A Missense Mutation in a Highly Conserved Alternate Exon of Dynamin-1 Causes Epilepsy in Fitful Mice

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Abstract

Dynamin-1 (Dnm1) encodes a large multimeric GTPase necessary for activity-dependent membrane recycling in neurons, including synaptic vesicle endocytosis. Mice heterozygous for a novel spontaneous Dnm1 mutation—fitful—experience recurrent seizures, and homozygotes have more debilitating, often lethal seizures in addition to severe ataxia and neurosensory deficits. Fitful is a missense mutation in an exon that defines the DNM1a isoform, leaving intact the alternatively spliced exon that encodes DNM1b. The expression of the corresponding alternate transcripts is developmentally regulated, with DNM1b expression highest during early neuronal development and DNM1a expression increasing postnatally with synaptic maturation. Mutant DNM1a does not efficiently self-assemble into higher order complexes known to be necessary for proper dynamin function, and it also interferes with endocytic recycling in cell culture. In mice, the mutation results in defective synaptic transmission characterized by a slower recovery from depression after trains of stimulation. The DNM1a and DNM1b isoform pair is highly conserved in vertebrate evolution, whereas invertebrates have only one isoform. We speculate that the emergence of more specialized forms of DNM1 may be important in organisms with complex neuronal function.

Introduction

Epilepsy affects about 1% of the population and approximately 30% of cases are idiopathic, with no obvious explanation such as head injury, stroke, lesions or tumors [1]. Genetic factors are thought to lie behind idiopathic epilepsy [1]. Several human epilepsy genes have been identified [2,3] but most genes for common idiopathic epilepsy remain unknown, in part due to the complex genetics. Employing a forward genetics approach, we have studied the mutant “fitful” mouse which exhibits spontaneous limbic and generalized tonic-clonic seizures upon routine handling, due to a spontaneous mutation in the gene encoding dynamin-1. Heterozygous fitful mice develop epilepsy by two to three months of age, but are otherwise outwardly normal. Homozygous mice have a more severe neurological phenotype at a much younger age - three weeks - including ataxia, hearing and vision defects and lethal seizures.

Dynamin-1 belongs to a family of large GTPases that function in endocytosis, vesicle scission, membrane recycling, organelle division, cytokinesis and antiviral activity [4–10]. In mammals, there are three dynamin genes (Dnm1, Dnm2, and Dnm3) each of which undergoes complex alternative splicing resulting in over 25 dynamin isoforms. Dnm2 is expressed in all tissues [11]. Dnm1 is expressed only in the brain, localizing to the presynaptic terminal [12,13]. Dnm3 is expressed in the brain (where it is associated with the postsynaptic compartment) and the testes [12,14]. Flies carrying mutant temperature-sensitive alleles of shibire, the Drosophila homolog of dynamin, exhibit paralysis at the restrictive temperature that is due to depletion of synaptic vesicles in a use-dependent manner [15].

Dynamin-1 has an established role in endocytic vesicle fission from the plasma membrane [16] and its expression is upregulated in the brain during postnatal development, concomitant with synaptogenesis. In primary neuronal culture, the expression and protein levels of dynamin-1 increase steadily in conjunction with the formation of neurites over time in culture, peaking as synapse formation occurs [9,12,17]. This expression pattern mimics that of other synaptic vesicle proteins such as synaptophysin and suggests a critical role for dynamin-1 in synaptic vesicle recycling based on its developmental expression pattern as well as localization to presynaptic compartments and known function in membrane recycling and endocytosis.

Dynamin molecules assemble into tetrameric structures that hydrolyse GTP to scission vesicle membrane, including synaptic vesicles which recycle after neurotransmitter release. Dynamin homologs are 100KD polypeptides containing 5 functional domains: a GTPase domain that binds and hydrolyzes GTP, a middle domain that is involved in oligomerization, a GTPase
Author Summary

Epilepsy, a group of chronic disorders characterized by recurrent seizures, results from abnormal, synchronized neuronal activity in the brain. The mouse represents a powerful system to study novel mutations that model neurological disease, including epilepsy. Here we describe a new mouse mutation ("fitful") in the gene encoding dynamin-1. Fitful mice have recurrent seizures and other neurological defects, including impaired hearing. Dynamin-1 is very well studied, but has yet to be linked to neurological disease. Dynamin-1 is a large multimeric enzyme that functions in membrane fission, primarily of vesicles after they release neurotransmitter at neuronal synapses. Fitful occurs in the region of dynamin-1 that is important for self-assembly of single dynamin subunits into the multimers required for enzymatic function. We show that fitful interferes with dynamin-1 self-assembly and with endocytosis. Moreover, the mutation resides in one of two alternate forms of dynamin-1 and affects what may be a necessary shift during brain development, with the expression of the mutated form being higher after maturation in fitful mice. This particular genetic specialization is unique to vertebrate dynamin. We speculate that specialized forms of dynamin-1 are important for modifying the self-assembly process to meet the demands of complex brain activity in higher organisms.

effector domain (GED) that is involved in oligomerization and stimulation of GTPase activity, and plextin-homology (PH) and proline-rich (PRD) domains that mediate lipid- and protein-protein interactions. In fitful mice, a single nucleotide change results in an amino acid substitution in the highly conserved GTPase domain of the protein. This domain is involved in the dimerization and higher order assembly of the dynamin tetramers [18–20]. Interestingly, mutations in the corresponding middle domain of dynamin-2 underlie human disease such as autosomal dominant centronuclear myopathy [21] and a canine mutation in Dnm1 located at the GTPase/middle domain border results in an exercise induced collapse disorder in Labrador retrievers [22].

Mice that completely lack Dnm1 survive into the second week of life demonstrating that Dnm1 is not necessary for embryonic development or for perinatal synaptic transmission [9]. In Dnm1 null mice, inhibitory neurons are more sensitive to the loss of Dnm1 and experience endocytic defects [9,10], but neither the homozygous nor the heterozygous Dnm1 null mice have seizures [9]. In cortical neuron cultures established from Dnm1 null mice, inhibitory neurons are shown to be the most sensitive to loss of Dnm1, acquiring a large build up of endocytic intermediates during spontaneous network activity [10]. Silencing transmission relieves the endocytic defect - suggesting that the high intrinsic level of tonic activity of these cortical inhibitory neurons makes them more vulnerable to the lack of Dnm1 [10].

Dnm1, as well as Dnm2 and Dnm3, is alternatively spliced at two locations in the transcript resulting in the possibility of nine Dnm1 isoforms. In this study, we show that the fitful mutation in Dnm1 interferes with the normal expression of the first alternatively spliced region during postnatal development. This region is located within the middle domain required for oligomerization, a process that is affected in fitful mutants.

Given that Dnm1 is essential for synaptic vesicle endocytosis, fitful mice are likely to have a lack of sufficient vesicles for extended synaptic transmission. This may be particularly problematic for the recycling of synaptic vesicles at tonically firing inhibitory synapses in light of findings in dynamin-1 null mice [9,10]. Disruption of this process would consequently upset the balance between inhibition and excitation resulting in abnormal propagation of neuronal excitability and recurrent seizures. Previous studies have extensively examined the function and mechanisms of action of Dnm1, but this study is the first to identify a Dnm1 mutation that leads to epilepsy. Furthermore, our study suggests that there are distinct roles for the alternatively spliced Dnm1 isoforms which have not previously been examined in vivo. The fitful mouse model provides a unique resource for understanding the role of the Dnm1 isoforms in brain as well as for studying the frequency-dependent weakening of synaptic inhibition as has been seen in other genetic models of generalized epilepsy, such as SCN1A [23,24].

Results

The “fitful” mouse as a model of generalized idiopathic epilepsy

The fitful mutation (allele symbol: Ftfl), arose spontaneously in C57BL/6J (B6) mice and was identified initially by the occurrence of recurrent, non-lethal seizures. The overall phenotype of Ftfl is semidominant. Heterozygotes develop partial and generalized tonic-clonic seizures upon routine handling from approximately two to three months of age (Figure 1A and 1D), but otherwise appear normal and have a lifespan similar to wildtype littermates. Heterozygotes have a modest reduction in seizure threshold to an acute electrical stimulus, about 0.25 mA lower than controls (Figure 1B). Although this effect is statistically significant, it is smaller than the difference (>0.5 mA) we have reported in other seizure-prone mice on the B6 background - some of which go on to develop epilepsy (Brunol4Ff [25], and others that do not (S2z2[26]). Nevertheless, heterozygous fitful mice develop kindled seizures much more readily than wildtype mice in response to repeated electrical stimuli, suggesting that they are more epileptogenic (Figure 1C).

Homozygous fitful mutants have more severe phenotypes, including ataxia and spontaneous convulsive seizures that usually result in death before weaning age (see Video S1). Homozygous pups are born with the expected Mendelian genotype ratio, but only survive into the second or third week of life depending on strain background. Mutant pups are viable and indistinguishable from their wildtype littermates at birth and for the first week of life. At approximately post-natal day 12 (P12), the mutants become discernible from wildtype as they develop a progressive ataxia characterized by an abnormal and uncoordinated stance and gait. Homozygotes show tonic-clonic seizures at P14–P16. A seizure episode typically lasts 30 seconds to one minute and is immediately followed by a clear diminishment in health and movement. Typically, mutants die before P18 either from a lethal seizure or lack of nourishment brought on by continued weakening of physical movement. Mutant pups that receive nutritional supplementation tend to survive about one to two days longer.

Routine histology showed no obvious brain abnormalities in homozygous mice (data not shown). However, when examined by immunofluorescence, Purkinje cell dendritic trees were markedly smaller in all homozygous mice examined at P17 (Figure 1E). In mutants, Purkinje cell dendrites were polydendritic and the size of the dendritic arbor and degree of branching was reduced compared to wildtype (Figure 1E, bottom panels). The wildtype Purkinje cell layer had more regular somas that are ordered in structure and dendrites that are noticeably smoother than the mutants that showed spiny dendrites along with a disorderly arrangement of soma.
Figure 1. **Fitful mice have seizures and neurosensory defects.** (A) Differential traces from EEG recording of fitful heterozygotes. Behavioral events associated with the EEG are shown. Vertical lines are 1s time markings; each channel is approximately 500μV high. (B) Average electroconvulsive thresholds to the minimal forebrain clonic seizure endpoint of B6-Ftfl/+ and B6-+/+ mice at two different stimulus durations (1.0s – 0.2s). (C) Average latency to first partial seizure. (D) Number of mice at first observed seizure age. (E) Images showing brain structures. (F) Auditory threshold and amplitude over time.
left; 0.2s – right). Female and male mice are shown separately. The combined data showed that this modest difference between genotypes in acute seizure threshold was statistically significant (Student’s t-test, p<0.001). (C) Average latency (# of daily tests) to the first kindled partial seizure following sequential stimulation in B6-/+ and B6-Ftfl/+ male mice (*Student’s t-test, p<0.0001). (D) Graph showing the age of onset of observed (behavioral tonic-clonic) seizures in fitful heterozygous mice in the B6 or FVB background. (E) Purkinje cell defect in fitful cerebellum. The upper panels show calbindin antibody staining of wildtype and fitful P15 cerebellum. Notice that the Purkinje cell dendrites are stunted (arrow) in the mutant as compared to wildtype and the soma are less ordered. The lower panels show B6.FVB fitful homozygous and heterozygous Purkinje cells expressing GFP at P17. (F) Auditory brainstem response in fitful and wildtype mice. Left panel: ABR audiograms with average thresholds ± SEM of fitful mouse mutants (open symbols, —/—, n = 14) and their wildtype (n = 5, +/+ ) and heterozygote (n = 9, +/—) littermates (closed symbols). Right panel: average ABR waveforms (±SEM) of fitful (light grey, n = 9) and wildtype/heterozygous mice (dark grey, n = 9) in response to click stimuli (86 dB, peak equivalent). Latin numbers demote the ABR peaks.

doi:10.1371/journal.pgen.1001046.g001

Hearing impairment in fitful homoygotes

We suspected that the homozygotes might have neurosensory defects. Given their lethality at three weeks of age, hearing was the most practical sense to test in detail. To examine hearing, we evaluated the auditory brainstem response (ABR) of P14–17 fitful homozygous mice and compared with wildtype and heterozygous littermates. Fitful homoygotes display a modest ABR threshold increase, approximately 15 dB, across all sound frequencies tested (Figure 1F, left panel). We also found that the latencies of most ABR peaks were progressively prolonged (Figure 1F, right panel) and the amplitude of some of the ABR peaks was reduced in the mutants. The first peak (peak I) was reduced and delayed reflecting impaired function of the inner hair cell-afferent synapse and a reduction in synchronous spiral ganglion activation [27,28]. To rule out a defect in outer hair cell function, we measured distortion product otoacoustic emissions. This showed intact outer hair cell function in fitful homoygotes (Figure S1). Thus, the hearing impairment is most likely due to a deficit in sound coding at the inner hair cell synapse that could be due to a lack of a rapid resupply of readily-releasable vesicles [29].

The Ftfl mutation resides in an alternative exon of Dnm1

Whole genome scans were done to map both the dominant seizure phenotype, and the recessive ataxia phenotype, to Chromosome 2. Further fine mapping narrowed the region to two Mb including the Dnm1 gene and several other candidate genes with high expression in the brain: Mapkap1, Fibd1, Pgapd1, and Stxbl1. Expression analysis of these genes did not reveal any differences between wildtype and mutant brain RNA, and further cDNA sequencing of Stxbl1 specifically revealed no mutations. In Dnm1 a single nucleotide change was discovered by sequence analysis of mutant cDNA, revealing a G-to-A nucleotide mutation at nucleotide 2284 (Figure 2A). This particular alternative exon pair of Dnm1 and Dnm2 is conserved in all vertebrates including mice (e.g. Dnm1ax and Dnm1bx) and humans (Dnm1 and Dnm1 “alternative”), but it is not present in Dnm3 nor is it in invertebrate dynamin (Figure 2A–2C; [30,31], reviewed in [32]; see Figure 2B legend for more details). The fitful missense mutation results in an alanine to threonine substitution at amino acid 408, an evolutionarily conserved residue (Figure 2A and 2B). Noticeably, fitful only affects the Dnm1ax isoform sequences; the “a” exon is spliced out in the Dnm1bx forms, resulting in potentially all three Dnm1 transcripts being altered.

To provide genetic confirmation that the Ffil phenotype is caused by the missense mutation in Dnm1, we crossed heterozygous Dnm1Ftfl mice to mice heterozygous for the null mutation Dnm1tm1Pdc; [9]). Compound heterozygous Dnm1tm1Pdc/Dnm1Ftfl pups were born very near to the expected Mendelian ratio of 1:4 (Figure 3). We observed 80 compound heterozygous out of 46 litters having a total of 334 pups. The average litter size was 6–8 pups depending on background strain. The phenotype of the resulting Dnm1tm1Pdc/Dnm1Ftfl compound heterozygous mice was in many ways similar to that of Ffil homozygotes, including seizures by the third week of life and death before weaning. We observed 15 compound heterozygous mice to have seizures during their shortened lifespan, while no such seizures were observed in wildtype, or deleted or fitful heterozygous littermates at this age (Figure 3). There were four confirmed deaths due to seizures, with three more suspected by virtue of finding carcasses with hindlimb or forelimb tonic extension. Although the onset and appearance of lethal seizures was very similar between fitful homozygotes and compound heterozygotes, these two classes had different locomotor phenotypes; the compound heterozygotes were not ataxic, although they had a slight tremor and hunched appearance that continually increased in severity. Together with the mapping and mutation analysis, these results provide strong evidence that fitful is an allele of Dnm1. They also reinforce the suggestion that wildtype Dnm1 is necessary for normal postnatal neurodevelopment.

Fitful alters the developmental expression of Dnm1 isoforms at RNA and protein level

Dynamin-1 increases in abundance during early postnatal brain development [9,12]. We examined the expression of Dnm1ax and Dnm1bx during brain development in mutants. cDNA amplification using a single set of primers for both isoforms, followed by restriction with HpaI which has a recognition site present only in exon 10b, allowing for direct visualization of the relative expression of exon 10a and exon 10b containing isoforms. From E17.5 through P14, the overall increase in Dnm1 is due to isoforms containing exon 10a, while isoforms containing exon 10b either remain constant or diminish slightly with age (Figure 4A). Interestingly, in homoygous mutant, there was a delay in this shift; isoforms containing exon 10b remained upregulated longer (compare at P0) during development. This was significant as P0 through P14 is the period of extensive synaptic maturation and regulated endocytosis. Quantification of exon 10b-containing isoforms relative to the total amount of Dnm1 mRNA in whole brain was calculated and showed a clear trend suggesting that Dnm1bx is more prominent in mutants than wildtype throughout development (Figure 4B, left panel). This shift was confirmed by quantification of relative isoform transcript levels using pyrosequencing (Figure 4B, right panel) and did not result in a significant change in overall Dnm1 transcript abundance.

The alteration in isoform expression was confirmed and extended at the protein level using isoform-specific antibodies
Figure 2. Conserved protein sequence alignment of Dynamins. (A) Conserved protein sequence alignment. The upper diagram shows DNM1 sequence conservation among various species and the location of the fitful mutation within the middle domain of the protein. The first splice region (amino acids 399–444) shows the location of the fitful mutation (*408) and the conservation within mammalian, fly and worm dynamins. Notice that
the fly and worm orthologs do not have splice variants in this region. The second alternative splicing region is also shown at the end of the PRD domain, GTPase domain, middle, middle domain, PH, pleckstrin homology domain, GED, GTPase effector domain, PRD, proline rich domain. The bottom diagram depicts the mutually exclusive alternative splicing of the Dnm1a and Dnm1b isoforms. Putative orthologues of mouse Dnm1, Dnm2, Dnm3 and Dpr (dynamin related protein – official mouse gene symbol, Dnm1b) from different species. For simplicity, the same generic gene symbol is used for all; for simpler eukaryotes, the symbol Dnm refers to their orthologue(s) most like the mammalian Dnm1, Dnm2 or Dnm3. The known or predicted alternate sequences corresponding to mouse Dnm1 exon 10 are also shown. The underlined amino acid symbols show where mouse Dnm1b differs from Dnm1a. The arrow at the bottom shows the highly conserved alanine residue that is mutated to threonine in the mouse Dnm1b allele. Residues colored in red show amino acid substitutions with respect to mouse Dnm1; for nematode (C.b. - C. briggsae; C.e. - c. elegans), differences between the two Dnm peptides are shown in blue. Peptide sequences were obtained as follows: mouse Dnm1a: GenBank AAA37318, Dnm1b: GenBank EDL03539; human Dnm1a: GenBank AAA20804, Dnm1b: GenBank AAA827228; fruit fly (D. melanogaster) EMBL CAA42068. The remaining predicted sequences were obtained from analysis using the USCS Genome Browser and draft genome sequence assemblies from the following respective genome centers: chicken (G. gallus), sea lamprey (P. marinus) and nematode (C. briggsae) - Genome Sequencing Center, Washington University School of Medicine; opposum (M. domestica) and lizard (A. carolinensis), The Broad Institute; xenopus (X. tropicalis), lancelet (B. ro白马gus), and sea squirt (C. intestinalis) - DoE Joint Genome Institute; sea urchin (S. purpuratus) - Baylor College of Medicine Human Genome Sequencing Center. The dynamin gene composition from sea lamprey was inferred from sequence alignment of draft 5.9-fold genome sequence (accessed via the UCSC browser - genome.ucsc.edu). When used as query, the respective mouse Dnm1, Dnm2 and Dnm3 peptide sequences corresponding to the assembly domain region each yielded significant alignments with only a single, approximately 20kb contig (Contig16000). This contig contained seven Dnm-like exons, with appropriate splice site recognition motifs, that was co-linear with mouse Dnm1. Proportional number of differences is estimated at the bottom. Note the closer relationship between the two isoforms from sea lamprey and the respective Dnm1a and Dnm1b branches from more complex vertebrates. Also note the closer relationship between Dnm2 isoforms and invertebrate dynamin.

\[\text{doi:10.1371/journal.pgen.1001046.g002}\]

(see Figure S3 for details). Figure 4C shows one of three replicate western blots demonstrating a decrease in protein containing exon 10a peptides (DNM1a) at each age examined in mutants as compared to wildtype. There is a parallel increase corresponding to exon 10b containing peptides (DNM1b). Least square regression analysis with age, genotype and replicates as independent variables revealed a significant (p<0.05) increase in the expression of DNM1b in mutant animals after two weeks of age. These results suggest the possibility that altered dynamin-1 isoform composition during this important period of synaptic maturation may contribute to the disease phenotypes.

**DMN1 protein assembly defect in fitful mice**

The fitful mutation resides within the “middle” domain of dynamin (Figure 2A), previously shown to be required for self-assembly into dimers and further higher-order assembly into tetrameric structures [18–20]. We examined the ability of DNM1Ftfl to properly assemble into dimers and further order oligomers using protein extracts from normal and fitful brain.

**Figure 3. Fitful and compound heterozygous Dnm1 seizure and locomotor phenotypes.** Shown is the frequency of fitful homogygous (Dnm1Ftfl/Dnm1Ftfl) and compound heterozygous (Dnm1Ftfl/Dnm1tm1Pdc) mutant mice and their respective seizure incidence and locomotor phenotypes. The fitful homogyzotes shown were from fully informative matings from the mapping cross used to map the recessive phenotype; the compound heterozygotes were from crosses between single heterozygotes from respective FVB-fitful and B6-null colonies. The asterisk is used to indicate that the latter population was observed almost daily from P12 through demise (P16–P23), whereas the former were observed at weaning age only – suggesting that death occurred in these mutants between P12 and P21. doi:10.1371/journal.pgen.1001046.g003

Protein extracts were incubated with a cross-linking agent, 1-ethyl-3-[3-dimethylaminopropyl]carboodimide hydrochloride (EDC), of zero length, to bind proteins intimately associated in dimer or tetramer formation [33]. Equal amounts of extract were treated with or without EDC and separated by SDS-PAGE. Protein from wildtype brains exhibited the ability to form dimers and trimers in the presence of cross-linker (Figure 5A, left panel). Over half the DNM1 protein detected was assembled, with 28% in tetrameric form and 36% in dimeric form. While the homozygous fitful brain extract appeared to contain DNM1 dimers upon the addition of EDC, the amount of higher order structures (trimers in this assay) was reduced with only 9% of detected DNM1 in tetrameric form (Figure 5A, left). This result indicates a defect in the ability of DNM1Ftfl to form multimeric DNM1 complexes.

The dimer band in the mutant extracts, which was 45% of the DNM1 signal, was possible due to the assembly of normal DNM1b. To explore this further, we analyzed the ability of exogenous DNM1 isoforms to self-assemble in cells. We made isoform-specific constructs that express GFP fused to the C-terminal tail of DNM1a or DNM1b, and used GFP or HA antibodies, we observed that the mutant DNM1Ftfl protein can dimerize with each wildtype isoform (Figure 5B), albeit to a lesser extent than wildtype DNM1a.

**DNM1Ftfl interferes with endocytic trafficking in cells**

Dynamins are catalytic for endocytosis. Extensive previous studies introducing various dynamin mutant constructs into mammalian cells have demonstrated that overexpression of mutant dynamin blocks endocytosis and causes an accumulation of endocytic intermediates [52]. Several of these mutations (e.g.
K44A) are dominant-negative, binding to endogenous dynamin and preventing it from properly functioning [4,16]. Specifically, dynamin GTPase domain mutations that inhibit endocytosis when expressed in COS-7 cells have a reticular type of localization as opposed to the more diffuse cytoplasmic localization of wildtype dynamin [34]. In prior studies to more closely examine these opposed to the more diffuse cytoplasmic localization of wildtype dynamin, these results suggest that unlike wildtype DNMIa, DNMIaFtfl cannot support endocytosis in COS-7 cells. Furthermore, DNMIaFtfl seems to act in a dominant-negative manner, interfering with the function of endogenous DNMI2 - the dynamin that normally carries out these functions in COS-7 cells - similar to previously described dynamin GTPase mutants that are not able to catalyze the fission of membrane and inhibit endocytosis [34]. Dominant-negative effects are currently thought to be due to hetero-oligomerization of the mutant oligomer with endogenous dynamin oligomers, perturbing normal function.
Prolonged inhibitory synaptic activity in cerebral cortex slices

We examined synaptic properties of cortical neurons in acute slices for evidence of abnormalities in fitful homozygotes. We focused on GABAergic transmission because recent studies using neuronal cell culture demonstrated that the loss of Dnm1 preferentially affects inhibition [9]. First, we recorded quantal GABAergic IPSCs (mIPSCs) in layer V pyramidal neurons in the somatosensory cortex at P14–15. Cells with comparable series resistance were analyzed (9.3±0.5 MW, n = 21 cells from three wildtype mice; 9.3±0.5 MW, n = 22 cells from three mutant mice). Samples of GABAergic mIPSCs recorded from a wildtype (upper trace) and a fitful (lower trace) layer V pyramidal neuron are shown in Figure 7A. Fitful neurons demonstrated more prolonged quantal events than those in wildtype mice (Figure 7B). The mean decay time constant was 3.2±0.2 ms (n = 22) for mutant cells, and 4.1±0.2 ms (n = 21) for wildtype (p<0.005, Mann-Whitney test). Quantal events in mutant also had slower rise time. The mean rise time (from 20 to 80% of the peak) was 0.49±0.01 ms for mutant, and 0.42±0.01 ms for wildtype, which was significantly different. There were no significant differences between mutant and wildtype in the frequency (10.8±0.8 Hz vs. 10.9±0.8 Hz; p>0.8) or peak amplitude of mIPSCs (42.9±3.8 pA vs. 34.5±3.2 pA; p>0.06; data not shown).

To determine whether the fitful mutation leads to a deficit in vesicle membrane recycling, we recorded monosynaptic GABAergic responses by applying current pulses through stimulation electrodes placed in layer V. There was no significant difference between mutant and wildtype cells in the maximum IPSC (1254±113 pA, n = ten cells from three mutant mice; 1209±132 pA, n = ten cells from three wildtype mice; p>0.5; data not shown). Trains of 10-Hz stimulation were applied at intensities that achieve 40 to 60% of the maximum response. As illustrated in Figure 7E and 7F, the amplitude of IPSCs decreased more rapidly in mutant than wildtype cells during 10-Hz stimulations. The recovery was tested by applying stimuli at 0.1 Hz immediately following 1000 stimuli at 10 Hz. The recovery of IPSCs was much slower in mutant than wildtype cells (Figure 7G; n = nine cells from two mutant mice; n = eight cells from two wildtype mice). The depression and the reduced rate of recovery in IPSC amplitude were affected in fitful, similar to the results in Dnm1 null primary neuron culture [9]. These differences in evoked GABAergic response are consistent with a likely deficit in vesicle membrane recycling in mutant cells and suggests that the mutation in dynamin-1 interferes with sustained evoked release.

Discussion

Fitful mutant mice reveal intriguing differences between underlying dynamin-1 mutations and the respective phenotypes. Alternative splicing in the molecular assembly-associated domain of dynamin-1 appears to play a key role in these phenotypes and in the proper function of dynamin-1. Fitful mice have a complex neurological disorder in which epilepsy is a major and consistent feature. Adult heterozygotes have recurrent limbic and tonic-clonic seizures, a modestly reduced seizure threshold, and a predisposition for becoming epileptic, but no other obvious phenotypic abnormalities. This dominant phenotype provides a new model for common idiopathic generalized epilepsy (IGE), for which few causative genes are known and progress is slowed by the complex genetic architecture underlying this disorder. In contrast, fitful homozygotes have multiple neurological deficits by three weeks of age, including delayed growth, cerebellar ataxia, hearing loss, and severe, ultimately lethal, tonic-clonic seizures. Fitful creates an amino acid substitution in the highly conserved...
DNM1ax isoform encoded by Dnm1 exon 10, leaving DNM1bx structurally intact. While DNM1a^Ftfl protein is expressed in mutant mice, it does not efficiently assemble into higher order oligomeric dynamin complexes. In addition, similar to previously described dominant-negative alleles of Dnm1, characterized only in vitro (e.g. K44A; [4]), DNM1a^Ftfl also interferes with normal endocytosis in COS-7 cells.

Our heterologous expression experiments provide insight into the biochemical consequences of the DNM1a^Ftfl mutation, with its dominant-negative effect on endocytosis evidenced by a lack of transferrin uptake and subsequent proper trafficking. We can envision three ways in which this effect is manifest. First, sequestration of interacting proteins necessary for endocytosis could reduce the pool available for endocytosis. Preliminary, however, we have found no evidence for altered abundance of several known dynamin-associated proteins, such as amphiphysin I and auxilin, in fitful homozygotes (our unpublished results). Second, assembly of non-functional DNM2 and DNM1a^Ftfl heterodimers could inhibit proper DNM2 function. This remains a possibility as we find that DNM1a can interact with DNM2 to some extent, both in COS-7 cells and also in brain (unpublished data), however we find no evidence of colocalization by immunofluorescence (unpublished data). We do find evidence for heterodimerization of DNM1a^Ftfl with each of the wildtype dynamin-1 isoforms (Figure 5B), suggesting a dominant negative effect whereby mutant protein binds with wildtype protein resulting in non-functional heterodimers that cannot fission membrane.

Third, the accumulation of unassembled DNM1a^Ftfl at sites of fission could stall endocytosis. This is an attractive possibility for fitful mice. The appearance of tubules in cells that overexpress DNM1a^Ftfl is reminiscent of previous dynamin mutations [34] and narrow tubules were also observed in dynamin-1 null neurons [9]. While tubules may represent a buildup of invaginating membrane that cannot be fissioned, they could alternatively result from stalled clathrin-mediated endocytosis at the invagination stage (a stage at which dynamin is thought to be a checkpoint; [35]). Membrane invagination and fission are mediated by two separate mechanisms carried out by dynamin [7] and both assembly-independent GTPase and assembly-stimulated GTPase activities are needed to carry out these process [35–37]. The assembly/disassembly cycle is required for association and dissociation with the membrane.

Figure 7. Altered GABAergic transmission in cortical neurons of fitful mice. (A) Samples of GABAergic mIPSCs recorded from a wildtype (upper trace) and a fitful (lower trace) layer V pyramidal neuron at P14 in the presence of DNQX, kynurenic acid, and TTX. The patch pipettes contained 130 mM KCl, and holding potential was at −70 mV. (B) Averaged mIPSCs from 21 wildtype cells (grey) and 22 fitful cells (red) with normalized peaks. (C,D) Histograms of the decay constant (C) and rise time (D) of mIPSCs for wildtype and fitful cells. The mean decay time constant was 5.2±0.2 ms (n=22) for mutant cells, and 4.1±0.2 ms (n=21) for wildtype (p<0.005, Mann-Whitney test). (E) Samples of evoked GABAergic IPSCs in response to 10-Hz stimulation recorded from a wildtype (upper black traces) and a fitful (lower red traces) cell at P14 in the presence of DNQX and kynurenic acid. For both cells, the three traces were responses to the first, 100th, and 500th stimulus. The patch pipettes contained 110 mM cesium methylsulfate, and holding potential was at 0 mV. A pair of twisted microwire was placed in layer V to evoke monosynaptic IPSCs. (F) Plots of evoked IPSCs in response to 400 stimuli at 10 Hz. For each cell, peak amplitudes of IPSCs were normalized to that of the baseline response. (G) Recovery after 1000 stimuli at 10 Hz. IPSCs were measured every 10 s after the 10-Hz stimulation. Peak amplitudes of IPSCs were normalized to that of the baseline response before the 10-Hz train stimulation.

doi:10.1371/journal.pgen.1001046.g007
Self-assembly defective DNM1aFtfl would presumably not have the assembly-stimulated activity required for fission, but would retain the assembly-independent activity necessary for membrane invagination. Furthermore, unassembled dynamin has been proposed to be the molecular checkpoint during early stages of clathrin-mediated endocytosis, potentially allowing the abortion or progression of clathrin-coated particle (CCP) intermediates through maturation [35,37]. Dynamin assembly is suggested to control the transition from early to late events in CCP maturation [37]. Such a model would imply that unassembled dynamin can monitor CCP formation and is a separate activity from membrane fission as catalyzed by assembled dynamin. Our data showing that unassembled DNM1aFtfl can tubulate membranes but cannot fuse them are consistent with this hypothesis.

We speculate that the seizure phenotype in fitful but not in Dnm1-null heterozygotes may result from the unique presence of mutant DNM1aFtfl stalling such an endocytic checkpoint. This would also provide an explanation for the reduced phenotype severity of compound heterozygous mice as compared with homozygous fitful (e.g. Figure 3A); homozygous fitful would have up to twice the amount of abnormal protein, accumulating and exerting its negative effect with a quicker timecourse. However, even the compound heterozygotes ultimately succumb to lethal seizures. This dominant effect of DNM1aFtfl presumably leads to a lack of sufficient vesicles for extended inhibitory transmission — particularly problematic for tonically firing inhibitory synapses [9,10].

The genetic and phenotypic differences between fitful and Dnm1-null mice - in particular, epilepsy in fitful heterozygotes but not in Dnm1 null mice of either genotype [9]; P. de Camilli, personal comm.; our unpublished observations) – further suggests that DNM1aFtfl protein confers unique properties relevant for disease. We examined both spontaneous and evoked mIPSCs in layer V pyramidal neurons in acute brain slices. The peak amplitude of spontaneous or mIPSCs was not significantly different between fitful mutant and wildtype mice. This is in contrast with the results obtained in Dnm1-null cortical cultures where Dnm1-null neurons show a large increase in the peak amplitude of mIPSCs [9]. The reason for this difference is unknown. Dnm1-null neurons have larger diameter synaptic vesicles, which may lead to an increase in quantal content. This increase in vesicle size observed in Dnm1-null neurons may be caused by direct or compensatory mechanisms. It is possible that the presence of the mutated dynamin-1 in fitful mice attenuates compensatory responses. Another possibility is that fitful causes changes in the cable (electrical) properties of neurons or the location of GABAergic synapses, so that recordings from the soma are unable to detect changes in mIPSCs at the dendrites. Indeed, the slower kinetics of mIPSCs observed in fitful neurons is consistent with this possibility.

However, the evoked mIPSC responses in fitful acute slice preparations were similar to those shown previously in Dnm1-null primary neurons [9], reflecting a loss-of-function. Compared with wildtype, both alleles confer a more rapid depression of evoked mIPSCs in response to 10-Hz stimulation, and a much slower recovery from depression. These findings, together with recent results obtained using a selective blocker of dynamin [38], demonstrate a critical role of dynamin in synaptic vesicle recycling. During repetitive stimulation, neurotransmission relies on the rapid reuse of synaptic vesicles endocytosed in a dynamin-dependent manner [9,38]. Without efficient recovery of these rapidly reused vesicles, there is very likely a frequency-dependent exhaustion of synaptic inhibition, such a seen in other models of generalized epilepsy [23,24].

The inability of DNM1aFtfl to assemble efficiently into oligomers is a plausible explanation for the biochemical defect, but to fully understand the development of disease, it may be important to consider functional differences between the two isoforms. Despite the high degree of conservation, functional differences conferred by the respective protein isoforms have not been demonstrated in vivo, but insight has been gained from heterologous systems [39,40]. The alternate splice in exon 10 that occurs in Dnm1 and Dnm2, but not Dnm3 or in the dynamin-related protein Dph, has been recognized previously. We observe that this diversification is present in all jawed vertebrates and is even present in sea lamprey - a primitive, jawless freshwater fish – which, despite having these alternate exons, unlike other vertebrates possesses only a single dynamin gene (Figure 2B). Furthermore, neighbor-joining best tree analysis suggests that vertebrate dynamins are closer to DNM1b (and DNM2), whereas DNM1a forms a more isolated phylogenetic group (Figure 2C). The divergent exon 10a may well be a specialization that is unique to vertebrates.

The developmental shift in isoform expression may also provide clues into function. DNM1b expression is highest during embryonic and early postnatal development and decreases with synaptogenesis as DNM1a expression increases (Figure 4A and 4C). This may be to accommodate changing requirements for endocytosis over the course of development. At the onset of synaptogenesis, basal endocytosis is down-regulated and stimulation-induced endocytosis takes over as the major form of SV recycling [41]. If one form of endocytosis relies more heavily on a specific DNM1 isoform, then the altered isoform expression in fitful may disrupt the changes in endocytosis necessary for maturation, resulting in a number of adverse consequences. Early endocytosis is required for many vital processes such as growth cone development, cellular migration, axonal arborization and dendritic branching. It is possible that the significantly stunted Purkinje cell arborization observed in the homozygous fitful mutants is one consequence of an early developmental abnormality. Alternatively, inappropriate expression of DNM1b in mature fitful neurons that should predominately express DNM1a, may adversely affect the kinetics of endocytosis, in a manner suggested above.

It is intriguing that a missense mutation in a discrete splice variant of Dnm1 can lead to significant neurological disease phenotypes. We speculate that Dnm1 isoforms confer specificity to both the developmental program of endocytosis and also to variant endocytic response to stimuli (e.g. clathrin-mediated vs. activity-dependent bulk endocytosis). In the future, studies using conditional isoform-specific expression will help to distinguish these possibilities.

## Materials and Methods

### Ethics statement

All animal procedures followed Association for Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by Institutional Animal Care and Use Committee.

### Mice

The fitful mice arose at The Jackson Laboratory (Bar Harbor, ME) as a spontaneous mutation on the C57BL/6j inbred strain in 2000. B6.129s1-Dnm1tm1PDC mice were a gift from P. De Camilli [9]. To generate GFP mice used for cerebellar histology, fitful heterozygotes were mated to a B6 mouse strain carrying a transgene with the parvalbumin promoter fused to the GFP gene (strain # B20; [42]). All strains were housed in the Research...
Animal Facility at The Jackson Laboratory where animal procedures were approved by the ACUC.

Genome scans
Two separate genome scans were used to map fitful to mouse Chromosome 2. To map the dominant seizure phenotype, B6-Ftfl heterozygotes were crossed to FVB/NJ mice. Two (of six total) resultant F1 hybrids were noted to have spontaneous limbic and tonic-clonic seizures after two months of age upon routine handling and weekly cage changes. The F1 hybrids were backcrossed to FVB/NJ mice, and the resultant N2 progeny were aged and observed at least weekly for seizures. Genomic tail DNA was prepared from 79 backcross mice, 22 of which showed at least two limbic and tonic-clonic behavioral seizures, and a genome scan was performed with seizure occurrence as a binary trait using microsatellite markers. The mutation was provisionally mapped to proximal Chromosome 2 and the critical interval for the recessive phenotype was ultimately refined to the genomic region between D2Mit152 and D2Mit203 after typing a total of 487 F2, F3 and F4 progeny.

Independently, the recessive ataxia and early onset seizure phenotypes were mapped after crossing B6-Ftfl heterozygotes to CAST/Ei, and subsequently crossing the F1 hybrids inter se. Provisional linkage to Chromosome 2 was established in 20 affected mice, and the critical interval for the recessive phenotype was ultimately refined to the genomic region between D2Mit80 and D2Mit72 after typing a total of 990 F2, F3 and F4 progeny.

Genotyping the Dnm1Ftfl allele
Genotyping Dnm1Ftfl (fitful) was done by PCR using the primers: Dnm1 MwoI F2 (5'-GGGACCGCGCCTCTTCACACCTG-3') and Dnm1 MwoI R (5'-GGGCCCTACCTTTTCTCATA-3'). The PCR product was digested for 2 hours at 60°C with the restriction enzyme MwoI (NEB). The digestion products were confirmed by direct sequencing. Primers used to amplify Dnm1 sequences were: Dnm1ex9F (5'-GAACTGCGAAGGGAGATCAG-3') and Dnm1ex12R (5'-GGGTGCAAAATTTCGCTCAGTCT-3') corresponding to Dnm1 exon 9 forward and exon 12 reverse, respectively. The PCR amplifications from three pairs of age-matched mice were run in triplicate. The PCR products were digested with the HphI restriction enzyme (NEB) overnight at 37°C and examined on a 2% agarose gel.

Pyrosequencing
Pyrosequencing of cDNA from wildtype, heterozygous and fitful P15 brains was carried out by the Transgenic Genotyping Services facility at The Jackson Laboratory. PCR amplification of Dnm1 was done with an initial denaturation step of 94°C for 5 min, followed by 50 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 10 s, and extension at 65°C for 30 s. Final termination of the elongation step was carried out at 65°C for 5 min. The sequences of all of the primers are listed below. The biotinylated PCR products were prepared for pyrosequencing analysis by the use of a Vacuum Pre Workstation (Biotage AB), and the sequencing reactions were carried out on a PSQ 96MA system (Biotage AB) as described by the manufacturer. Sequence analysis software (Biotage AB) was used for measurement of the peak heights. Primers:
- pMR220F1, AGCTATGCTATCAAAAATATCCA; pMR221R1, Biotin ACCATGTCACACACTCTGA; pMR222S1b, CTCTTTCGCCCAGACCAG; pMR223S1a, CTCTTCGGTACCGTACCTC.

Western blot
Protein extracts were made in IP lysis buffer (20mM Tris, 0.5mM EDTA, 100mM NaCl, 0.5% NP-40) with Complete-mini protease inhibitor mix (Roche) added fresh. Extracts were quantified using the Bradford reagent (Bio-Rad). Extracts (50–100µg protein) were diluted in Laemmli buffer, incubated at 95°C for 5 minutes, resolved by SDS-PAGE and transferred to nitrocellulose membrane. All membrane blotting steps were carried out in TBS plus Tween (TBST) with 5% non-fat dry milk. Blots were incubated at RT with primary antibody (for specific dilutions see below) for 1–2h, HRP-conjugated secondary antibody (1:5000) for 1h and visualized with the ECL plus kit (VWR Scientific). Membranes were incubated with Restore Western blot stripping buffer (Fisher) at 37°C for 15 min while shaking to remove antibodies for subsequent hybridization. Primary antibodies used were dynamin-1 (1:1000; Affinity BioReagents, PAI-660), dynamin-1 (1:500; Chemicon, MAB5402), dynamin 2 (1:200; Santa Cruz Biotechnology, sc-6400), dynamin 2 (1:250; BD Biosciences, 61025), dynamin 3 (1:1000; Affinity BioReagents, PAI-662), dynamin-1a (1:500; Affinity BioReagents), dynamin-1b (1:1000; Affinity BioReagents).
Secondary antibodies used were HRP anti-goat (1:5000; Santa Cruz Biotechnology), HRP anti-mouse (1:5000; Thermo Scientific), HRP anti-rabbit (1:5000; BioRad) and HRP anti-chicken (1:5000; Santa Cruz).

Oligomerization

Protein extracts made with IP lysis buffer were used for oligomerization assays. 50 or 100 μg of protein was incubated with 0 or 20 mM EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride; Pierce) in a final constant volume between samples for 45 minutes at RT in the dark. Samples were then diluted in Laemmli buffer, incubated at 95°C for 5 minutes, resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed as for Western blots (see above).

Immunofluorescence microscopy

For cryosection staining, sections were fixed with 4% paraformaldehyde, 4% sucrose in PBS for 15 minutes at RT. Slides were washed 3 times with PBS and blocked and permeabilized in PBS, 3% BSA and 0.5% Triton-X. The slides were incubated with primary antibody (anti-Calbindin; Swant, CB38) diluted in block overnight at 4°C, washed three times with PBS-T and incubated with secondary antibodies diluted in block for 1 hour at RT. Cells were washed three times with PBS, stained for 5 minutes with DAPI, washed and mounted with GelMount (Biomeda).

To analyze the GFP-Purkinje cells, brains from F2 homozygous fitful and controls were dissected at 17 days of age for fixation in Z-fix buffer (Anatech, Battle Creek, MI, USA) for 3–4 h. Vibratome sagittal sections (60–100 μm) were mounted onto slides with Clear-mount (Zymed, San Francisco, CA, USA). The sections were viewed using a Leica SP5 AOBS spectral confocal microscope, with a 63×, 1.3 NA glycerol immersion objective. Excitation was at 488 nm, with emission collection optimized to detect green fluorescence.

Electroconvulsive testing

For electroconvulsive testing, we followed procedures described previously with some modifications [43,44]. Briefly, mice were anesthetized with tribromoethanol (250 mg/kg, i.p.) and decapitated. Brains were quickly removed and transferred into ice-cold solution containing (in mM): 210 sucrose, 3.0 KCl, 1.0 CaCl₂, 3.0 MgSO₄, 1.0 NaH₂PO₄, 26 NaHCO₃, 10 glucose, saturated with 95% O₂ and 5% CO₂. Coronal slices were cut at 300 μm on a vibratome (VT 1000s, Leica) and kept in artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 3.0 KCl, 1.5 CaCl₂, 1.3 MgSO₄, 1.0 NaH₂PO₄, 26 NaHCO₃, and 20 glucose, saturated with 95% O₂ and 5% CO₂ at room temperature (21–23°C). Slices were allowed to recover for at least 1 hr before any recording. Recordings were made at 32–34°C using whole-cell patch-clamp techniques. Each slice was transferred to a submerge-type chamber where it was continuously exposed to ACSF heated to 32–34°C, saturated with 95% O₂ and 5% CO₂, and flowing at a rate of 2.0±0.2 ml/min. Whole-cell patch clamp recordings were made at the soma of layer 5 pyramidal neurons of the somatosensory cortex using a 40× water immersion objective (40×/0.80W, Nikon) and infrared Nomarski optics. Patch pipettes were pulled from thick wall borosilicate glass (1.5/0.84 mm, WPI) on a horizontal puller (P-97, Sutter Instruments). Resistance of electrodes was between 2 and 4 MΩ. Liquid junction potential was not corrected. Seal resistance was greater than 2 GΩ. Recordings were made with a Multiclamp 700B amplifier (Molecular Devices). The series resistance (Rs), usually between 8 and 14 MΩ, was monitored throughout the recording, and data were discarded when Rs varied by 20% or more over the course of the recording. GABAergic IPSCs were selectively recorded by blocking ionotropic glutamate receptors with 20 μM DNQX (6,7-dinitro-quinoxaline-2,3-dione) and 1 mM kynurenic acid. For evoked IPSCs, the pipette solution contained (in mM): 110 Cs methylsulfate, 20 TEA-Cl, 15 CsCl, 4 ATP-Mg, 0.3 GTP-Na, 4 QX-314, 0.5 EGTA, and 20 HEPES (pH 7.2, 270–280 mOsm). A pair of twisted nichrome microwires (38 mm in diameter, A-M Systems) were placed in layer V about 200 mm away from the recorded neuron. IPSCs were evoked by current pulses (20–400 μA, 50 ms). For mIPSC recording, the pipette solution contained (in mM): 130 KCl, 4 ATP-Mg, 0.3 GTP-Na, 0.5 EGTA, and 20 HEPES (pH 7.3, 270–280 mOsm with sucrose). Quantal events were recorded in the presence of tetrodotoxin (TTX, 0.4 μM). DNQX and QX-314 were obtained from Tocris; all other chemicals were obtained from Sigma-Aldrich USA.

Auditory brainstem response

Experiments were performed as described in [46], complied with national animal care guidelines and were approved by the University of Goettingen Board for animal welfare and the animal welfare office of the state of Lower Saxony. In brief, animals were anaesthetized intraperitoneally with a combination of ketamine (125 mg/kg) and xylazin (2.5 mg/kg) and the core temperature was maintained constant at 37°C. For stimulus generation, and data acquisition we used the TDT III Systems (Tucker-Davis Technologies, Ft Lauderdale, FL) run by BioSig12 software (TDT). Tone bursts (4/0/12/16/24/32 kHz, 10 ms plateau, 1 ms cos’ rise/fall, calibrated and provided in dB SPL) were applied at 20 Hz in the free field ipsilaterally using a Monacor DT-119 (Monacor, Bremen, Germany). The difference potential between vertex and mastoid intradural needles was amplified (5×10⁴ times), filtered (low pass: 4 kHz, high pass: -400 Hz) and sampled at a rate of 50 kHz for 20 ms, 2×2000 times to obtain two mean ABR traces for each sound intensity. Hearing threshold was determined with 10 dB precisions as the lowest stimulus intensity that evoked a reproducible response waveform in both traces, as judged by visual inspection.

Patch-clamp electrophysiology

Acute brain slices were prepared from Fit/fit homoyzygotes and wildtype littermates at P14 using methods described previously [47]. Briefly, mice were anesthetized with tribromoethanol (250 mg/kg, i.p.) and decapitated. Brains were quickly removed and perfused with 2% v/v 3% glutaraldehyde, 4% sucrose, 3.0 KCl, 1.0 CaCl₂, 3.0 MgSO₄, 1.0 NaH₂PO₄, 26 NaHCO₃, 10 glucose, saturated with 95% O₂ and 5% CO₂. Whole-cell slices were cut at 300 μm on a vibratome (VT 1000s, Leica) and kept in artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 3.0 KCl, 1.5 CaCl₂, 1.3 MgSO₄, 1.0 NaH₂PO₄, 26 NaHCO₃, and 20 glucose, saturated with 95% O₂ and 5% CO₂ at room temperature (21–23°C). Slices were allowed to recover for at least 1 hr before any recording. Recordings were made at 32–34°C using whole-cell patch-clamp techniques. Each slice was transferred to a submerge-type chamber where it was continuously exposed to ACSF heated to 32–34°C, saturated with 95% O₂ and 5% CO₂, and flowing at a rate of 2.0±0.2 ml/min. Whole-cell patch clamp recordings were made at the soma of layer 5 pyramidal neurons of the somatosensory cortex using a 40× water immersion objective (40×/0.80W, Nikon) and infrared Nomarski optics. Patch pipettes were pulled from thick wall borosilicate glass (1.5/0.84 mm, WPI) on a horizontal puller (P-97, Sutter Instruments). Resistance of electrodes was between 2 and 4 MΩ. Liquid junction potential was not corrected. Seal resistance was greater than 2 GΩ. Recordings were made with a Multiclamp 700B amplifier (Molecular Devices). The series resistance (Rs), usually between 8 and 14 MΩ, was monitored throughout the recording, and data were discarded when Rs varied by 20% or more over the course of the recording. GABAergic IPSCs were selectively recorded by blocking ionotropic glutamate receptors with 20 μM DNQX (6,7-dinitro-quinoxaline-2,3-dione) and 1 mM kynurenic acid. For evoked IPSCs, the pipette solution contained (in mM): 110 Cs methylsulfate, 20 TEA-Cl, 15 CsCl, 4 ATP-Mg, 0.3 GTP-Na, 4 QX-314, 0.5 EGTA, and 20 HEPES (pH 7.2, 270–280 mOsm). A pair of twisted nichrome microwires (38 mm in diameter, A-M Systems) were placed in layer V about 200 mm away from the recorded neuron. IPSCs were evoked by current pulses (20–400 μA, 50 ms). For mIPSC recording, the pipette solution contained (in mM): 130 KCl, 4 ATP-Mg, 0.3 GTP-Na, 0.5 EGTA, and 20 HEPES (pH 7.3, 270–280 mOsm with sucrose). Quantal events were recorded in the presence of tetrodotoxin (TTX, 0.4 μM). DNQX and QX-314 were obtained from Tocris; all other chemicals were obtained from Sigma-Aldrich USA.

Experiments were conducted using the AxoGraph X program (AxoGraph Scientific) with a PowerMac G5 connected to an ITG-18 interface. Data were filtered at 4 kHz and digitized at 16 kHz. Data were analyzed using AxoGraph X and IgorPro (WaveMetrics). Quantal IPSCs were detected using variable amplitude
template functions with the rise time set at 0.5 ms and decay times. The detection threshold was set at four times the standard deviation of baseline noise. At least 100 isolated events for each cell were aligned and averaged to give the mean response.

Supporting Information

**Figure S1** Distortion product otoacoustic emissions (DPOAE) in *Fit* and wildtype/heterozygous and mice. DPOAE at 2f1-f2 were observed. The flat lines represent the noise floor+2 SEM (obtained from frequencies neighboring 2f1-f2). (A) No significant differences in DPOAE levels were observed when testing different primary tone frequencies at stimulus levels of 60dB. (B) No significant differences in amplitude growth functions at 16 kHz were observed. The flat lines represent the noise floor+2 SEM (obtained from frequencies neighboring 2f1-f2).

**Figure S2** Developmental expression of dynamin-1 isoforms. Shown are three separate PCR amplifications of cDNA from wildtype and homozygous Fitful whole brains at the time points indicated above the gels. The variant isoform region is amplified with common primers and the two transcripts are distinguished by a diagnostic HphI restriction enzyme site specific for the b isoform. The two bands representing the *Dnm1b* transcript cDNA run as one band and lower on the gel than the *Dnm1a* transcript cDNA. The actin transcript is amplified as control for cDNA levels.

**Figure S3** Isoform specific antibody production. Custom antibodies were produced by Affinity BioReagents “Antibody on Demand” production services. Protein sequences from the two alternative exon 10 alleles were used to design two peptide antigens for each region. The two *Dnm1a* specific peptides were used to immunize rabbits and the two *Dnm1b* specific peptides were used to immunize chickens. The serum was collected and affinity purified. The antibodies were first analyzed for their isoform specificity by western blots using protein extracts from Cos-7 cells containing isoform specific constructs.

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Acknowledgments

We are grateful to Drs. Shawn Ferguson and Pietro De Camilli for providing *Dnm1* null mutant mice for these studies, to Caroline Dunbar and Nathalie Berube for excellent animal care and technical assistance, and to the Jackson Laboratory shared Scientific Services for sequencing and pyrosequencing. We also thank Drs. Susan Ackerman and Gregory Cox for their consultation throughout the project and for critical reading of this manuscript. This paper is dedicated to the memory of Monica Roberts, a postdoctoral fellow who passed away shortly after her critical contributions to this study.

Author Contributions

Conceived and designed the experiments: RMB TM WNF. Performed the experiments: RMB VAL MCR CL CLM ZwZ. Analyzed the data: RMB WNF. Contributed reagents/materials/analysis tools: RMB VAL MCR CL ZwZ WNF. Wrote the paper: RMB WNF.
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