery resting time	ns
012_001	Periphery permanence time	ns
013_001	Periphery average speed	ns
014_001	Center distance travelled	ns
015_001	Center resting time	ns
016_001	Center permanence time	ns
017_001	Center average speed	ns
018_001	Latency to center entry	ns
019_001	Number of center entries	ns
020_001	Distance travelled – total	ns
022_001	Percentage center time	ns

Figure 4B: Open field (7W).

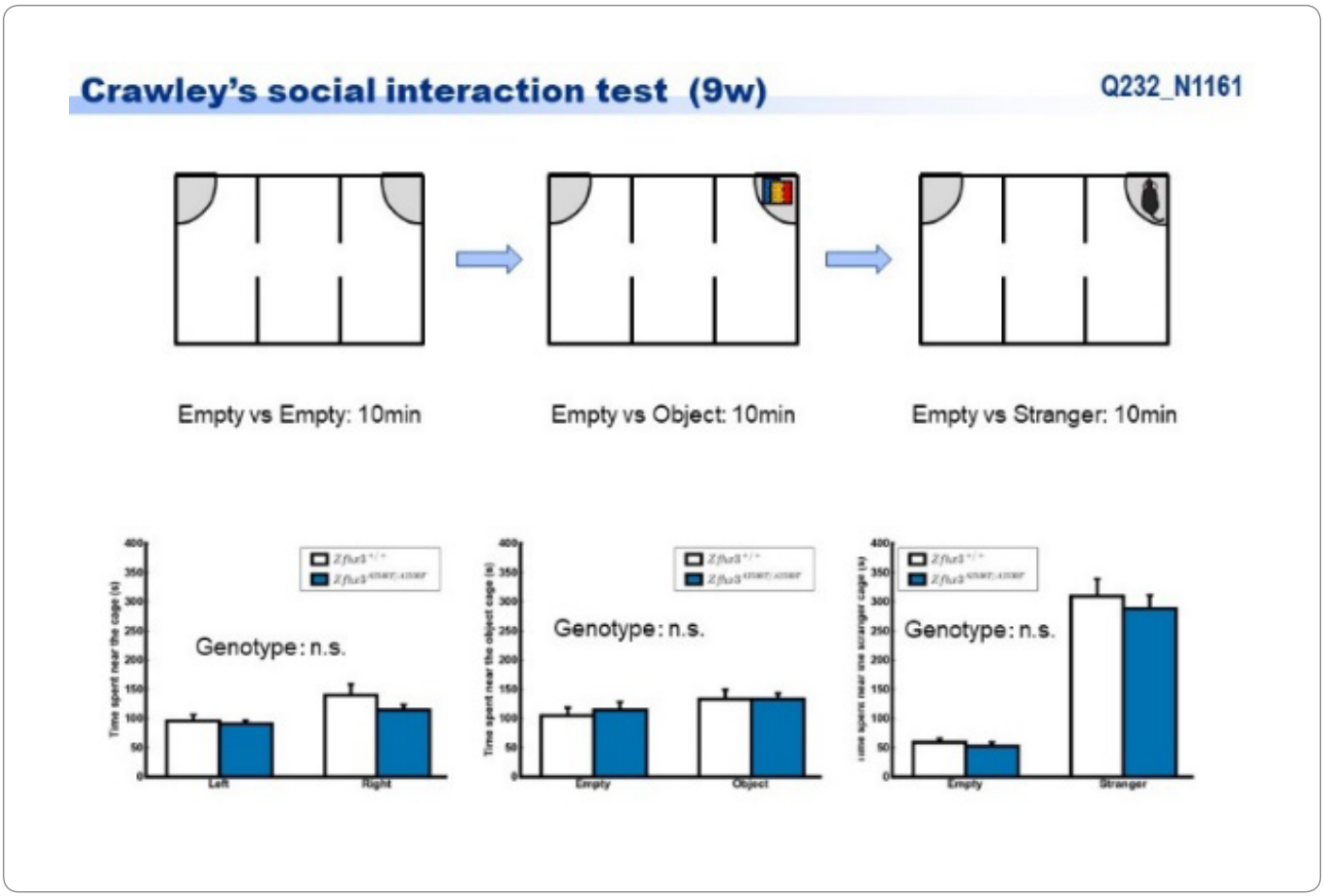


Figure 4C: Crawley's social interaction test (9w).

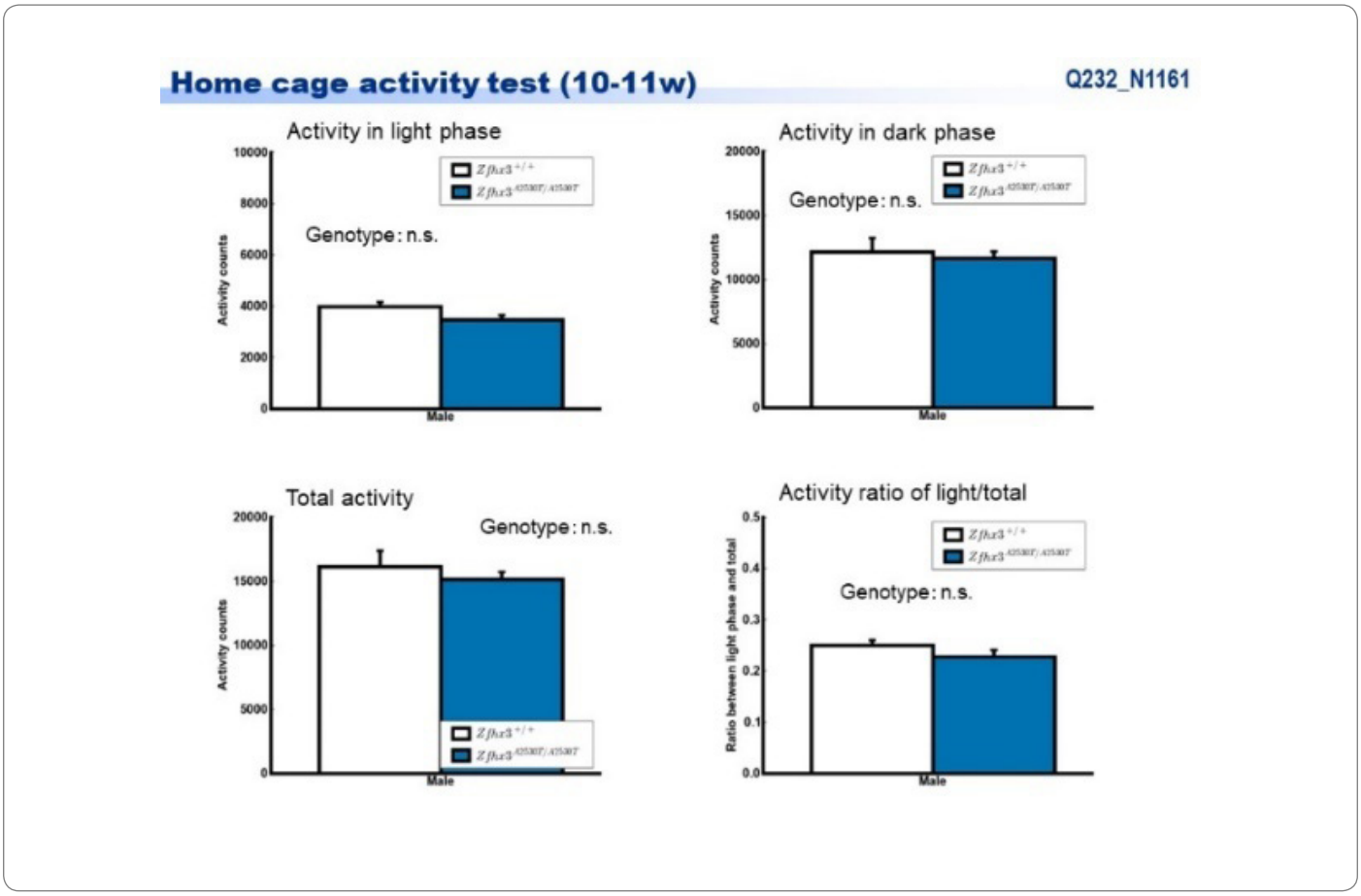


Figure 4D: Home cage activity test (10w – 11w).

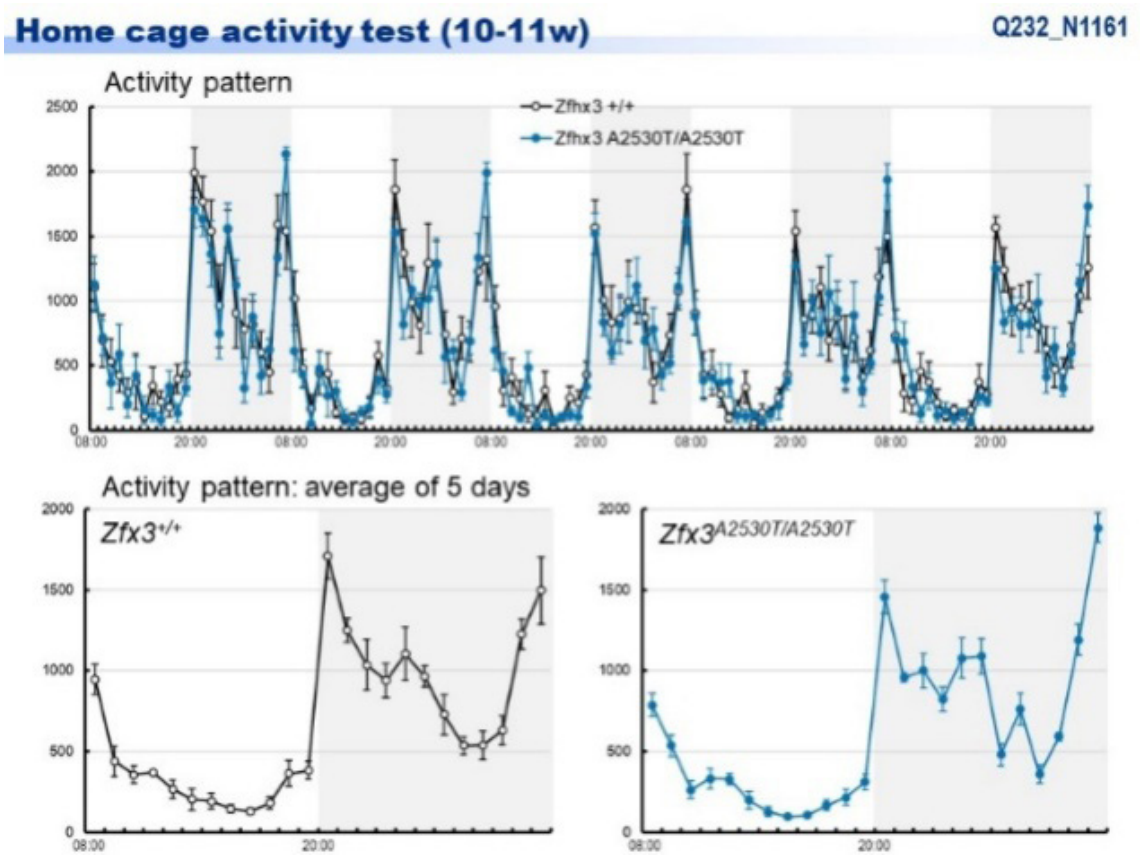
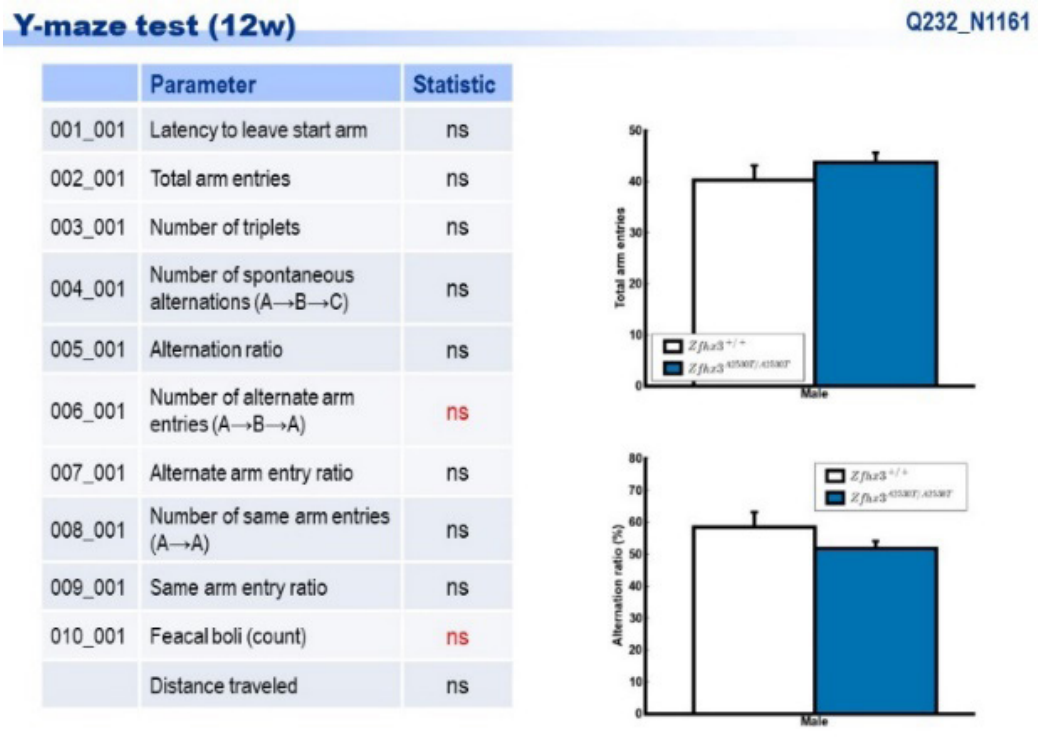


Figure 4E: Home cage activity test (10 w–11w) – Graph.



	Parameter	Statistic
001_001	Latency to leave start arm	ns
002_001	Total arm entries	ns
003_001	Number of triplets	ns
004_001	Number of spontaneous alternations (A→B→C)	ns
005_001	Alternation ratio	ns
006_001	Number of alternate arm entries (A→B→A)	ns
007_001	Alternate arm entry ratio	ns
008_001	Number of same arm entries (A→A)	ns
009_001	Same arm entry ratio	ns
010_001	Feacal boli (count)	ns
	Distance traveled	ns

Figure 4F: Y-maze test (12w).

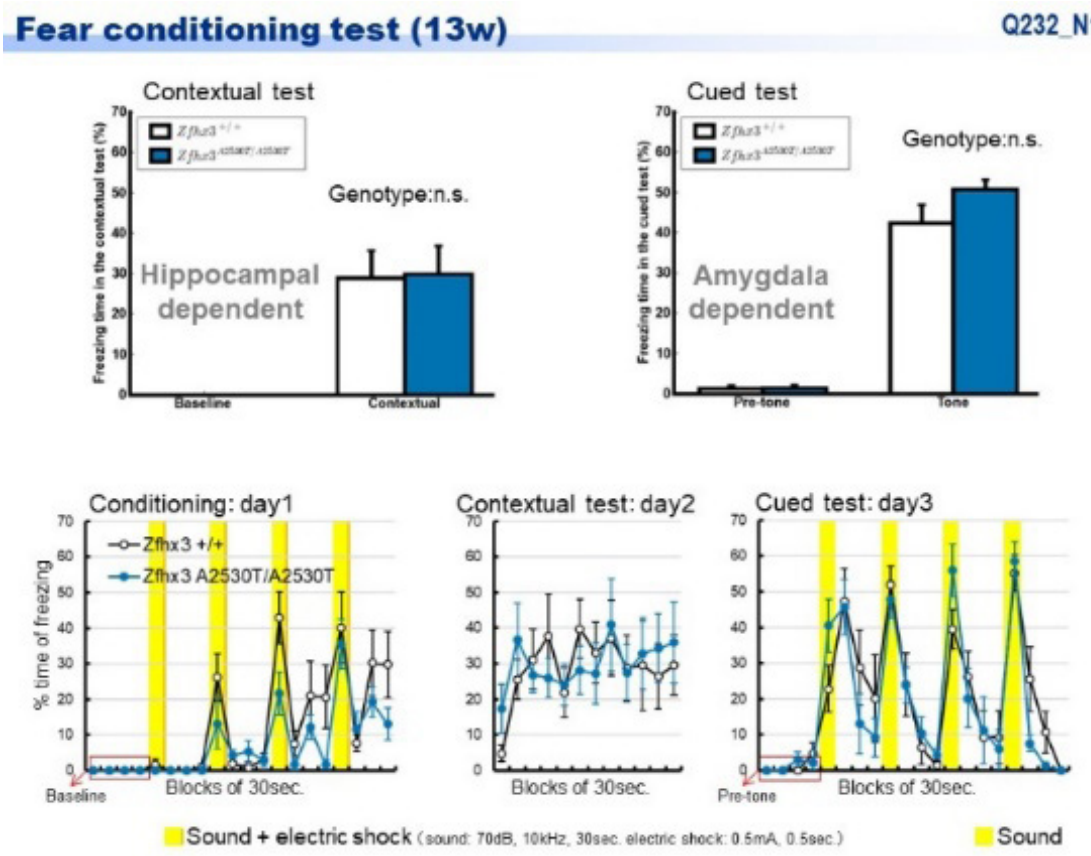
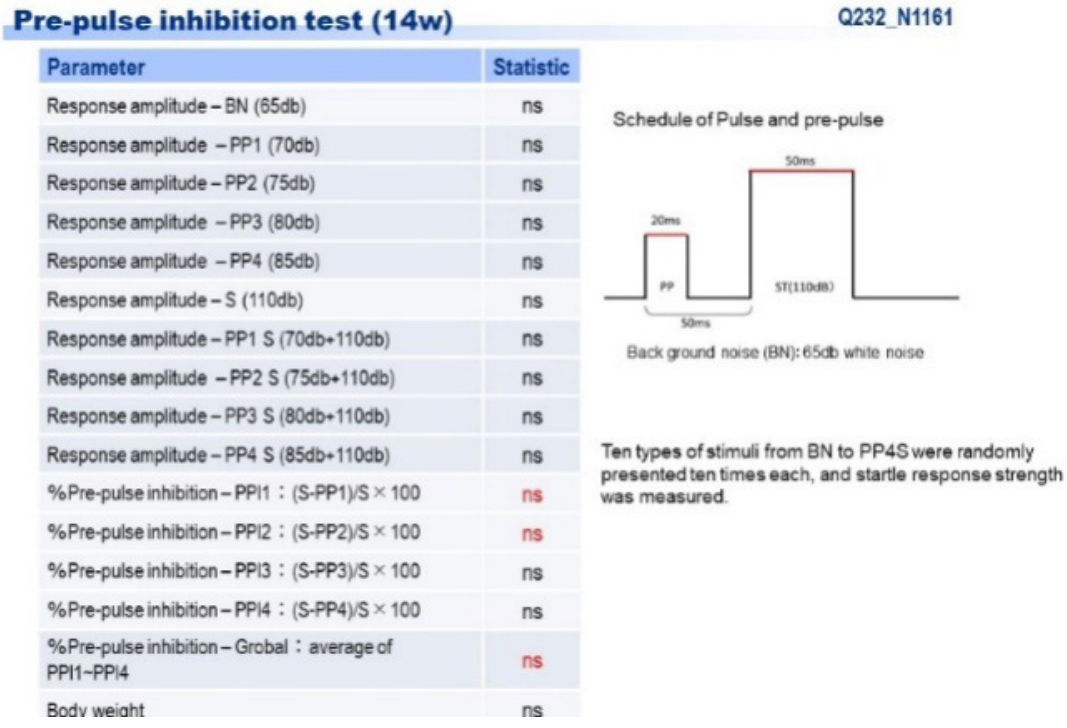


Figure 4G: Fear conditioning test (13w).



Parameter	Statistic
Response amplitude – BN (65db)	ns
Response amplitude – PP1 (70db)	ns
Response amplitude – PP2 (75db)	ns
Response amplitude – PP3 (80db)	ns
Response amplitude – PP4 (85db)	ns
Response amplitude – S (110db)	ns
Response amplitude – PP1 S (70db+110db)	ns
Response amplitude – PP2 S (75db+110db)	ns
Response amplitude – PP3 S (80db+110db)	ns
Response amplitude – PP4 S (85db+110db)	ns
%Pre-pulse inhibition – PPI1 : (S-PP1)/S × 100	ns
%Pre-pulse inhibition – PPI2 : (S-PP2)/S × 100	ns
%Pre-pulse inhibition – PPI3 : (S-PP3)/S × 100	ns
%Pre-pulse inhibition – PPI4 : (S-PP4)/S × 100	ns
%Pre-pulse inhibition – Grobal : average of PPI1~PPI4	ns
Body weight	ns

Figure 4H: Pre-pulse inhibition test (14w).

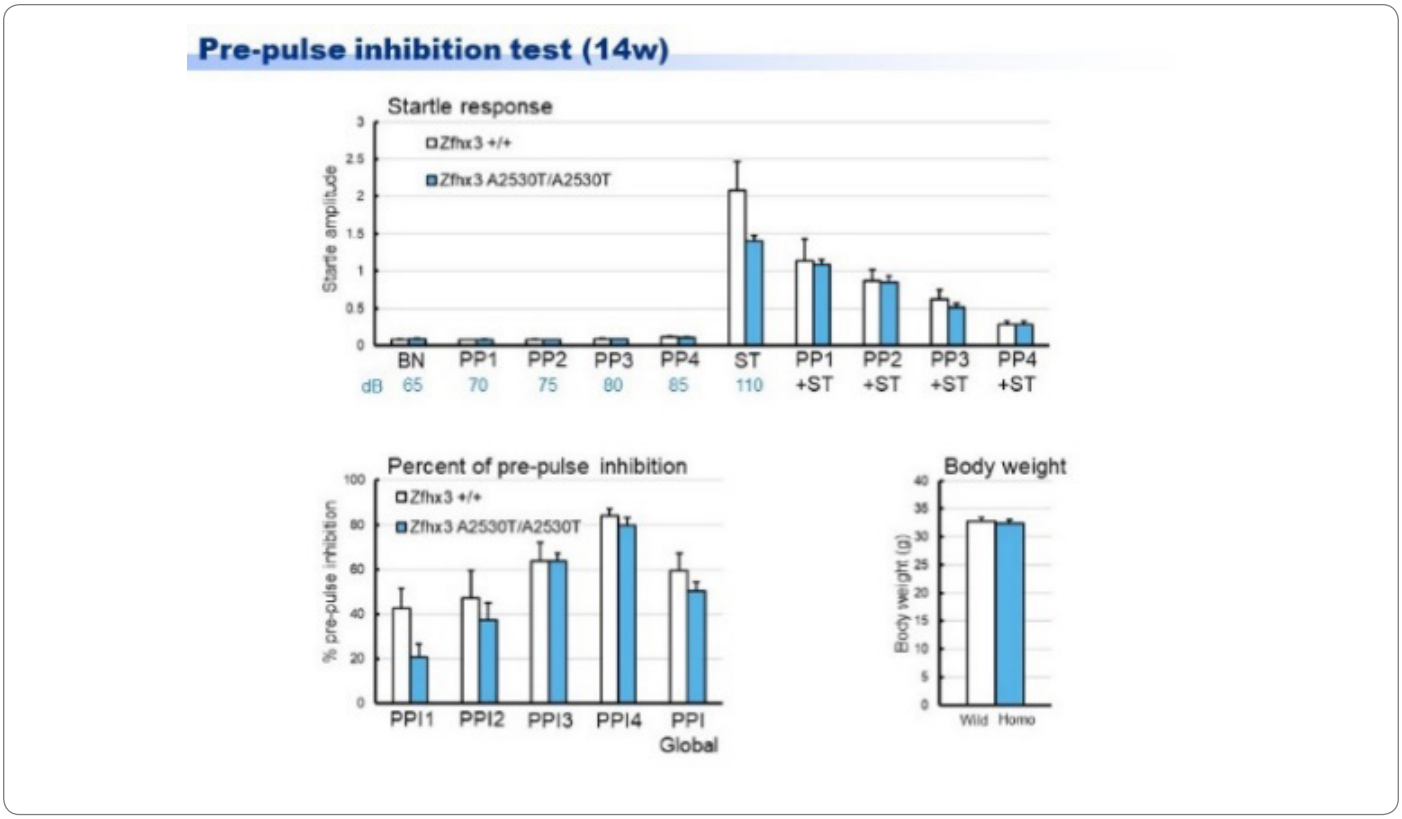


Figure 4I: Pre-pulse inhibition test (14w) –Graph.

Discussion

AAS, which is a rare syndrome with a recessive mode of inheritance, was first described by Aarskog in 1970, and then further described in detail by Scott in 2 different families. The characteristics of AAS individuals were reported as short-statured with craniofacial anomalies (hypertelorism, short nose, ptosis, and genital dysmorphism, such as shawl scrotum and cryptorchidism) [9,10]. Various other characteristics, such as clinodactyly, brachydactyly, long philtrum, widow's peak, camptodactyly, interdigital webbing, and inguinal/umbilical hernia have also been reported [11]. Fifty-two pathogenic variants of FGD1, which is the responsible gene for the X-linked form of AAS, have been reported [12]. However, no clear phenotype-genotype correlation has been found with mutations in FGD [13]. Although AAS is clinically and genetically heterogeneous, causative genes other than FGD1 have not yet been identified. In this study, we identified *ZFHX3* as a new candidate causative gene of AAS. Zhang et al. performed a whole-exome sequencing study and isolated a panel of genes as candidate causative genes of Hirschsprung's disease, and reported that *ZFHX3* was prioritized for follow-up studies: both the time-space expression patterns in the mouse and human colon showed that it is a strong candidate gene for Hirschsprung's disease [14]. Zhang et al. demonstrated that the striatum, which is the major component of the basal ganglia, consists of the caudate-putamen, nucleus accumbens, and olfactory tubercle. The striatal principal projection neurons are comprised of Medium Spiny Neurons (MSNs) with 2 dopamine receptors, namely DRD1 (Dopamine receptor D1) (D1 MSNs) and DRD2 (D2 MSNs). *ZFHX3* is strongly expressed in the boundary of the sub ventricular zone / mantle zone of the lateral ganglionic eminence and its expression in the striatum is down regulated during the first postnatal week. At the cellular level, *ZFHX3* is selectively expressed in immature D1 MSNs. Moreover, a significant reduction in the number of D1 MSNs in the striatum of *ZFHX3* conditional knockout mice was observed. They concluded that *ZFHX3* plays a crucial role in the differentiation and survival of late-born D1 MSNs [15]. Regarding the neurological and psychological manifestations of individuals with AAS, individuals have been reported to show an average IQ in most cases. However, various degrees of neurocognitive disabilities and/or behavior disorders, ranging from attention-deficit/hyperactivity disorder to severe intellectual disability were reported [16–19]. *ZFHX3* is also known as ATBT/ATBF1, and has been reported in several studies of the nervous system especially neuronal differentiation [20,21]. Further genomic and pathological studies should be performed in the future. We could not find any study reporting neurological manifestations in *ZFHX3* knockout mice, nor a pathology similar to Hirsch sprung disease in AAS patients. From these reports, *ZFHX3* is assumed to be associated with neuronal differentiation. In the present study, all affected individuals had mental retardation. Other effects caused by having a megacolon for a long time, including effects on the nutritional environment, might occur as the individuals grow and develop. In the present study, mice with a frame shift in *ZFHX3* were fetal lethal, whereas mice with A2530T (which corresponds to human A2521T) knockout did not show lethality. We speculated that the frame shift mutation may have a more severe effect on the protein's function than the A2530T mutation. However, further research is needed to confirm this hypothesis. To our knowledge,

there are no direct lines of evidence to date that *ZFHX3* can cause specific diseases. Somatic mutations in *ZFHX3* have been detected in prostate cancers. The most frequently mutated genes in prostate cancer are *KMT2D* (26.45%), *FOXA1* (16.13%), *ATM* (15.81%), *ZFHX3* (9.35%), *TP53* (8.06%), and *APC* (5.48%). Hotspot mutations in *ZFHX3* have been identified in human prostate cancer [22]. Variants of *ZFHX3* have also been reported to be associated with atrial fibrillation, cerebral infarction, and lung thromboembolism. A recent study identified a loss-of-function variation in *ZFHX3* as a novel cause of syndromic Intellectual Disability (ID). They found that the loss-of-function variation of *ZFHX3* consistently associates with (mild) ID and/or behavioral problems, postnatal growth retardation, feeding difficulties, and recognizable facial characteristics, including the rare occurrence of cleft palate [23]. Our present patients with variants of *ZFHX3* are similar to their cases. Unfortunately, we did not investigate pathological changes occurring in the central nervous system of A2530T knockout mice. Further studies on these effects are needed to make any conclusions regarding the association between *ZFHX3* and neuronal differentiation.

Conclusion

We found a new candidate disease-causing gene in 3 siblings who have Aarskog-Scott syndrome with mental retardation and megacolon. Mice with a frame shift mutation in *ZFHX3* were fetal lethal. However, mice with a knockout mutation in *ZFHX3* were viable and did not have any obvious neurological abnormalities. An association between the *ZFHX3* mutation and their symptoms was not clear.

Author Contributions

H.K. and N.S. designed the study; SS. and MY. Performed the exome analysis, TN. and YK. Collected the data, and HK. Wrote the manuscript; AT., TM., AA and NK. Performed the knockout mouse experiments, including the behavioral analysis. All authors read and approved the final manuscript. H.K. critically reviewed the manuscript and supervised the whole study process. All authors read and approved the final manuscript.

Acknowledgment

Not applicable.

Funding

This research received no specific grants from any funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. Informed consent was obtained for this publication.

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