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Glial overexpression of *Dube3a* causes seizures and synaptic impairments in *Drosophila* concomitant with down regulation of the Na⁺/K⁺ pump ATP α

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Abstract

Duplication 15q syndrome (Dup15q) is an autism-associated disorder co-incident with high rates of pediatric epilepsy. Additional copies of the E3 ubiquitin ligase *UBE3A* are thought to cause Dup15q phenotypes, yet models overexpressing *UBE3A* in neurons have not recapitulated the epilepsy phenotype. We show that *Drosophila* endogenously expresses *Dube3a* (fly *UBE3A* homolog) in glial cells and neurons, prompting an investigation into the consequences of glial *Dube3a* overexpression. Here we expand on previous work showing that the Na⁺/K⁺ pump ATP α is a direct ubiquitin ligase substrate of *Dube3a*. A robust seizure-like phenotype was observed in flies overexpressing *Dube3a* in glial cells, but not neurons. Glial-specific knockdown of ATP α also produced seizure-like behavior, and this phenotype was rescued by simultaneously overexpressing ATP α and *Dube3a* in glia. Our data provides the basis of a paradigm shift in Dup15q research given that clinical phenotypes have long been assumed to be due to neuronal *UBE3A* overexpression.

Keywords

Duplication 15q syndrome; seizure; epilepsy; UBE3A; Dube3a; ATP α ; glia; mushroom bodies; synaptic transmission

Introduction

Duplication 15q syndrome (Dup15q) is caused by either interstitial or isodicentric duplications of the chromosomal region 15q11.2-q13.1. Dup15q is characterized by autism,

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developmental delay, mental retardation, hypotonia, and epilepsy. The most devastating feature of Dup15q is difficult to control seizures (Finucane et al., 2016). Approximately 65% of isodicentric Dup15q individuals have seizures that often begin as infantile spasms that progress to Lennox-Gastaut type syndrome (Battaglia, 2008, Finucane et al., 2016). Poorly controlled epilepsy severely impacts the quality of life of both affected individuals and their caregivers. Current treatment options for Dup15q-associated epilepsy are often ineffective. GABAergic promoting antiepileptics are typically ineffective while broad-spectrum antiepileptic medications such as valproic acid and rufinamide provide some relief (Conant et al., 2014). Elevated levels of *UBE3A* in neurons or duplication of a cluster of GABA receptors (*GABRA5*, *GABRB3*, and *GABRG3*) located within Chr 15q11.2-q13.1 have been proposed as the etiology of Dup15q epilepsy, although 15q copy numbers do not correlate with GABA receptor transcript levels (Scoles et al., 2011), and there is no robust experimental proof connecting increased expression of GABA receptors to seizure susceptibility.

Multiple mouse models have been generated to investigate Dup15q pathobiology, yet no model has recapitulated the seizure phenotypes observed in Dup15q individuals. Mice harboring a 6.3 Mb duplication of Chr 7 (syntenic to the 15q11.2-q13.1 region in humans) displayed social abnormalities and behavioral inflexibility when duplications were paternally inherited (Nakatani et al., 2009), although Dup15q syndrome results primarily from maternally inherited duplications. Another mouse model duplicating only *Ube3a* recapitulated deficits in social interaction, social communication, and increased repetitive behavior but it is likely that the addition of a 3xFLAG tag to the C-terminus of *Ube3a* rendered the ubiquitin ligase activity of Ube3a-FLAG protein non-functional (Smith et al., 2011). More recently, overexpression of *Ube3a* limited to neurons was not associated with seizures in mice but was linked to seizure-induced decreased sociability (Krishnan et al., 2017). Elevated levels of *Ube3a* in excitatory neurons in mice caused increased anxiety-like behaviors, cognitive impairments, and reduced seizure threshold to pharmacologically induced seizures with pentylenetetrazol, however spontaneous seizures have not been observed in these mice (Copping et al., 2017). In summary, mouse models of Dup15q which focus on neuronal overexpression of *Ube3a* have failed to recapitulate the spontaneous Dup15q seizure phenotype.

In contrast to neurons, glial cells biallelically express *Ube3a* (Yamasaki et al., 2003, Dindot et al., 2008, Judson et al., 2014, Grier et al., 2015). Analysis of post-mortem brain tissue from Dup15q individuals found elevated levels of *UBE3A* transcript and protein (Scoles et al., 2011). Even though glia play a key role in epileptogenesis through regulation of ion homeostasis (Chvatal and Sykova, 2000, D'Ambrosio, 2004, Devinsky et al., 2013), the functional consequence of increased glial UBE3A has been unexplored in Dup15q syndrome. Due to the absence of seizures in Dup15q mouse models, which have primarily focused on elevating *Ube3a* expression in neurons, we hypothesized that increased glial expression of UBE3A may contribute to the pathogenesis of epilepsy in Dup15q syndrome.

A related neurogenetic disorder, Angelman syndrome (AS), is also characterized by a high rate of epilepsy (Williams et al., 2006, Thibert et al., 2009). AS is caused by reciprocal deletions of the maternal 15q11.2-q13.1 region, methylation defects, or loss-of-function

point mutations which result in non-functional UBE3A (LaSalle et al., 2015). Due to the well-established link between *UBE3A* loss and seizures in AS, we predict that *UBE3A* may similarly underlie seizures in Dup15q.

The primary role of UBE3A, an E3 ubiquitin ligase, is to transfer ubiquitin moieties from an E2 ligase to substrate proteins, targeting them for degradation by the ubiquitin proteasome system (Finley, 2009, Zheng and Shabek, 2017). Several investigative teams, including our own, have long been searching for UBE3A substrates relevant to Dup15q and AS phenotypes. However, due to the transient nature of the interaction between an E3 ubiquitin ligase and its substrate proteins, only a handful of confirmed UBE3A substrates have emerged thus far (LaSalle et al., 2015). As a result of the limited number of UBE3A substrates currently identified, the molecular mechanism of seizure production in Dup15q (and AS) individuals remains largely unknown.

Our laboratory previously used the genetically tractable model organism *Drosophila melanogaster* to identify Dube3a (the fly homolog of *UBE3A*) substrates in whole head fly extract following a transient elevation of Dube3a levels in all cell types (Jensen *et al.*, 2013). We identified approximately 50 proteins whose expression was altered by increased *Dube3a* expression. One of these proteins was the sodium/potassium (Na^+/K^+) exchanger ATP α , and we confirmed that ATP α is ubiquitinated in a Dube3a-dependent manner *in vitro* (Jensen *et al.*, 2013). Prior work demonstrated that *Drosophila* with reduced levels of ATP α protein display a bang-sensitive seizure phenotype (Schubiger et al., 1994, Sun et al., 2001, Palladino et al., 2003). Glial specific knockdown of the β subunit of the Na^+/K^+ pump *nrv2* also results in seizure-like behaviors (Ng et al., 2016), thereby supporting the idea that impaired glial-dependent regulation of extracellular ion concentration contributes to the pathogenesis of seizures. Na^+/K^+ homeostasis is critically important for proper central nervous system function, while perturbation of Na^+/K^+ balance has been identified as an underlying cause of epilepsy in humans (Somjen, 2002, Scharfman, 2007). Here, we follow up on the consequences of the interaction between Dube3a and ATP α in an attempt to understand the underlying mechanism of seizures in our novel fly model of Dup15q epilepsy.

Materials and Methods

Fly Stocks

All flies were maintained at 25°C on a 12-hour light/dark cycle and raised on standard corn meal media (Bloomington Stock Center). The following stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN): UAS-*ATP α -RNAi* (BDSC# 32913, RRID:BDSC_32913), *10XUAS-IVS-myr::tdTomato* (referred to as UAS-*tdTomato*, BDSC#32222, RRID:BDSC_32222) and *repo-GAL4* (BDSC# 7415, RRID:BDSC_7415). The *ATP α ::GFP* reporter line was generated by the FlyTRAP project (Morin et al., 2001), and the UAS-*ATP α* line (RRID:FlyBase_FBst0500173) was obtained from FlyORF (Bischof et al., 2013). The following *Dube3a* and *UBE3A* lines were used in this study and described previously (Reiter et al., 2006): UAS-*Dube3a*, UAS-*Dube3aC/A*, and UAS-*UBE3A*. *elav-GAL4* was provided by Dr. Hugo Bellen and UAS-*GFP* was provided by Dr. Cynthia Hughes. See Table S1 for a complete list of stocks.

Quantitative Western Blot Analysis

30–40 fly heads were removed and homogenized in RIPA buffer containing complete protease inhibitor cocktail (Roche). Samples were spun at $9,500 \times g$ for 1 minute to remove debris and the supernatant containing protein was used for analysis. Samples were resolved by loading 20 μg of protein into each lane of a 1.5 mm NuPAGE Bis-Tris 4–12% gradient gel (Invitrogen) and transferred to Immobilon-FL PVDF membrane (Millipore). Membranes were blocked with 5% bovine serum albumin, 3% milk, and 0.1% Tween-20 in phosphate buffered saline (PBS). Primary antibodies used were α -GAPDH (1:5000, IMGEX Cat# IMG-3073, RRID:AB_316883), α -ATP α (1:5000, Developmental Studies Hybridoma Bank Cat# A5-S, RRID:AB_2166869) and α -ELAV (1:200, Developmental Studies Hybridoma Bank Cat# 7E8A10, RRID:AB_528218). α -goat (LI-COR Cat# 925-68074, RRID:AB_2650427 or LI-COR Cat# 926-32214, RRID:AB_621846), α -rat (LI-COR Cat# 926-68076, RRID:AB_10956590), and α -mouse (LI-COR Cat# 926-32212, RRID:AB_621847) infrared secondary antibodies were used at 1:7500. The blot was imaged using the Odyssey Infrared Imaging System (Li-Cor) and all lanes were normalized to a GAPDH loading control as the reference for calculating adjusted relative signal intensities.

Immunohistochemistry and Image Acquisition

Flies were briefly anesthetized with CO₂, heads were removed and submerged in a dissecting dish containing phosphate buffered saline (PBS). Brains were dissected and fixed in 4% formaldehyde for 1 hour. Following 3 washes for 5 minutes in PBS, brains were blocked and permeabilized with 5% bovine serum albumin and 0.1% Triton X-100. Primary antibodies used in this study were mouse α -repo (Developmental Studies Hybridoma Bank Cat# 8D12, RRID:AB_528448, 1:100), mouse α -brp (Developmental Studies Hybridoma Bank Cat# NC82, RRID:AB_528108, 1:100), rat α -Dube3a (Gift from Dr. Janice Fischer, 1:100), mouse α -FasII (Developmental Studies Hybridoma Bank Cat# 1D4, RRID:AB_528235, 1:100), and rabbit α -GFP (Abcam Cat# ab6556, RRID:AB_305564, 1:1000). Secondary antibodies used were AlexaFluor 594 goat α -mouse secondary antibody (ThermoFisher Cat# A11005, RRID:AB_141372, 1:500), AlexaFluor 488 goat α -rat (ThermoFisher Cat# A11006, RRID:AB_2534074, 1:500), and AlexaFluor 488 goat α -rabbit (ThermoFisher Cat# A11008, RRID:AB_143165, 1:500). Slides were mounted with Vectashield mounting medium (Vector Labs Cat# H-1200).

Images were captured on a Zeiss 710 confocal microscope (Zeiss) in the UTHSC Neuroscience Institute Imaging Core at 1024×1024 resolution with a 63X objective, numerical aperture 1.4. Z-sections were acquired at 1 μm optical section thickness. Detector gain and offset were optimized to the control group and were not changed between brains once the configuration was set to allow for direct comparisons. Images were acquired with ZEN Software (Zeiss).

Co-localization Analysis

Co-localization analysis was performed using the ImageJ plugin Coloc 2 (Schneider et al., 2012). A $50 \mu m \times 50 \mu m$ section was selected from ATP α ::GFP brains stained with α -GFP and α -Dube3a antibodies for analysis.

Seizure Susceptibility Assays

For all behavioral tests, flies were not exposed to CO₂ for at least two days prior to testing. BSA was performed as previously described (Benzer, 1971, Ganetzky and Wu, 1982, Stone et al., 2014). Flies were transferred via mouth pipette to empty standard fly vials and subjected to mechanical stress (“bang”) by vortexing the vial on a standard laboratory vortexer (LabNet) at top speed for 10 seconds. At the end of the “bang”, a timer was started and the duration of paralysis and uncontrolled movements was recorded. Flies were considered “recovered” when they righted themselves and were able to walk or groom.

For heat induced paralysis assays, flies were transferred to empty plastic fly vials and the vials were submerged in a 39°C water bath. The number of immobile flies was recorded in 30-s intervals and the assay continued for a total of 6 min.

For photogenic-induced paralysis, flies were transferred to empty plastic fly vials and placed in a dark room for 30 min prior to testing. Vials were placed 5 cm from a strobe light (C4162 High Power Strobe, Chaney Electronics) and subjected to 10 Hz stimulation for 10 s. Recovery time was recorded and was defined by the same parameters as for BSA.

Chemical Inhibition of ATP_α Function

Flies were fed ouabain (Sigma, 03125) using a previously described method (Sun et al., 2001). Briefly, three-day old flies were fed 10 mM ouabain dissolved in water containing 5% sucrose for three hours by placing flies in an empty vial with containing a Kimwipe saturated with ouabain solution. Green food coloring (1%) was included in the feeding solution to ensure flies consumed the ouabain solution, as indicated by their abdomens turning green. Following three hours of feeding, flies were tested for seizure susceptibility in the BSA.

Glial Cell K⁺ Content Assay

Stock solutions of 1 mM Asante Potassium Green 2 (APG-2, TEFlabs, a fluorescent cell membrane permeable K⁺ indicator) were prepared in DMSO. Three-day old *repo>tdTomato* or *repo>Dube3a+tdTomato* flies were briefly anesthetized with CO₂ and their heads were removed and submerged in room temperature Drosophila saline consisting of the following (in mM): 128 NaCl, 1.8 CaCl₂, 2 KCl, 5 MgCl₂, 36 sucrose, 5 HEPES, pH 7.2. Brains were dissected from the head case and incubated with 7.5 μM APG-2 in Drosophila saline at room temperature with gentle agitation for 1 hour. Brains were washed 2X with Drosophila saline and wet mounted on a microscope slide with Drosophila saline.

Images were captured from the optic lobe on a Leica DM6000B microscope (Leica) using a 63X oil immersion lens using the N3 and L5 filters for tdTomato and APG-2 respectively. Exposure times were calibrated on *repo>tdTomato* brains and microscope settings remained constant between *repo>tdTomato* and *repo>Dube3a+tdTomato* groups to allow for direct comparison of fluorescence intensity. APG-2 fluorescence intensity was calculated offline using ImageJ (Schneider et al., 2012). The APG-2 channel was converted to 8-bit greyscale, and tdTomato-positive glial cells were selected for analysis. Cells were selected using the “freeform” tool to outline the cell body in the APG-2 channel. Using the “measure”

command, the mean fluorescence value for each cell was recorded. A total of 40 cells was analyzed per group from at least 3 images per group and 3 brains per group.

Electroretinogram Analysis

Electroretinograms (ERGs) were performed as previously described (Hope et al., 2016). Briefly, custom-made tungsten wire electrodes were gently inserted into the right eye (recording) and abdomen (ground) of immobilized flies. Fly eyes were stimulated using 1-sec light pulses from a 5-mm LED (RadioShack). Data was collected with a Model 1800 Microelectrode AC Amplifier (A-M Systems), digitized with a Micro3 1401 digitizer (Cambridge Electronic Design), and analyzed with Spike2 software (Cambridge Electronic Design).

Electron Microscopy

Fly heads were dissected and fixed in 2.5% glutaraldehyde solution overnight at 4°C. Fixed heads were processed, resin embedded, and sectioned (50 nm) by the UTHSC Neuroscience Institute Core. A JEM-2000EXII microscope (JEOL USA, Inc.) at 60 kV was used to capture transmission electron microscopy images at 10,000X.

Statistical Analysis

All statistical tests were performed using Prism version 6.0 (GraphPad). To test for statistical significance of two samples, Student's *t*-test was used. To determine statistical significance among multiple samples, we used a one-way ANOVA with Tukey's multiple comparisons. An alpha (α) of 0.05 was chosen for statistical significance.

Results

Glia endogenously express both *Dube3a* and ATP α proteins

Dube3a is expressed broadly in neurons of the fly larval and adult brain (Wu et al., 2008). Our laboratory recently demonstrated that *Dube3a* is not subjected to the complex imprinting observed in mammals and is biallelically expressed in fly heads (Hope et al., 2016). *UBE3A* is known to be expressed solely from the maternal allele in mature mammalian neurons and biallelically expressed in mammalian glial cells (Yamasaki et al., 2003, Dindot et al., 2008, Judson et al., 2014, Grier et al., 2015). To determine if glial cells express *Dube3a*, we performed immunohistochemistry for the glial-specific transcription factor reversed polarity (Repo) and *Dube3a* in wildtype adult fly brains. We observed co-localization of Repo and *Dube3a* signals indicating that glial cells endogenously express *Dube3a* (Fig. 1A).

Our laboratory previously found that the Na⁺/K⁺ pump ATP α is ubiquitinated in a *Dube3a*-dependent manner using an *in vitro* ubiquitination assay (Jensen et al., 2013). For *Dube3a* to ubiquitinate ATP α *in vivo*, the two proteins must be present in the same cell. Therefore, we performed immunohistochemistry on brains from ATP α ::GFP flies with antibodies against both GFP and *Dube3a* to determine if ATP α and *Dube3a* proteins co-localize *in vivo*. We observed a lattice-like staining pattern of ATP α ::GFP in the optic lobes and relatively similar, yet more diffuse *Dube3a* staining patterns which overlapped with ATP α ::GFP in

several regions of the brain (Fig. 1B and 1C). Quantitative co-localization analysis indicated strong overlap of ATP α and Dube3a proteins ($r = 0.78$) (Fig. S1). These data suggest that ATP α and Dube3a are present in at least some of the same cells in adult fly brain.

To determine whether Dube3a is able to regulate ATP α *in vivo*, we utilized the GAL4/UAS system (Brand and Perrimon, 1993) to overexpress *Dube3a* in either neurons with *elav-GAL4* or glia with *repo-GAL4*. We compared ATP α levels from fly heads via western blot from control *uas-Dube3a* alone, *elav>Dube3a*, and *repo>Dube3a* animals (Fig. 1D). There was a significant overall effect of genotype on ATP α levels (Fig. 1E, One-way ANOVA, $p = 0.001$). Post-hoc comparisons indicated that ATP α levels were significantly reduced in *elav>Dube3a* compared to *uas-Dube3a* alone ($p = 0.05$) and in *repo>Dube3a* compared to *uas-Dube3a* alone ($p = 0.001$). These results are consistent with our previous studies indicating that Dube3a can regulate ATP α levels in the brain in a ubiquitin dependent manner.

Overexpression of *Dube3a* in glia using *repo-GAL4* results in seizure susceptibility

During our *repo>Dube3a* experiments, we serendipitously observed a bang-sensitive phenotype in *repo>Dube3a* flies (Supplemental Movie 1). To quantify this observation, we performed a series of seizure assays in both *repo>Dube3a* and *elav>Dube3a* animals. Utilizing the well-characterized bang sensitivity assay (BSA) (Benzer, 1971, Ganetzky and Wu, 1982, Stone et al., 2014), we subjected flies to mechanical stress and measured time to recovery. Control *repo-GAL4* alone or neuronal overexpression *elav>Dube3a* flies did not display a bang-sensitive phenotype, whereas glial *Dube3a* overexpression with *repo>Dube3a* resulted in robust bang-induced paralysis (Fig. 2A). We did not observe bang-sensitivity when we expressed catalytically-inactive ligase-dead *Dube3a* in glia using *repo>Dube3aC/A* (Fig. 2A), indicating that bang sensitivity requires the ubiquitin ligase activity of *Dube3a*. Additionally, we observed both heat-induced and photogenic paralysis in *repo>Dube3a* animals (Fig. S2A and S2B), indicating that seizures may be initiated by multiple modalities in *repo>Dube3a* flies.

To evaluate the developmental time course of seizure susceptibility in *repo>Dube3a* we measured bang-sensitivity at 0–2 days, 3–6 days, and 7–10 days post-eclosion. We found that approximately 50% of *repo>Dube3a* flies were bang-sensitive at 0–2 days post-eclosion and by 3–6 days all *repo>Dube3a* flies showed bang-sensitivity. By 7–10 days, no *repo>Dube3a* flies recovered from the bang within the 2-min assay period (Fig. 2B). These results indicate that seizures develop over time in adult flies overexpressing Dube3a in glia, and are therefore not developmentally fixed when flies eclose from their pupal cases.

Because fly *Dube3a* and human *UBE3A* are greater than 70% homologous in the C-terminal HECT domain but display less homology at the N-terminus (Reiter et al., 2006), we next wanted to determine if overexpression of human *UBE3A* in fly glia also resulted in seizure susceptibility. In *repo>UBE3A* flies, we observed a similar but less severe bang-sensitivity that progressively worsened as flies aged just as *repo>Dube3a* seizure severity progressively worsened in adult animals (Fig. 2C). Moreover, *repo>UBE3A* flies also demonstrated heat-induced paralysis, although heat-sensitivity was less severe than in *repo>Dube3a* animals (Fig. S2A). These experiments demonstrate that human UBE3A can emulate fly Dube3a in

our Dup15q model implying shared substrate specificity between flies and humans for this E3 ubiquitin ligase.

Genetic and Pharmacologic manipulation of ATP α modifies seizure susceptibility

In order to establish that Dube3a mediated degradation of ATP α is the primary cause of seizures, we investigated ATP α protein stability and abundance in the brain under various conditions. We performed quantitative western blot analysis of ATP α levels in flies overexpressing human *UBE3A* in glial cells to determine if ATP α is downregulated by human UBE3A protein, and on flies co-overexpressing *ATP α* on a *repo>Dube3a* background to determine whether downregulation of ATP α could be genetically rescued (Fig. 3A). There was a marked effect of genotype (Fig. 3B, One-way ANOVA, $p = 0.0001$) but no significant differences between control *repo-GAL4* alone and ligase-dead overexpression *repo>Dube3aC/A*, indicating the downregulation of ATP α is dependent upon the ubiquitin ligase activity of Dube3a. Again, we observed significant downregulation of ATP α in *repo>Dube3a* animals ($p = 0.01$), similar to our earlier results (Fig. 1D and 1E). No significant differences were observed in ATP α levels between control and ATP α overexpression animals (*repo>Dube3a+ATP α*) indicating that ATP α can be restored to near normal protein levels by simultaneously overexpressing *ATP α* in *repo>Dube3a* flies (Fig. 3A and 3B). Overexpression of human *UBE3A* in *repo>UBE3A* flies resulted in a similar downregulation of ATP α (Fig. 3A and 3B, $p = 0.001$) again suggesting conserved ubiquitin ligase substrates between fly *Dube3a* and human *UBE3A*.

We next performed BSA on *repo>Dube3a+ATP α* flies to determine if restoring ATP α levels specifically within glial cells of *repo>Dube3a* animals was capable of rescuing seizure behavior. Compared to *repo>Dube3a* flies, *repo>Dube3a+ATP α* animals had significantly reduced seizure severity as indicated by reduced seizure time on the BSA (Fig. 3C, t -test, $p = 0.005$). Therefore, reduced ATP α protein levels are necessary for seizure susceptibility in *repo>Dube3a* flies.

The cardiotonic steroid ouabain is a known allosteric inhibitor of the Na⁺/K⁺ pump function of ATP α . We predicted that *repo>Dube3a* animals with reduced ATP α levels would be more susceptible to chemical ATP α inhibition and display longer recovery times on the BSA. We fed flies 10 mM ouabain for 3 hours (a dose low enough to have no effect on control flies) and performed BSA (Fig. 3D, t -test, $p = 0.05$). In *repo>Dube3a* animals there was a significant increase in recovery time on the BSA (Fig. 3D, t -test, $p = 0.005$). These experiments confirm the connection between seizure susceptibility and ATP α function.

If Dube3a-dependent reduction in ATP α is responsible for seizures in *repo>Dube3a* flies, then glial-specific knockdown of ATP α should also result in seizure susceptibility. Loss-of-function ATP α mutants are known to show seizure susceptibility, but these mutants have reduced ATP α throughout all tissues in the body (Schubiger et al., 1994). To determine if glial-specific knock down of ATP α is sufficient to cause seizures, we crossed *repo-GAL4* flies to *UAS-ATP α -RNAi* flies and performed the BSA. We observed significantly increased recovery times in *repo>ATP α -RNAi* flies compared to controls (Fig. 3E, t -test, $p = 0.005$), showing that glial-specific reduction of ATP α levels is sufficient to generate a seizure

phenotype, albeit less severe than that found in *repo>Dube3a* animals. This also implies that additional Dube3a glial substrates may contribute to the seizure phenotype.

Glial *Dube3a* overexpression alters neuronal architecture

We examined the mushroom body (MB) in adult flies to investigate the morphological effects of glial *Dube3a* overexpression. Immunohistochemistry was performed with an α -FasII antibody in *repo>GFP* and *repo>GFP+Dube3a* animals. The α lobe of the mushroom body normally extends dorsally in a single column and the γ lobe displays FasII immunoreactivity as seen in control *repo>GFP* brains (Fig. 4A, top row). In the MB of *repo>GFP+Dube3a* flies we observed a loss of FasII immunoreactivity in the γ lobe and a misdirected portion of the α lobe (Fig. 4A, bottom row, arrows). These results indicate that elevated levels of Dube3a in glia disrupt proper formation of the MB.

To further investigate neuronal architecture in glial *Dube3a* overexpression animals we stained adult fly brains with α -brp antibody to visualize synaptic organization (Wagh et al., 2006). The optic lobe (OL) of *Drosophila* displays a laminar structure with the outer medulla (OMe) and inner medulla (IMe) separated by a column of glial cells from the lobular plate (LP) (Fig. 4B, top row). Upon overexpression of *Dube3a* in glia we observed a similar laminar structures in the OL of *repo>GFP+Dube3a* brains, however α -brp immunoreactivity was observed in the GFP⁺ column of glial cells separating the IMe from the LP which was not observed in control flies (Fig 4B, bottom row, arrow). Again, these results indicate that overexpression of *Dube3a* in glial cells results in abnormal development of neuroanatomical structures.

Western blot analysis of the neuronal-specific transcription factor embryonic lethal abnormal vision (*elav*) was used to determine if reduced levels of ATP α in *repo>Dube3a* flies was a consequence of reductions in the total number of fly neurons in the fly brains. We did not observe a difference in *elav* protein levels between *repo-GAL4* and *repo>Dube3a* animals (Fig. S2, *t*-test, *p* = 0.05), suggesting that neuronal loss is not the primary cause of seizures in *repo>Dube3a* flies.

Overexpression of *Dube3a* in glia, but not neurons, impairs photoreceptor neuron function

Seizures are thought to be primarily caused by excitatory/inhibitory imbalances in neurons. Functional changes in neuronal activity due to changes in *Dube3a* have been studied at the fly larval neuromuscular junction (Valdez et al., 2015), but synaptic transmission defects caused by overexpression of Dube3a have not been investigated in adult flies. Since seizures are caused by abnormal neuronal activity (Wu et al., 2015), we next wanted to directly measure neuronal function in the fly. Electroretinograms (ERGs) have long been used to measure synaptic activity and photoreceptor neuron function in the fly eye (Ugur et al., 2016). Using ERGs, we observed a significant effect of *Dube3a* or *UBE3A* overexpression in both “on” transients (Fig. 5A and 5B, One-way ANOVA, *p* = 0.0001) and “off” transients (Fig. 5C, One-way ANOVA, *p* = 0.0001), as well as photoreceptor potentials (Fig. 5A and 5D, One-way ANOVA, *p* = 0.0001). Tukey’s multiple comparison test (α = 0.05) indicated no effect of neuronal overexpression of *Dube3a* in *elav>Dube3a* animals on any ERG component, though there was a significant difference between *repo>Dube3a* and

repo>UBE3A compared to control *repo-GAL4* alone flies in on/off transients and photoreceptor potentials. Again, no differences were observed between *repo>Dube3aC/A* in any ERG component (Fig. 5A–5D), indicating the effects of *Dube3a* overexpression in glia are due to the ubiquitin ligase activity of Dube3a. Thus, *Dube3a* overexpression in glia, but not neurons, causes downstream synaptic defects in neurons in a cell non-autonomous manner.

To further investigate these ERG defects, we conducted an ultrastructural analysis of the fly eye using transmission electron microscopy. Ommatidial cross-sections of control *repo-GAL4* and *repo>Dube3a* animals revealed that both groups had similar stereotypic arrangements of photoreceptor neurons (Fig. S3). In addition, secondary pigment glial cells are present and directly contact photoreceptor neurons in both *repo-GAL4* and *repo>Dube3a* flies (Fig S3, **arrows**), indicating abnormal ERGs do not arise from gross photoreceptor defects or a lack of physical interaction between supporting glial cells and photoreceptor neurons.

Glial-specific overexpression of Dube3a causes reduced intercellular K⁺ in glial cells

The function of ATPα is to pump K⁺ into the cell while pumping Na⁺ into the extracellular space in an ATP-dependent manner. Since *repo>Dube3a* animals have reduced levels of ATPα, we predicted that glial cells in the fly brain would have reduced intracellular K⁺ concentrations. We dissected whole brains from *repo>tdTomato* or *repo>tdTomato+Dube3a* animals to visualize glia and incubated them with the cell-permeant fluorescent K⁺ indicator Asante Potassium Green 2 AM (APG-2). We then quantified the fluorescence of APG-2 in tdTomato-positive cells to determine the relative concentration of K⁺ in *repo>tdTomato* and *repo>tdTomato+Dube3a* glial cells. We observed a significant reduction in APG-2 fluorescence in *repo>tdTomato+Dube3a* compared to control *repo>tdTomato* glial cells (Fig. 5E and 5F, *t*-test, *p* = 0.01). These data indicate that elevated Dube3a levels within glia reduce internal K⁺, consistent with the hypothesis that Dube3a downregulates ATPα levels causing a disruption of Na⁺/K⁺ homeostasis.

Discussion

To date, animal models of Dup15q focused on neuronal overexpression of *UBE3A* have failed to recapitulate the seizure phenotypes observed in Dup15q. Our fly model of Dup15q with elevated levels of *Dube3a* in glia, but not neurons, displays a robust seizure phenotype, highlighting the previously unappreciated role that glia play in Dup15q pathophysiology. Our lab previously demonstrated that *Dube3a* is biallelically expressed in the *Drosophila* brain (Hope et al., 2016), and here we demonstrate that *Drosophila* glia endogenously express *Dube3a* in addition to expression in neurons. Studies in mammalian systems have indicated biallelic expression of *Ube3a/UBE3A* in glial cells (Yamasaki et al., 2003, Dindot et al., 2008, Judson et al., 2014, Grier et al., 2015), yet until now the functional consequence of overexpression of *Ube3a/Dube3a* in glial cells has been largely unexplored. Increased dosage of the E3 ubiquitin ligase UBE3A is thought to be the underlying cause of at least the autistic features of Dup15q syndrome (LaSalle et al., 2015), and evidence presented here suggests that elevated UBE3A contributes to epilepsy in Dup15q as well.

Drosophila harboring loss-of-function mutations in ATPa have previously shown a bang-sensitive phenotype (Schubiger et al., 1994, Sun et al., 2001, Palladino et al., 2003), and bang-sensitivity is a simple yet robust measure of seizure-like activity in flies (Benzer, 1971, Ganetzky and Wu, 1982, Stone et al., 2014). Here we utilized the GAL4/UAS system (Duffy, 2002) to selectively knockdown ATPa levels in glial cells. This approach revealed the importance of Na⁺/K⁺ regulation within glial cells on proper central nervous system function, as glial-specific knockdown of ATPa generated a bang-sensitive seizure phenotype. Glial dysfunction is well-recognized as a major contributor to epilepsy (Wetherington et al., 2008, Devinsky et al., 2013), and the data presented herein suggests that glial dysfunction contributes to the pathogenesis of epilepsy in Dup15q.

One role of glial cells is to maintain ion homeostasis in the central nervous system. Normally, K⁺ is pumped into glial cells via ATPa to maintain relatively low concentrations of K⁺ in the extracellular space (Fig. 6A). When Dube3a (or UBE3A) is elevated in glia, ATPa levels are reduced, thus reducing the amount of intracellular K⁺ within glial cells and increasing the concentration of K⁺ in the extracellular space (Fig. 6B). We propose that elevated extracellular K⁺ leads to the neuronal dysfunction and seizure behavior observed in *repo>Dube3a* flies. Na⁺/K⁺ dysregulation has emerged as a potential cause of epilepsy as elevated levels of extracellular K⁺ lead to more positive neuronal membrane potentials, tipping neuronal networks into hyper-excitable states and precipitating seizure activity (Scharfman, 2007, Seifert and Steinhauser, 2013). Our data indicate both reduced levels of the Na⁺/K⁺ pump and reduced intracellular K⁺ levels in glial cells of *repo>Dube3a* animals which is likely the underlying mechanism for seizure susceptibility in our fly model, and potentially Dup15q syndrome where UBE3A levels are known to be elevated (Scoles et al., 2011).

The glial specific transcription factor *reversed polarity (repo)* was initially identified in a screen for mutants affecting the physiology of the ERG signal, and *repo* mutants displayed an ERG with a positive polarity rather than the typical negative polarity upon photoreceptor stimulation (Xiong et al., 1994). Pigment glial cells of the eye ensheath photoreceptor neuron cell bodies while epithelial glia surround axon terminals (Edwards and Meinertzhagen, 2010). These glial cells in the *Drosophila* eye strongly express ATPa (Gorska-Andrzejak, 2013). It is not surprising then that overexpression of *Dube3a* specifically within glial cells using the *repo*-GAL4 driver line causes defects in the ERG signal, potentially through the downregulation of ATPa.

Identifying disease-relevant substrates of UBE3A has remained elusive. We previously identified ATPa as a ubiquitin ligase substrate of Dube3a (Jensen et al., 2013), and here we demonstrate that overexpression of *Dube3a* in both glia and neurons results in reduced levels of ATPa, confirming our previous work. Glial specific knockdown of ATPa recapitulates a portion of the seizure phenotype observed in *repo>Dube3a* animals, indicating that reduced levels of ATPa in glia alone is sufficient to generate seizures. Even though we did not detect a significant difference in ATPa protein levels between *repo*-GAL4 and *repo>Dube3a* +ATPa, we did not fully rescue the seizure phenotype in *repo>Dube3a*+ATPa compared to *repo>Dube3a* flies. It is possible that slight decreases in ATPa protein levels undetectable by western blot may still result in seizure susceptibility, or that other unidentified proteins and

pathways regulated by Dube3a contribute to seizure. These other targets may contribute substantially to the variability in seizure severity in Dup15q syndrome (Battaglia, 2008, Hogart et al., 2009). Taken together, reduced levels of ATP α due to elevated levels of *Dube3a* seems to be driving a significant portion of the seizure phenotype observed in *repo>Dube3a* flies.

Human UBE3A and Drosophila Dube3a are approximately 70% homologous at the C-terminus. The two proteins are less conserved in the N-terminus which is believed to determine substrate protein binding (Cooper et al., 2004, Scheffner and Kumar, 2014). We observed reductions in ATP α levels in both *repo>Dube3a* and *repo>UBE3A* flies, suggesting that ATP α is a substrate of both fly Dube3a and human UBE3A. The ability of UBE3A to downregulate ATP α is similar to or possibly exceeds the ability of Dube3a to downregulate ATP α , yet we observed less severe seizure susceptibility in *repo>UBE3A* compared to *repo>Dube3a* flies. As stated above, this discrepancy in seizure severity may result from other substrate proteins or pathways that are not conserved between Dube3a and UBE3A which contribute to seizure susceptibility and variability. Indeed, we observed a partial rescue of seizure susceptibility when ATP α levels were restored to near normal levels in *repo>Dube3a* flies, indicating that downregulation of ATP α plays a large role in seizure susceptibility in *repo>Dube3a* flies. However, seizures were not fully rescued by restoring ATP α levels, suggesting that mechanisms other than the downregulation of ATP α , which may not be shared by Dube3a and UBE3A, contribute to seizure susceptibility. This data is further supported by our observations that glial specific knockdown of *ATP α* generates a seizure phenotype similar in severity to *repo>UBE3A* flies, both of which are less severe than *repo>Dube3a* flies. It is reasonable to propose, therefore, that mechanisms contributing to seizure other than downregulation of ATP α remain to be identified.

Mammals have 3 homologs to *ATP α* : *ATP1A1*, *ATP1A2*, and *ATP1A3*. While *ATP1A1* is ubiquitously expressed and *ATP1A3* is primarily found in neurons, *ATP1A2* is predominantly expressed in astrocytes (McGrail et al., 1991). *ATP1A2* mutations cause familial hemiplegic migraine type 2 with epileptic seizures (OMIM 602481) (De Fusco et al., 2003, Vanmolkot et al., 2003, Spadaro et al., 2004, Swoboda et al., 2004, Jurkat-Rott et al., 2004, Deprez et al., 2008, Gallanti et al., 2008), providing further evidence that impaired Na⁺/K⁺ pump function in glial cells causes epilepsy. It remains to be determined whether Na⁺/K⁺ pump levels are affected in the brains of individuals with Dup15q.

In *repo>Dube3a* flies we observed abnormal neuroanatomical structures in both the MB and OL. Previous reports have indicated abnormal MB morphology in homozygous *Dube3a* loss-of-function flies (Chakraborty et al., 2015). Here we demonstrate that glial overexpression of *Dube3a* causes MB defects in a cell non-autonomous manner. Hippocampal heterotopias and malformations have been observed in Dup15q individuals antemortem via MRI and postmortem (Wegiel et al., 2012, Boronat et al., 2015). Our data suggests that elevated glial expression of UBE3A may contribute to the development of hippocampal heterotopias and malformations observed in Dup15q syndrome. Glia play a crucial role in the development and organization of neural circuits (Stiles and Jernigan, 2010), such that altering glial function could profoundly impact neuronal pathfinding,

altering the structure of the central nervous system and affecting neuro-behavioral phenotypes such as autism or intellectual disability in Dup15q individuals.

This is the first model of Dup15q syndrome that recapitulates the seizure phenotype observed in Dup15q individuals as currently available mouse models do not develop epilepsy (Nakatani et al., 2009, Smith et al., 2011, Krishnan et al., 2017). Dup15q mouse models have thus far focused on overexpression of *Ube3a* in neurons, since *Ube3a* is only imprinted in neurons while glia, which biallelically express *Ube3a*, have been largely ignored. During early experiments we serendipitously observed a bang-sensitive seizure phenotype in *repo>Dube3a* animals simply by placing a fly vial on the bench top or opening the incubator door, indicating a relatively severe seizure phenotype. Individuals with Dup15q syndrome often manifest a pharmacologically resistant form of epilepsy that progressively worsens over time, exhibiting infantile spasms which progress to Lennox-Gastaut type syndrome (Finucane et al., 2016). *Repo>Dube3a* animals displayed a relatively mild seizure phenotype at 0–2 days of age but by 7–10 days displayed a severe seizure behavior, similar to the progression of seizures in Dup15q syndrome. These observations imply that seizures are not fixed but have a developmental trajectory, suggesting that early intervention strategies may be capable of preventing or reversing seizures.

The data presented here indicate that glial cells, not neurons, are the key cell type responsible for seizure development in Dup15q syndrome through dysregulation of ATP α -mediated K⁺ homeostasis. Recognition that glial cells contribute to the pathophysiology and clinical manifestations of Dup15q syndrome represents a paradigm shift in the study of Dup15q syndrome, UBE3A, and autism in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of interest: none.

Abbreviations

Dup15q	Duplication 15q syndrome
GABA	Gamma-aminobutyric acid
<i>Ube3a</i>	Ubiquitin-protein ligase E3A (also known as E6AP)
<i>Dube3a</i>	Drosophila homolog of <i>Ube3a</i>

AS	Angelman syndrome
ATPα	Na ⁺ , K ⁺ -ATPase α -subunit
MB	Mushroom body
ERGs	Electroretinogram
APG-2	Asante potassium green

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Highlights

- *Drosophila* glia endogenously express *Dube3a*
- Flies overexpressing *Dube3a* in glia are susceptible to seizures
- Overexpression of *Dube3a* downregulates the Na⁺/K⁺ pump ATP α and reduces glial K⁺ content
- Glial *Dube3a* overexpression alters mushroom body structure and impairs neuronal activity

Figure 1.

Glia endogenously express *Dube3a* and overexpression of *Dube3a* reduced ATP α protein levels. **A)** Wild type adult fly brains stained for the glial specific transcription factor repo and E3 ubiquitin ligase Dube3a reveals a strong co-localization of the two signals, *Drosophila* glia endogenously express *Dube3a*. **B) and C)** Immunohistochemistry of adult wild type fly brains for ATP α and Dube3a revealed that Dube3a and ATP α co-localize. Cellular co-localization of the two proteins indicates the potential for Dube3a to directly ubiquitinate ATP α *in vivo*. Scale bar is 20 micrometers (Image **C** is a zoomed region indicated by the box in **B**). **D)** Western blot for ATP α levels from control (UAS-*Dube3a*), neuronal overexpression (*elav*>*Dube3a*), or glial overexpression (*repo*>*Dube3a*) fly heads. **E)** Quantification of signals from **D)** indicate a significant decrease in ATP α levels in the

brain. Note that Dube3a levels from *repo>Dube3a* are reduced by greater than 50%. A one-way ANOVA revealed a significant effect of genotype on ATP α levels ($p = 0.001$). Tukey's multiple comparisons test ($\alpha = 0.05$) indicated reduced levels of ATP α in *elav>Dube3a* compared to *uas-Dube3a* ($p = 0.05$) and also in *repo>Dube3a* compared to *uas-Dube3a* ($p = 0.001$). $n = 3$ for all samples, and data are presented as mean $\pm$ SEM.

Figure 2.

Elevated levels of Dube3a in glia result in seizure susceptibility in the bang-sensitivity seizure assay. **A)** 3–5 day old flies from each genotype were tested in the BSA. Control (*repo-GAL4*, $n = 15$), overexpression of ligase dead *Dube3a* in glia (*repo>Dube3a C/A*, $n = 10$), neuronal overexpression of Dube3a (*elav>Dube3a*, $n = 12$), had no effect on seizure susceptibility (100% recovery at 10 seconds). Only overexpression of *Dube3a* in glia with *repo>Dube3a* ($n = 18$) caused bang sensitivity (80% recovery at 120 seconds). **B)** Seizures in *repo>Dube3a* flies progressively worsened as flies aged. Control *repo-GAL4* flies did not display seizure activity at 0–2 days ($n = 20$), 3–6 days ($n = 24$), or 7–10 days ($n = 24$) of age (100% recovery at 10 seconds). Approximately 50% of *repo>Dube3a* flies display seizures at 0–2 days ($n = 17$), 100% of *repo>Dube3a* flies had seizures at 3–6 days ($n = 22$) and recovered steadily over 2 minutes, and all *repo>Dube3a* flies at 7–10 days ($n = 4$) had immediate seizure-like activity after BSA leading to paralysis and death (note the 3–6 day

time point includes data from **2A**). **C**) Overexpression of human *UBE3A* in glia with *repo>UBE3A* resulted in a similar but less severe seizure phenotype. At 0–2 days (n = 27), *repo>UBE3A* flies displayed little seizure activity. However, by 3–6 (n = 39) and 7–10 (n = 34) days, *repo>UBE3A* flies displayed seizures similar to that of 3–6 day old *repo>Dube3a* flies.

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Figure 3.

Genetic or pharmacologic manipulation of ATPα modulates seizure severity. **A) and B)** We observed a significant effect of genotype on ATPα protein levels (One-Way ANOVA, $p = 0.0001$). ATPα protein levels are partially restored in *repo>Dube3a* flies by concurrent overexpression of *ATPa* ($p = 0.30$). We observed no reduction in ATPα when we overexpressed catalytically inactive *Dube3a* in glial cells ($p = 0.09$), but overexpression of human *UBE3A* in glia similarly resulted in reduced levels of ATPα ($p = 0.0001$). **C)** Overexpression of *ATPa* in *repo>Dube3a* animals rescues seizure susceptibility ($p = 0.005$, $n = 14$ all groups). **D)** Feeding *repo>Dube3a* flies the Na^+/K^+ pump inhibitor ouabain increases seizure severity ($p = 0.01$, $n = 9$ for all groups) likely due to the reduced levels of ATPα. **E)** Glial specific knockdown of *ATPa* results in seizure susceptibility ($p = 0.005$, $n = 14$ all groups). Data are presented as mean $\pm$ SEM.

Figure 4.

Overexpression of *Dube3a* in glia alters mushroom body and optic lobe morphology. **A)** Adult *repo>GFP* or *repo>GFP+Dube3a* fly brains stained with α -FasII antibody to visualize the mushroom body (MB). In *repo>GFP* animals (top row), α -FasII marks α , β , and γ lobes, with the α lobe making a single dorsal projection. In *repo>GFP+Dube3a* (bottom row) flies we observed a loss of α -FasII immunoreactivity in the γ lobe (dashed circle) and a portion of the α lobe was misdirected (arrows). **B)** Adult *repo>GFP* or *repo>GFP+Dube3a* fly brains stained with α -Brp antibody to visualize neuronal organization in the optic lobe. In *repo>GFP* animals (top row), α -Brp reveals the laminar organization of the optic lobe consisting of the outer medulla (OMe), inner medulla (IMe), and lobula plate (LP), with the IMe and LP separated by a band of GFP⁺ glial cells. In *repo>GFP+Dube3a* animals (bottom

row) the laminar structure of the optic lobe is conserved, however Brp⁺ projections are observed in the glial cell layer between the IMe and LP. Scale bar is 20 micrometers. **C) and D)** Western blot analysis of the neuronal marker elav in fly heads revealed no change in *repo>Dube3a* (n = 3) vs. *repo-GAL4* (n = 3) alone brains (p = 0.42). Data are presented as mean $\pm$ SEM.

Figure 5.

Overexpression of *Dube3a* in glia alters ERG signals and reduces internal K^+ levels in glial cells. **A)** Control *repo>GAL4* alone ($n = 10$) ERGs display robust on/off transients and photoreceptor potentials. Overexpression of *Dube3a* in neurons with *elav>Dube3a*, ($n = 8$) or overexpression of ligase dead *Dube3a* in glia (*repo>Dube3aC/A*, $n=8$) has no effect on the ERG signal. However, ERGs of glial overexpression of *Dube3a* (*repo>Dube3a*, $n = 11$) or human *UBE3A* (*repo>UBE3A*, $n = 8$) flies show reduced on/off transients and reduced photoreceptor potentials in 3–5 day old flies. **B)** Quantification of on transients. **C)** Quantification of photoreceptor potentials. **D)** Quantification of off transients. Data is presented as mean $\pm$ SEM. **E)** Using the fluorescent K^+ indicator Asante Potassium Green 2

(APG-2) and tdTomato to mark glial cells, we quantified internal potassium levels of control (*repo>tdTomato*) and glial overexpression of Dube3a (*repo>tdTomato+Dube3a*) glia. **F**) Quantification of the APG-2 fluorescence revealed a significant reduction in fluorescence signal ($p = 0.01$), indicating reduced intracellular K^+ levels. Scale bar is 20 micrometers, and $n = 40$ cells from 3 flies per group. Data are presented as mean $\pm$ SEM.

Figure 6.

Proposed mechanism of seizure in *repo>Dube3a* animals. **A)** In control flies, relatively low levels of Dube3a are present in glia while high levels of ATPα are present in the glial membrane. As K⁺ effluxes from neurons, ATPα removes K⁺ from the synaptic cleft, restoring K⁺ gradients required for proper neuronal function. **B)** In *repo>Dube3a* animals, elevated levels of Dube3a in glia reduce ATPα levels in the membrane. As K⁺ effluxes from neurons, it is not cleared from the extracellular space, leading to hyperpolarized neurons and increasing neuronal activity, resulting in epileptiform activity and seizures.