Genome-wide screen for modifiers of Na⁺/K⁺ ATPase alleles identifies critical genetic loci

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Abstract

Background: Mutations affecting the Na⁺/K⁺ ATPase (a.k.a. the sodium-potassium pump) genes cause conditional locomotor phenotypes in flies and three distinct complex neurological diseases in humans. More than 50 mutations have been identified affecting the human ATP1A2 and ATP1A3 genes that are known to cause rapid-onset Dystonia Parkinsonism, familial hemiplegic migraine, and variants of familial hemiplegic migraine with neurological complications including seizures and various mood disorders. In flies, mutations affecting the ATPalpha gene have dramatic phenotypes including altered longevity, neural dysfunction, neurodegeneration, myodegeneration, and striking locomotor impairment. Locomotor defects can manifest as conditional bang-sensitive (BS) or temperature-sensitive (TS) paralysis: phenotypes well-suited for genetic screening.

Results: We performed a genome-wide deficiency screen using three distinct missense alleles of ATPalpha and conditional locomotor function assays to identify novel modifier loci. A secondary screen confirmed allele-specificity of the interactions and many of the interactions were mapped to single genes and subsequently validated. We successfully identified 64 modifier loci and used classical mutations and RNAi to confirm 50 single gene interactions. The genes identified include those with known function, several with unknown function or that were otherwise uncharacterized, and many loci with no described association with locomotor or Na⁺/K⁺ ATPase function.

Conclusions: We used an unbiased genome-wide screen to find regions of the genome containing elements important for genetic modulation of ATPalpha dysfunction. We have identified many critical regions and narrowed several of these to single genes. These data demonstrate there are many loci capable of modifying ATPalpha dysfunction, which may provide the basis for modifying migraine, locomotor and seizure dysfunction in animals.

Keywords: Drosophila melanogaster, ATPalpha, Sodium pump, Temperature-sensitive paralysis, Conditional paralysis, Seizure, Migrane, Screen, Genome-wide, Seizure suppressor

Background

In many organisms, highly conserved Na⁺/K⁺ ATPases are responsible for maintaining ion gradients across the plasma membrane through ATP-dependent asymmetric translocation of Na⁺ and K⁺ ions. These ion gradients maintain the resting potential of cells, which facilitates neural signaling and many essential secondary processes. Mature Na⁺/K⁺ ATPase complexes are heteromultimers of alpha, beta, and gamma subunits in mammals. Flies express only the alpha and beta subunits, the former of which is known as ATPalpha. Like its mammalian homologue, ATPalpha contains ten transmembrane domains and has the ATP-dependent catalytic activity essential for pump function [1-3].

Mutations affecting the alpha subunit of the Na⁺/K⁺ ATPase in humans are associated with at least three human diseases: Rapid-onset Dystonia Parkinsonism (RDP), Familial Hemiplegic Migraine (FHM), and Alternating Hemiplegia of Childhood (AHC) [4]. RDP is a severe DOPA non-responsive form of dystonia Parkinsonism the etiology of which is poorly understood [5]. FHM, possibly the most severe form of migraine, is associated with a debilitating partial paralysis, and currently is largely untreatable [6]. AHC is a severe childhood locomotor disease associated with recurring acute bouts of paralysis and muscle weakness, and general developmental delays (reviewed by [7]). Recently Sasaki and colleagues have described several...
children who seem to have a disease intermediate to AHC and RDP [8]. All of these diseases are complex neuromuscular conditions associated with marked locomotor dysfunction and for which the underlying pathogenesis is poorly understood.

*Drosophila* conditional mutants have been isolated based upon temperature-sensitive (TS) or bang-sensitive (BS) paralysis phenotypes over the past many decades. TS mutants generally become paralyzed in less than five minutes at 38°C and BS mutants paralyze in response to 20 seconds of mechanical stress. These classes of mutants have proven informative and have defined many essential components of neural signaling [9-15]. Conditional TS mutations typically affect critical neural proteins and include well-studied genes such as *para* (voltage-dependent NaCH), *NapTS* (RNA helicase affecting *para* transcripts), *caccomony* (a voltage-gated calcium channel), *ATPalpha* (Na+/K+ ATPase), *comatose* (dNSF1), *shibire* (Dynamin), *syntaxin*, *synaptobrevin*, and *dao* (regulator of Erg-type K-channels), to name a few [15-24]. Conditional BS mutations can also affect important neural signaling and ion homeostasis proteins, such as para and *ATPalpha* [23,25]. They also affect many proteins with integral roles in bioenergetics and mitochondrial function, such as *sesB*, *ATP6*, *kdn*, *eas*, and *SOD2* [26-30]. Interestingly, numerous BS mutants have been shown to exhibit seizures and model epilepsies (e.g. *para* thoroughly, *ATP6*, and *Kazachoc*; [25,31,32]). BS and TS conditional mutants have proven incredibly important to our understanding of neurobiology and previous studies have successfully used them to identify genes that modify these behaviors (e.g. [33-35]). However, there are no reports of genome-wide screens for modifier loci using these behavioral phenotypes in *Drosophila* or studying *ATPalpha* in any model system. This suggests that such an approach could yield novel loci involved in regulating ion homeostasis or neural excitability.

It has previously been shown that mutations in *ATPalpha* result in profound neural and locomotor dysfunction in *Drosophila* [23,36-40]. Hypomorph *ATPalpha* alleles, such as *ATPalpha* DTS1, display BS paralysis and phenocopy injection of the selective Na+/K+ ATPase inhibitor, ouabain [39]. The *ATPalpha* DTS1 mutation is a dominant, conditional, gain-of-function, missense mutation [23]. The mutation results in an E982K substitution near the protein’s C-terminus (short isoform numbering). *ATPalpha* DTS1 heterozygotes exhibit rapid paralysis at 38°C with complete penetrance. This is thought to be a result of conditional neuronal hyperexcitability caused by the mutation [23]. *ATPalpha* CJS and *ATPalpha* CJ10 are also dominant missense mutations affecting evolutionarily conserved amino acids [36]. However, they each exhibit unique locomotor phenotypes. *ATPalpha* CJS behaves like a loss-of-function allele of *ATPalpha*, exhibiting haploinsufficiency and BS paralysis [36]. *ATPalpha* CJ10 exhibits BS and progressive TS phenotypes, suggesting this is a loss-of-function allele that exhibits weak gain-of-function features, which are uncovered with age [36]. Thus, *ATPalpha* DTS1, *ATPalpha* CJS, and *ATPalpha* CJ10 are all dominant, phenotypically well-characterized, and possibly functionally distinct, conditional locomotor mutants. Such alleles are ideally suited for a modifier screen. Using multiple alleles of *ATPalpha* increases the power of the screen and affords the likelihood of identifying allele-specific modifiers. Furthermore, to our knowledge, this is the first report of a genome wide genetic screen in any animal system using three distinct alleles of the same gene in parallel to identify allele-specific interactions.

Deficiency screens have been effectively used for elucidating novel gene interactions in *Drosophila* using various phenotypes [41-43]. Deficiency (Df) strains each have a unique deletion of a segment of the genome. Phenotypically screening for genetic interactions between defined point mutations and an individual defined deficiency is an efficient way to identify modifier loci. Using a collection of Dfs covering a high percentage of the genome (95-98%), one can identify critical modifier loci anywhere in the genome. This provides an efficient yet powerful and unbiased forward genetic approach. Critical loci can often be narrowed to single genes using smaller deficiencies and single gene disruptions. We have performed such a screen using *ATPalpha* DTS1, *ATPalpha* CJS and *ATPalpha* CJ10 identified 64 critical modifier intervals, and successfully confirmed 50 single-gene modifiers, including numerous novel loci of interest. These data suggest the existence of many susceptibility loci capable of modifying migraine, locomotor and seizure dysfunction in animals and provide a rich data set from which new targets for anti-migraine or anti-epileptic drugs could be drawn.

**Results**

**Primary genetic modifier screen**

To identify new genes that interact with *ATPalpha* we performed a deficiency screen using three characterized alleles: *ATPalpha* DTS1, *ATPalpha* CJS and *ATPalpha* CJ10. We used the Bloomington Stock Center deficiency (Df) kit that covers approximately 98% of the *Drosophila* genome. All

| Table 1 Primary screen summary | DTS1 | CJS | CJ10 |
|-------------------------------|------|-----|------|
| Number tested in primary screen | 386  | 393 | 358  |
| % of Kit tested               | 83%  | 84% | 77%  |
| Avg. Response (Sec.)          | 34   | 88  | 42   |
| St. Dev. (Sec.)               | 26.4 | 74.6| 46.1 |
| Normal Range (Sec.)           | 20-60| 20-190| 10-150|
| Number selected for verification | 89 | 69 | 78 |
| Screened phenotype            | TS   | BS  | BS   |
Figure 1 (See legend on next page.)
of the 467 Df strains we received were tested with at least one ATPalpha mutant allele and the vast majority of strains were tested with multiple alleles (see Table 1). Each of the three ATPalpha mutants was mated to each Df line. F1 progeny bearing ATPalphaDTS1 and each deficiency were subjected to TS assays while progeny bearing ATPalphaCJS or ATPalphaCJ10 and each Df were assayed for BS. The average response for ATPalphaDTS1, ATPalphaCJS and ATPalphaCJ10 Df double mutants was 34.8+/−25.3, 89.9+/−53.6 and 41.5+/−34.8 seconds, respectively (Additional file 1). We used these values to identify putative genetic interactions. Df(3R)BSC819 contains a deletion of the ATPalpha locus and failed to complement each mutant allele, as expected.

The data from the primary screen were organized graphically by average time to recovery or paralysis for each double mutant (Figure 1). In each case, the resulting data formed a largely normal distribution. Double mutants that deviated significantly from the mean were termed putative enhancers or suppressors and were tested again in a verification screen. The workflow for the genetic screen is depicted in Figure 2. In the primary screen, 1137 interactions were examined for the three conditional locomotor mutants identifying 117 putative enhancer, suppressor, or synthetic lethal regions. These interactions were examined further in the verification screen.

**Verification screen**

To mitigate the effect of false positives and confirm that interactions were reproducible before pursuing them further, we performed a verification screen (an independent experiment) with the putative modifiers. We began the verification with 89 ATPalphaDTS1 modifiers (Figure 1A), 69 ATPalphaCJS modifiers (Figure 1B), and 78 ATPalphaCJ10 modifiers (Figure 1C). After verification, we took advantage of having two data sets (primary and verification screen) and created a formula to determine the reproducibility of each putative genetic interaction (see Materials and Methods). We calculated a reproducibility index (RI) and used it to help us identify the most promising critical intervals. Dfs with the highest RIs were prioritized for mapping and secondary screening. This approach yielded 7 putative ATPalphaDTS1 enhancers, 12 suppressors, and five synthetic.
lethal (enhanced to lethality) combinations (Table 2). The ATPalpha<sup>CJ5</sup> screen yielded 13 enhancers, 10 suppressors, and four synthetic lethal combinations (Table 3). The ATPalpha<sup>CJ10</sup> primary screen yielded 17 enhancers, 11 suppressors, and one synthetic lethal (Table 4).

**Single gene identification and testing**

After the verification of critical intervals, genes contained within these intervals were selected for testing. Where practical large intervals were narrowed using smaller Dfs. We obtained classical alleles for integral genes from Bloomington, when possible. Each single gene mutant was mated to the ATPalpha allele it putatively modified and to w<sup>1118</sup>. All single gene mutants displayed no BS or TS phenotype as heterozygotes (data not shown). Heterozygous double mutants were again assayed for TS or BS with age matched controls. Significant interacting single gene mutants were also tested with the other ATPalpha alleles (Figure 2). Twenty single gene interactions were found using classical mutants for ATPalpha<sup>DTS1</sup> including 19 single gene enhancers and one single gene suppressor. Ten single gene suppressors were found for ATPalpha<sup>CJ5</sup>. Twenty-four single gene interactions were found with ATPalpha<sup>CJ10</sup>, all but one of which showed suppression of the mutant phenotypes. In total, 35 single gene interactions were found and, importantly, 14 different genes had effects with more than one ATPalpha allele (Table 5).

**Table 2 Confirmed ATPalpha<sup>DTS1</sup> interacting deficiencies**

| Df Name     | Enh/Sup | Mean +/- SEM | Total N | RI | Hits in region       | Coincidence |
|-------------|---------|--------------|---------|----|----------------------|-------------|
| Df(3 L)Exel6092 | Sup     | 76.4 +/- 33.4 | 11      | 6.27 | spz5, FMRFaR, scramb2, aly | C10         |
| Df(2R)ED1275 | Sup     | 209.8 +/- 57.0 | 6       | 4.87 |                       |             |
| Df(2R)BSC361 | Sup     | 87.8 +/- 10.6  | 16      | 4.68 | Stj                   | C10         |
| Df(3 L)BSC33 | Sup     | 103.9 +/- 34.4 | 14      | 4.64 |                       |             |
| Df(3 L)Exel8104| Enh     | 27.3 +/- 27.3  | 11      | 3.18 |                       |             |
| Df(3 R)BSC486 | Enh     | 17.2 +/- 1.7   | 19      | 2.65 |                       | C10         |
| Df(2 L)BSC180 | Sup     | 85.3 +/- 16.5  | 25      | 2.13 | Rbp9                  | C10         |
| Df(3 R)Exel6210| Sup     | 151.3 +/- 42.9 | 11      | 2.02 |                       |             |
| Df(2 R)BSC383 | Sup     | 129.1 +/- 40.9 | 11      | 1.90 |                       |             |
| Df(2 L)BSC278 | Sup     | 52.3 +/- 14.9  | 25      | 1.54 |                       |             |
| Df(3 L)BSC23  | Enh     | 11.2 +/- 1.4   | 14      | 1.53 | spz5, scramb2, rasp, aly | C15, C10    |
| Df(2 L)Exel6005| Sup     | 73.9 +/- 23.1  | 19      | 1.51 |                       |             |
| Df(3 R)BSC650 | Enh     | 22.1 +/- 2.3   | 13      | 1.20 |                       |             |
| Df(2 L)ED1203 | Enh     | 21.2 +/- 2.1   | 13      | 0.92 | Ham                   | C15         |
| Df(3 R)ED2   | Enh     | 20.0 +/- 2.6   | 25      | 0.88 |                       |             |
| Df(2 R)ED3728| Sup     | 46.3 +/- 8.2   | 15      | 0.86 |                       |             |
| Df(1)BSC767  | Sup     | 138.2 +/- 26.7 | 13      | 0.82 |                       |             |
| Df(2 R)M60E  | Sup     | 48.1 +/- 5.9   | 25      | 0.74 | Rpl19, pain           |             |
| Df(2 L)ED629 | Enh     | 27.1 +/- 2.9   | 13      | 0.49 | Glutactin, sema-1a    |             |
| Df(3 R)ED7665| Enh/Leth | -              | -       | -   |                      | C10         |
| Df(3 R)ED6361| Enh/Leth | -              | -       | -   |                      |             |
| Df(3)BSC375  | Enh/Leth | -              | -       | -   |                      |             |
| Df(3 R)BSC467| Enh/Leth | -              | -       | -   |                      | C10         |
| Df(1)BSC708  | Enh/Leth | -              | -       | -   |                      |             |
| Df(3 R)BSC819| Enh/Leth | -              | -       | -   | ATPalpha              | All Enh/Leth|

Gal4 driven RNAi strains result in a loss-of-function phenotype and are well-suited to confirm the hypomorphic effect of a heterozygous Df. RNAi knockdown was driven with da-Gal4 in ATPalpha mutant backgrounds. Daughterless transcripts are stably expressed throughout the life of a fly and are detectable in every tissue by the FlyAtlas affymetrix array analysis [72,73]. We used this driver to ubiquitously express the RNAi.
constructs and mimic the effect observed with the Df. RNAi mediated knockdown of candidate genes was compared to age matched controls lacking the UAS-RNAi construct. Twenty-five different genes showed interactions using this method, including 10 genes already identified in the classical mutant screen. Fourteen interactions were identified with ATPalpha\textsuperscript{DTS1}, with nine enhancers and five suppressors. Seventeen interactions, with two enhancers and 15 suppressors, were identified for ATPalpha\textsuperscript{CJ5}. Thirteen interactions, all suppressors, were confirmed with ATPalpha\textsuperscript{CJ10}. In total 15 different genes showed a genetic interaction with two or more ATPalpha alleles (Table 6). In total we have identified 50 genes that interact with ATPalpha, 25 of which were confirmed to interact with at least two independent alleles.

**Discussion**

The Na\textsuperscript{+}/K\textsuperscript{+}ATPase is central to maintaining cytosolic ion homeostasis suggesting that many of the genes identified in our screen would encode proteins that affect cytosolic ion concentrations and, indeed, this was the case (Figure 3A). Nearly 25% of the genes we identified encode proteins with a known function in ion transport. In our search for single gene modifiers we selected genes known to be expressed in the nervous system. Unsurprisingly, ~50% of our hits are known to cause some neuronal defect when knocked out (Figure 3B). For example, most of the cell adhesion and paracrine signaling molecules we found, such as Galectin (Tables 5 and 6, Figure 4), Glt (Tables 5 and 6), and Sema-1a (Table 5) were previously known to cause malformed or improperly targeted synapses [45,48-50]. However, about
half of our genes were not previously linked to neuronal function. Additionally, many genes we identified encode proteins implicated in signaling pathways. In particular we found proteins involved in developmental signaling pathways, such as **Wingless** and **Hedgehog** (Tables 5 and 6) and **spz5** (Tables 5 and 6).

**Spz5** (Figure 5) is especially interesting because it has recently been identified as a **Drosophila** neurotrophin that signals through a Toll receptor [61,62]. Both Slmb and Cact (Table 5) were also identified by our screen and both may function downstream of Spz5. In mammals and flies, Toll signaling activates NF-κB transcription factors, typically through the degradation of an inhibitor of NF-κB (I-κB), such as Cact. Phosphorylated I-κB is targeted for degradation, allowing NF-κB-like transcription factors to translocate to the nucleus. Slmb and its mammalian homolog β-TrCP regulate phospho-I-κB, likely Slmb, target an E3 ubiquitin ligase complex to phospho-I-κB and mediate its degradation via ubiquitin proteasome system [68]. Interestingly, we have also identified Uch-L5 (Table 6) in our screen, a member of the 26S regulatory complex which is likely responsible for the deubiquitylation of proteins as they enter the 26S proteasome [81].

Previously published studies of **Slmb** and **Spz5** show that they play an important role in neural development. Slmb is involved in pruning dendrites and axons during

| Table 4 Confirmed ATPalpha<sup>C10</sup> interacting deficiencies | Df Name | Enh/Sup | Mean +/- SEM | Total N | RI | Hits in region | Coincidence |
|---|---|---|---|---|---|---|---|
| CJ10 Control | En | 50.3 +/- 7.1 | 17 | - | - | - | |
| Df(3R)BSC486 | Enh | 168.5 +/- 38.8 | 6 | 4.92 | DTS1 |
| Df(3 L)Exel16112 | Enh | 144.6 +/- 15.4 | 18 | 4.20 | CJS |
| Df(2 L)BSC180 | Enh | 151.7 +/- 34.5 | 9 | 2.93 | Rbp9 | DTS1 |
| Df(2 L)TW161 | Enh | 103.1 +/- 18.2 | 12 | 2.89 | CJS |
| Df(3R)BSC469 | Enh | 96.5 +/- 22.9 | 11 | 2.59 | CJS |
| Df(3R)BSC681 | Enh | 98.7 +/- 49.4 | 6 | 2.32 | |
| Df(3R)A113 | Enh | 92.4 +/- 8.8 | 14 | 2.16 | |
| Df(3R)BSC501 | Enh | 91.8 +/- 7.8 | 14 | 2.10 | CG14508 |
| Df(3R)ED5495 | Enh | 139.6 +/- 34.5 | 7 | 1.98 | |
| Df(3 L)Exel6092 | Enh | 142.8 +/- 31.5 | 20 | 1.85 | DTS1 |
| Df(2 R)BSC664 | Enh | 60.2 +/- 14.1 | 11 | 1.77 | |
| Df(3 R)Exel6196 | Enh | 109.1 +/- 28.5 | 11 | 1.74 | |
| Df(3 L)BSC410 | Enh | 85.3 +/- 11.1 | 12 | 1.54 | |
| Df(3 L)ED4475 | Sup | 8.0 +/- 1.6 | 7 | 1.48 | CJS |
| Df(3 L)BSC23 | Sup | 8.0 +/- 3.0 | 18 | 1.43 | DTS1, CJS |
| Df(2 L)BSC240 | Enh | 91.8 +/- 10.9 | 24 | 1.43 | Nckx30C, ppk11, nACHR-alpha6, FKBP59 |
| Df(2 L)J39 | Sup | 7.9 +/- 2.2 | 25 | 1.43 | FKBP59 | CJS |
| Df(2 R)BSC361 | Enh | 114.0 +/- 26.3 | 8 | 1.29 | Stj | DTS1 |
| Df(2 R)BSC661 | Enh | 78.0 +/- 10.9 | 23 | 1.25 | |
| Df(3 R)ED5577 | Sup | 14.0 +/- 2.0 | 13 | 1.20 | |
| Df(2 L)ED489 | Sup | 12.1 +/- 2.6 | 25 | 1.19 | Ndæ1 | CJS |
| Df(3 L)ED230 | Sup | 13.7 +/- 3.7 | 10 | 1.17 | |
| Df(4 L)ED6380 | Sup | 12.6 +/- 3.6 | 25 | 1.14 | |
| Df(3 L)BSC113 | Sup | 14.3 +/- 1.7 | 15 | 1.13 | aay |
| Df(2 L)ED793 | Sup | 16.2 +/- 4.1 | 25 | 1.09 | Dyrk2, NimBS, nACHRα5 |
| Df(2 L)BSC149 | Sup | 16.1 +/- 3.3 | 14 | 1.09 | |
| Df(3 R)ED7665 | Sup | 16.5 +/- 5.1 | 21 | 1.06 | DTS1 |
| Df(3 L)BSC442 | Enh | 79.1 +/- 10.4 | 15 | 1.02 | |
| Df(3 R)BSC467 | Enh/Leth | - | - | - | DTS1 |
| Df(3 R)BSC819 | Enh/Leth | - | - | - | ATPalpha |

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| Cytological region | Gene | Genotype | Putative function | ATPα Allele | Nature of interaction | Significance |
|--------------------|------|----------|------------------|-------------|----------------------|--------------|
| 10B3               | l(1)10Bb | EO4588 | Spliceosome component [44] | CJ10 | Suppressor * |             |
| 21B1-21B1          | Galectin | DG25505 | Cell surface protein, galactoside binding [45] | DT51 | Enhancer *** |             |
| 23C9-23C9          | Rbp9 | Δ1 | RNA binding [46] | DT51 | Enhancer * |             |
| 23C9-23C9          | Rbp9 | Δ1 | * | CJ5 | Suppressor **** |             |
| 27E-28B1           | Ndae1 | MB05294 | Sodium driven anion exchanger[47] | CJ5 | Suppressor * |             |
| 27E-28B1           | Ndae1 | MB05294 | * | DT51 | Enhancer * |             |
| 27E-28B1           | Ndae1 | MB05294 | * | CJ10 | Suppressor * |             |
| 29B4-29E4          | Sema-1a | K13702 | Axon guidance signal and receptor [48,49] | DT51 | Enhancer * |             |
| 29B4-29E4          | Sema-1a | K13702 | Axon guidance signal and receptor [48,49] | DT51 | Enhancer * |             |
| 30C7-30F2          | Nck30C | E00401 | Sodium/Calcium/Potassium exchanger [51] | CJ10 | Enhancer * |             |
| 30C7-30F2          | Ppk11 | MB02012 | Excitatory sodium channel [52] | CJ10 | Suppressor *** |             |
| 30C7-30F2          | Ppk11 | MB02012 | * | DT51 | Suppressor **** |             |
| 30C7-30F2          | Ppk11 | MB02012 | * | CJ5 | Suppressor **** |             |
| 30C7-30F2          | Ppk11 | MB02012 | * | CJ5 | Suppressor **** |             |
| 30C7-30F2          | nAChRa6 | MB06675 | Ach receptor subunit | CJ10 | Suppressor * |             |
| 30C7-30F2          | nAChRa6 | MB06675 | * | CJ5 | Suppressor **** |             |
| 31C-32E            | FKBP59 | E03538 | Calcium channel regulator [53] | DT51 | Enhancer * |             |
| 31C-32E            | FKBP59 | E03538 | * | CJ5 | Suppressor *** |             |
| 31C-32E            | FKBP59 | E03538 | * | CJ10 | Suppressor *** |             |
| 32E-33B1           | Pdelen | C04487 | cAMP/cGMP phosphodiesterase [54] | CJ5 | Suppressor **** |             |
| 34E4-35B4          | Dyrk2 | 1 | Serine/Threonine kinase [55] | DT51 | Enhancer *** |             |
| 34E4-35B4          | Dyrk2 | 1 | Serine/Threonine kinase [55] | DT51 | Enhancer *** |             |
| 34E4-35B4          | Nimb5 | Mio1793 | Bacterial defense | CJ10 | Suppressor * |             |
| 34E4-35B4          | nAChRa5 | MB11647 | ACh Receptor subunit | CJ10 | Suppressor * |             |
| 35 F1-36A1         | Cact | 7 | Inhibitor of NF-kB [56] | CJ10 | Suppressor **** |             |
| 36A8-36F1          | Beat-la & Fas3 | 3/E25 | Neuronal immunoglobulin-like proteins | CJ5 | Suppressor * |             |
| 25 F1-36A1         | CGS888 | MB00188 | Toll 3 like receptor | DT51 | Enhancer **** |             |
| 25 F1-36A1         | CGS888 | MB00188 | * | CJ5 | Suppressor *** |             |
| 46 F1-47A9         | CG42732 | MB04544 | Predicted potassium channel | DT51 | Enhancer **** |             |
| 46 F1-47A9         | CG42732 | MB04544 | Predicted potassium channel | DT51 | Enhancer **** |             |
| 46 F1-47A9         | Gaoo | Mio0833 | Heterotrimeric G-protein subunit | CJ10 | Suppressor **** |             |
| 46 F1-47A9         | Gaoo | Mio0833 | Heterotrimeric G-protein subunit | CJ10 | Suppressor **** |             |
| 50B1               | CG33156 | MB05931 | Predicted NAD⁺ kinase | DT51 | Enhancer **** |             |
| 57CS-57F6          | Pu | r1 | GTP cyclohydrolase [58] | CJ5 | Suppressor * |             |
| 57CS-57F6          | Pu | r1 | * | CJ10 | Suppressor **** |             |
| 60E6-60E11         | Pain | EP2451 | TRP calcium channel [59] | DT51 | Enhancer ** |             |
| 60E6-60E11         | Pain | EP2451 | * | CJ10 | Suppressor **** |             |
| 60E6-60E11         | Pain | EP2451 | * | CJ10 | Suppressor **** |             |
| 62E8-63B6          | Spaz | EO3444 | Neurotrophin [61,62] | DT51 | Enhancer ** |             |
| 62E8-63B6          | Spaz | EO3444 | * | CJ10 | Suppressor **** |             |
| 62E8-63B6          | Spaz | EO3444 | * | CJ10 | Suppressor **** |             |
| 62E8-63B6          | Spaz | EO3444 | * | CJ10 | Suppressor **** |             |
| 62E8-63B6          | Aly | 1 | Regulator of transcription [63,64] | DT51 | Enhancer **** |             |
| 62E8-63B6          | Aly | 1 | Regulator of transcription [63,64] | DT51 | Enhancer **** |             |
pupation [84] and Spz5 is a neurotrophic signal and axon guidance cue in the embryonic nervous system [61]. Interestingly, animals heterozygous for a loss of function allele of either gene displayed no phenotype in neurons [61,85]. In contrast, our screen examined heterozygous double mutants and found large effects, suggesting ATPalpha mutants are sensitive to otherwise inconsequential changes in neuronal development or another unappreciated function of these proteins. Furthermore, a seemingly insignificant disruption of neuronal survival signals early in development may have dramatic phenotypic effects for ATPalpha mutants since heterozygosity of Slmb, or Spz5 suppressed the loss-of-function ATPalpha phenotype. Additionally, numerous developmental genes were identified that suggest modulated locomotor function.

Another interesting possibility is that loss-of-function ATPalpha mutations are disrupting neuronal development through alterations in NF-kB signaling. It has been shown that sub-inhibitory concentrations of ouabain activate NF-kB via an Na\(^+/K^+\) ATPase dependent mechanism in rat kidney cells. The effect is mediated by slow, inositol triphosphate-dependent, calcium oscillations likely caused by shifting electrochemical gradients [86]. More recently, agrin, a protein involved in synapse formation at NMJs and in the CNS, has been shown to bind to and inhibit the mammalian Na\(^+/K^+\) ATPase α3 isoform. Furthermore, agrin seems to bind at the same site as ouabain because a protein fragment can prevent ouabain inhibition of the Na\(^+/K^+\) ATPase [87]. Thus it is possible that agrin exerts its effects through NF-kB. If a similar pathway exists in flies it would likely be constitutively active in our loss-of-function mutants and its dysregulation could cause developmental changes, which might increase seizure susceptibility. This is consistent with our finding that knockdown of proteins required for NF-kB activation suppresses seizures in our loss-of-function mutants. NF-kB activation may be caused by calcium oscillations [86], making it possible that some of the calcium channels we found also play a role in this pathway. FKBP59 (Figure 6) is particularly interesting because it inhibits an inositol triphosphate sensitive, non-specific calcium channel, TrpL [53]. Inhibition of calcium channels would likely be required in calcium oscillations. The preponderance of hits related to the NF-kB pathway suggests a possible role for this pathway in seizure pathogenesis.

In most cases the ATPalpha\(^{GR}\) and ATPalpha\(^{CH10}\) mutant phenotypes were modified in the same direction (enhancement or suppression) and they never had opposite phenotypes in our screen. This is consistent with the finding that both exhibit loss-of-function characteristics. The ATPalpha\(^{DTS1}\) phenotype, however, usually contrasted with the phenotypes of ATPalpha\(^{GR}\) and ATPalpha\(^{CH10}\). This is intriguing as ATPalpha\(^{DTS1}\) is a gain-of-function mutation that can be reverted by a second site mutation to give the characteristic ATPalpha loss-of-function phenotype [23]. In accord with this fact the vast majority (~80%) of the single gene interactions with ATPalpha\(^{DTS1}\) modified the loss-of-function alleles in the opposite direction or not at all. Reduction of Ppk11, Ppk21, and Ppk24 function all suppressed the ATPalpha\(^{DTS1}\) paralysis phenotype and that a reduction in the ability of

### Table 5 Single gene effects confirmed for ATPalpha alleles using classical mutants (Continued)

| Gene       | m47  | Predicted phosphatidyl serine scramblase | CJ10 | Suppressor  |
|------------|------|----------------------------------------|------|-------------|
| 62E8-63B6  | m47  | Predicted phosphatidyl serine scramblase | CJ10 | Suppressor  |
| 63A3-63A3  | EY01180 | Predicted phosphatidyl serine scramblase | CJ10 | Suppressor  |
| 63A3-63A3  | EY01180 | Predicted phosphatidyl serine scramblase | CJ10 | Suppressor  |
| 67A2-67D13 | Slm  | 295 | Ubiquitin ligase [67,68] | DT51 | Enhancer |
| 69B9-93D4  | Slm  | 295 | " | DT51 | Enhancer |
| 69B9-93D4  | Sec  | 2 | Protein trafficking [69,70] | CT10 | Suppressor |
| 69B9-93D4  | Sec  | 2 | " | CT10 | Suppressor |
| 69B9-93D4  | RhoGAP93B | EY07136 | Rac1 GAP [71] | DT51 | Enhancer |
| 93B9-93D4  | G9163 | Predicted cytochrome C | DT51 | Enhancer |
| 93B9-93D4  | G9163 | Predicted cytochrome C | CT10 | Suppressor |
| 93B9-93D4  | RhoGAP93B | EY07136 | Rac1 GAP [71] | DT51 | Enhancer |
| 98 F10-99B9 | CG14508 | G9163 | Predicted cytochrome C | DT51 | Enhancer |
| 98 F10-99B9 | CG14508 | G9163 | Predicted cytochrome C | CT10 | Suppressor |
| 99E1-3Rt   | Sro  | 1 | Ecdysone biosynthetic pathway | CT10 | Suppressor |

Many genes had an interaction with more than one allele, although some appear to be allele specific. Double mutants were compared to ATPalpha\(^+/+\) and heterozygous classical mutant controls. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

*Function per flybase.org and / or listed citation.
Table 6  Single gene effects confirmed for ATPalpha alleles using RNAi

| Cytological region | Gene       | Putative function*                                      | ATPα Allele | Nature of interaction | Significance |
|--------------------|------------|--------------------------------------------------------|-------------|-----------------------|-------------|
| 21A1-21B1          | Galectin   | Galactoside binding [45]                               | CJ10        | Suppressor            | **          |
| 21A1-21B1          | Galectin   | *                                                      | CJ5         | Suppressor            | ***         |
| 22 F4-22 F4        | CG3528     | Unknown                                                | DTS1        | Enhancer              | *           |
| 22 F4-22 F4        | CG3528     |                                                        | CJ10        | Suppressor            | *           |
| 22 F4-22 F4        | CG3528     |                                                        | CJ5         | Suppressor            | *           |
| 27E-28B1           | Ndai1      | Na + driven anion exchanger [47]                       | CJ5         | Enhancer              | *           |
| 29B4-29E4          | Glt        | Cell surface glycoprotein [50]                         | CJ10        | Suppressor            | *           |
| 30C8-30C9          | Ppk11      | Sodium channel [52]                                    | CJ5         | Suppressor            | **          |
| 31C-32E            | FKBP59     | Calcium channel regulator [53]                         | CJ5         | Suppressor            | ***         |
| 31C-32E            | FKBP59     |                                                        | DTS1        | Enhancer              | *           |
| 33A1-33A1          | Vha100-5   | ATPase, proton transport                               | DTS1        | Enhancer              | *           |
| 33A2-33A2          | Esc        | Histone methyltransferase component [74]               | DTS1        | Enhancer              | ***         |
| 34E4-35B4          | Dyrk2      | Serine/Threonine kinase [55]                           | DTS1        | Enhancer              | *           |
| 34E4-35B4          | Dyrk2      |                                                        | CJ5         | Suppressor            | ***         |
| 37A2-37A4          | Ham        | Transcription factor [75]                              | DTS1        | Suppressor            | *           |
| 50C5-50C6          | Stj        | Voltage-gated calcium channel regulatory subunit [77,78]| DTS1        | Enhancer              | ****        |
| 50C5-50C6          | Stj        |                                                        | CJ5         | Enhancer              | **          |
| 51D1-51D1          | Cyp6a19    | Cytochrome P450                                         | CJ10        | Suppressor            | *           |
| 62E8-63B6          | Spz5       | Neurotrophin [61,62]                                   | DTS1        | Suppressor            | **          |
| 62E8-63B6          | Spz5       |                                                        | CJ10        | Suppressor            | *           |
| 62E8-63B6          | Rasp       | Palmitoyl transferase [65,66]                          | CJ5         | Suppressor            | ***         |
| 63A3-63A3          | FMRaR      | Neuropeptide receptor [79]                             | DTS1        | Enhancer              | **          |
| 63A3-63A3          | FMRaR      |                                                        | CJ10        | Suppressor            | **          |
| 63A3-63A3          | FMRaR      |                                                        | CJ5         | Suppressor            | ****        |
| 64C2-64C5          | Con        | Homophilic cell adhesion [80]                           | DTS1        | Suppressor            | *           |
| 64C2-64C5          | Con        |                                                        | CJ5         | Suppressor            | **          |
| 67A2-67D13         | Aay        | Predicted phosphoserine phosphatase                    | CJ10        | Suppressor            | *           |
| 67A2-67D13         | Aay        |                                                        | CJ5         | Suppressor            | **          |
| 67B9-67B9          | Uch-L5     | 26S Proteasome component [81]                           | DTS1        | Enhancer              | *           |
| 67D11-67D11        | Scramb1    | Phosphatidyl serine scramblase                          | CJ10        | Suppressor            | **          |
| 99B6-99B6          | Ppk21      | Sodium channel                                         | DTS1        | Suppressor            | **          |
| 99B6-99B6          | Ppk21      |                                                        | CJ10        | Suppressor            | *           |
| 100B9-100B9        | Ppk24      | Sodium channel                                         | DTS1        | Suppressor            | **          |
| 100B9-100B9        | Ppk24      |                                                        | CJ5         | Suppressor            | *           |
| 100B9-100B9        | Ppk24      |                                                        | CJ10        | Suppressor            | **          |
the double mutant animals to sense the elevated temperature is sufficient to suppress the TS paralysis. This possibility is consistent with the kinetics of recovery after animals are returned to the permissive temperature. This is also intriguing as the locomotor dysfunction resulting in hemiparalysis in FHM patients has been reported to be associated with sensory dysfunction and FHM patients report having prolonged visual auras [90-92].

Conclusions

FHM, RDP, and AHC are complex human neurological diseases associated with mutations affecting the catalytic alpha subunit of the Na\(^{+}/K\(^{+}\) ATPase [4-6]. Currently, there is no cure or effective treatment for these diseases. Using three *Drosophila* strains with different missense mutations in *ATPalpha* we have performed a large-scale deficiency screen to identify novel genes that interact with the gene encoding the Na\(^{+}/K\(^{+}\)ATPase alpha subunit. In total, we have identified 50 genes that interact with *ATPalpha*, 25 of which were demonstrated to interact with at least two independent alleles. We have also implicated 50 critical intervals/deficiency regions for which we have yet to identify individual genes that interact with *ATPalpha* (Tables 2, 3 and 4). Modifier loci that encode proteins expressed in the adult, especially those that phenotypically suppress *ATPalpha* dysfunction, provide proteins/pathways that could be viable targets for the development of new migraine or anti-epileptic drugs. Additionally, studies of these loci and how they modify *ATPalpha* dysfunction will help us understand epilepsy, hemiplegia and migraine disease pathogenesis in animals.

Materials and methods

*Drosophila* strains

Flies were maintained on standard cornmeal-molasses agar medium at 21-22°C. Chromosomal deficiencies were obtained from the Bloomington Deficiency Kit from the Bloomington Stock Center (order date January 2010). The *Df* Kit we received contained 467 stocks with deletions spanning 97.8% of the *Drosophila* genome. Three Na\(^{+}/K\(^{+}\)ATPase alpha subunit mutants were used: *ATPalpha*\(_{DTS1}\) [23], *ATPalpha*\(_{CJ5}\) and *ATPalpha*\(_{CJ10}\) [36]. The other *Drosophila* strains used were obtained from the Vienna Drosophila RNAi Center (VDRC) or Bloomington Stock Center.

Locomotor assays

F\(_1\) offspring heterozygous for an *ATPalpha* allele and each individual *Df* were collected upon eclosion (day 0) and aged at 25°C on cornmeal-molasses medium. Temperature sensitivity (TS) was assayed on day 1 and bang sensitivity (BS) was assayed on day 15 as described previously [23]. Aged flies were moved to an empty vial in groups of 5 or fewer using an aspirator. For TS, the vial was submerged in a water bath at 38°C such that the flies were restricted to space in the vial below the waterline. A timer was started when the vial was submerged and time to paralysis was recorded for each fly. For BS, the vial was mechanically shaken using a standard lab
Vortex Genie 2 (Daigger, IL) on the highest setting for 20 seconds. Time to recovery for each fly was recorded. Both conditional locomotor assays were stopped after 300 seconds.

**Df Interaction screen**

Males with autosomal deficiencies were mated to ATPalpha<sup>DTS1</sup>, ATPalpha<sup>C5</sup>, and ATPalpha<sup>C110</sup> virgin females, and X-linked deficiency virgin females were mated with ATPalpha<sup>DTS1</sup>, ATPalpha<sup>C5</sup>, and ATPalpha<sup>C110</sup> males. F<sub>1</sub> progeny representing a total of 386 deficiency interactions were tested with ATPalpha<sup>DTS1</sup> animals (83% of Df kit), 393 were tested with ATPalpha<sup>C5</sup> (84% of Df kit), and 358 were tested with ATPalpha<sup>C110</sup> animals (77% of Df kit). Each of the 467 Dfs we received was tested with at least one ATPalpha allele, the vast majority were tested with multiple alleles and >55% were tested with all three alleles. Assays were performed as described above.

**Verification screen**

Putative modifier Df strains identified in the initial screen were retested in an independent experiment to verify the findings and reduce the rate of false positives. In selecting Df strains to test again, we favored Dfs that suppressed ATPalpha mutant phenotypes and/or interacted with more than one ATPalpha allele. During the verification screen all three ATPalpha alleles were investigated.
Single gene identification

We developed an analysis called the Reproducibility Index (RI) in order to guide our search for single gene modifiers of the ATPalpha alleles. The goal of this index was to rank the most promising Df intervals based on the magnitude and reproducibility with which they modified an ATPalpha allele phenotype. To this end, we first calculated the number of standard deviations of the Df, ATPalpha* double mutant mean from the total mean of the primary screen of each ATPalpha mutant using:

\[
\text{Num.Std.Dev.(SD)} = \frac{\text{Mean}_{\text{total}} - \text{Mean}_{\text{Df}}}{\text{StdDev}_{\text{total}}}
\]

where StdDev_{total} is the standard deviation of all deficiencies in the primary screen, Mean_{total} is the mean of all deficiencies in the primary screen, and Mean_{Df} is the mean response of a Df/ATPalpha double mutant. Num.Std.Dev (#SD) was calculated for the mean response of a Df double mutant in the primary (#SD_{prim}) and verification (#SD_{veri}) screen. We reasoned that these values provide a normalized metric of how much a Df modified an ATPalpha phenotype in each trial. We used these values to calculate the RI:

\[
\text{RI} = |\#SD_{\text{prim}} + \#SD_{\text{veri}}| - AV / 2
\]

where

\[
\text{Absolute Variance}(AV) = |\#SD_{\text{prim}} - \#SD_{\text{veri}}|
\]

The RI increases for Dfs that were further from the total mean and decreases for Dfs that varied more across the two trials. Thus, a high RI suggests that a region is more likely to contain a gene that interacts with and modifies an ATPalpha allele in a reproducible manner. In some intervals we were able to use small Dfs to narrow the interval further. We, again, prioritized strongly suppressing intervals over enhancing intervals and intervals that interacted with multiple alleles. Single genes were selected from critical intervals using the G-Browse feature (an annotated genome) of flybase.org. In some very small intervals all genes in the region were tested. In large intervals we necessarily focused on genes with described expression within the nervous and or muscular systems, introducing a noted bias. Many of the alleles chosen were P-element or classical mutations reported to knockout the genes of interest. The stocks of interest were ordered from the Bloomington Stock Center.

RNAi analysis

When classical mutants were unavailable for certain loci or to confirm an interaction found using a classical mutant, RNAi analysis was used to examine the gene in question. RNAi stocks were ordered from the VDRC. The RNAi transgenes were driven using daughterless Gal4 strains (daGal4) in each ATPalpha mutant background. RNAi male flies were mated to ATPalpha*, daGal4 virgin females. Progeny were raised at 25°C, and TS and BS tests were performed as described previously.

Data collection and statistics

Data were collected and organized using Microsoft Excel (Redmond, WA). Data were analyzed in GraphPad Prism 5 (San Diego, CA). We used ANOVA to compare the ATPalpha mutant heterozygotes, the classical mutant heterozygotes, and flies heterozygous for both alleles. Tukey's multiple comparison test was performed to determine if the double mutants were significantly different from the ATPalpha mutant heterozygote and the classical mutant heterozygote. Adjusted p-values are
reported in Table 5. The effect of RNAi transgenes was analyzed using a Student’s t-test to determine if single gene knockdowns significantly modified the phenotype of ATP6alpha, daGal4 controls. Significant interactions are reported in Table 6.

Additional file

Additional file 1: Data from the primary and verification of D sequences.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ADT, JFC, AL and EDW performed the experiments. ADT, JFC, EDW and MJP analyzed the data. ADT, JFC, EDW and MJP wrote the manuscript. MJP designed and coordinated the study. ADT, JFC, AL, EDW and MJP reviewed, edited and approved the manuscript.

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