Neurobiology of Disease

An *In Vivo* Pharmacological Screen Identifies Cholinergic Signaling as a Therapeutic Target in Glial-Based Nervous System Disease

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The role that glia play in neurological disease is poorly understood but increasingly acknowledged to be critical in a diverse group of disorders. Here we use a simple genetic model of Alexander disease, a progressive and severe human degenerative nervous system disease caused by a primary astroglial abnormality, to perform an *in vivo* screen of 1987 compounds, including many FDA-approved drugs and natural products. We identify four compounds capable of dose-dependent inhibition of nervous system toxicity. Focusing on one of these hits, glycopyrrolate, we confirm the role for muscarinic cholinergic signaling in pathogenesis using additional pharmacologic reagents and genetic approaches. We further demonstrate that muscarinic cholinergic signaling works through downstream Gαq to control oxidative stress and death of neurons and glia. Importantly, we document increased muscarinic cholinergic receptor expression in Alexander disease model mice and in postmortem brain tissue from Alexander disease patients, and that blocking muscarinic receptors in Alexander disease model mice reduces oxidative stress, emphasizing the translational significance of our findings. We have therefore identified glial muscarinic signaling as a potential therapeutic target in Alexander disease, and possibly in other gliopathic disorders as well.

Key words: Alexander disease; chemical screen; cholinergic signaling; *Drosophila*; glia

Significance Statement

Despite the urgent need for better treatments for neurological diseases, drug development for these devastating disorders has been challenging. The effectiveness of traditional large-scale *in vitro* screens may be limited by the lack of the appropriate molecular, cellular, and structural environment. Using a simple *Drosophila* model of Alexander disease, we performed a moderate throughput chemical screen of FDA-approved drugs and natural compounds, and found that reducing muscarinic cholinergic signaling ameliorated clinical symptoms and oxidative stress in Alexander disease model flies and mice. Our work demonstrates that small animal models are valuable screening tools for therapeutic compound identification in complex human diseases and that existing drugs can be a valuable resource for drug discovery given their known pharmacological and safety profiles.

Introduction

The current drug discovery pipeline has been inefficient in the context of neurological disease. Approaches to therapeutic compound identification that complement the conventional large-scale *in vitro* compound screens are therefore warranted. Proper functioning of the nervous system relies on complex interdependent cellular and anatomic relationships among diverse cell types. Particularly for diseases affecting the brain, drug screening *in vivo* may therefore be advantageous. Further, *in vivo* screens will select for compounds able to pass the blood–brain barrier,

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which is a major hurdle in drug development for diseases affecting the CNS. The time and expense involved in testing compounds directly in the widely used murine models of disease makes such *in vivo* screens difficult despite their theoretical advantages. In contrast, *Drosophila* are small and cheap to culture. Many basic neuronal and glial functions are conserved in flies; and, accordingly, useful *Drosophila* models of a number of human neurological diseases have been developed (Feany and Bender, 2000; Wittmann et al., 2001; Jackson et al., 2002; Bonini and Fortini, 2003; Wang et al., 2011).

Here we use a model of Alexander disease to perform a moderate throughput *in vivo* chemical screen in *Drosophila*. Alexander disease is a rare neurological disorder caused by autosomal dominant mutations in the gene encoding the glial intermediate filament, glial fibrillary acidic protein (GFAP). Clinically, Alexander disease manifests with symptoms ranging from prominent seizures in younger patients to movement disorders in adults (Li et al., 2005; Messing et al., 2012). The disease is inexorably progressive, and most patients die of end-stage neurological deterioration. There is no effective treatment. Pathologically, varying degrees of white matter loss and neurodegeneration are found. Of note, Alexander disease provides a unique example of glial-mediated neurodegeneration because GFAP expression is predominantly astrocytic. In addition to variable degrees of neuronal and glial cell loss, all patients show aggregation of GFAP into eosinophilic, beaded inclusions, termed Rosenthal fibers, in astrocytes.

Given the autosomal dominant pattern of inheritance, the presence of abnormal protein aggregates, and recapitulation of disease-related phenotypes by overexpression (Messing et al., 1998; Brenner et al., 2001), but not knock-out (Gomi et al., 1995; Pekny et al., 1995) of GFAP in mice, a dominant gain-of-function mechanism, perhaps related to toxic protein aggregation, is postulated in Alexander disease (Messing et al., 2012). Thus, we modeled the disease in *Drosophila* by expressing wild-type and Alexander disease-linked mutant human GFAP in flies. To recapitulate the cellular specificity of GFAP expression, we expressed human GFAP in fly glia using the bipartite UAS/GAL4 expression system (Brand and Perrimon, 1993) and the glial driver *repo-GAL4*.

Our model faithfully recapitulates multiple aspects of human Alexander disease, including aggregation of GFAP into inclusions strongly resembling Rosenthal fibers at the light and electron microscopic level, glial toxicity, non–cell-autonomous neuronal cell death, and seizures (Wang et al., 2011). Mutant GFAP, including the GFAP^{R79H} variant used in the current work, is significantly more toxic than wild-type GFAP, although, as in mice, wild-type GFAP will aggregate and produce toxicity at higher levels of expression (Messing et al., 1998).

Similarities to vertebrate systems are also apparent at the cell biological level. Both human and fly Rosenthal fiber inclusions contain αB-crystallin. As in mouse models of Alexander disease, overexpression of the fly homolog of αB-crystallin strongly ameliorates the behavioral and neuropathological defects of GFAP transgenic flies (Hagemann et al., 2009; Wang et al., 2011). Other pathways previously implicated in Alexander disease or in vertebrate models of the disorder, including oxidative stress, autophagy, JNK signaling, and glial glutamate transport, also show altered activity and contribute to pathology in the *Drosophila* model. These similarities support the use of our *Drosophila* model in the chemical screen described here aimed at identifying therapeutic compounds and pathways in Alexander disease.

Materials and Methods

Drosophila *stocks.* All fly crosses were performed at 25°C; adults were aged at 29°C to increase transgene expression. *repo-GAL4*, *da-GAL4*, and *UAS-Gαq RNAi* #1 (#30735) were from the Bloomington *Drosophila* Stock Center. The following stocks were obtained from Vienna *Drosophila* RNAi Center: *UAS-mAChR-A RNAi* #1 (33123), *UAS-mAChR-A RNAi* #2 (101407), and *UAS-Gαq RNAi* #2 (50729). Additional stocks used include *UAS-CD8-PARP-Venus* from D. Williams (Williams et al., 2006) and *GstD1-lacZ* from D. Bohmann (Sykiotis and Bohmann, 2008).

Transgenic mice. Six-month-old *Gfap*$^{R236H/+}$ mice in the B6 background (Hagemann et al., 2006) were used for immunofluorescence staining and Western blotting of muscarinic receptor expression (see Fig. 6). Age-matched wild-type littermates were used as controls.

For chronic drug treatment, 6.5-week-old *Gfap*$^{R236H/+}$ mice in the FVB background (Hagemann et al., 2006) were injected subcutaneously with saline or pirenzepine (20 mg/kg) twice daily for 10 d; tissues were collected on day 11 when mice were ~8 weeks old (Ishibashi et al., 2014). Age-matched wild-type littermates were used as controls and treated with either saline or pirenzepine (see Fig. 8). For quantification of NRF2-positive astrocytes, at least 50 astrocytes from the hippocampal CA1 region (coronal section 23 following the Mouse Brain Library, bregma: −2.92 mm; http://www.mbl.org/atlas170/atlas170_frame.html) were counted; 5 or 6 animals per genotype/treatment, both male (12 total) and female (11 total), were used.

All procedures were approved by the Institutional Animal Care and Use Committee of the Graduate School of the University of Wisconsin-Madison.

Human samples. Frozen frontal cortex white matter from 3 controls (mean age 14 years, range 1–28 years; 2 females and 1 male) and 3 Alexander disease patients (mean age 18 years, range 6–27 years; 1 female and 2 males) were obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore. GFAP mutations in the Alexander disease patients included R239C, K63E, and L359V. All cases had typical neuropathology of Alexander disease, including multiple Rosenthal fibers. Postmortem intervals were comparable between cases and controls and were <24 h in all cases.

Drug feeding in Drosophila. All the compounds from the Spectrum Collection (MicroSource Discovery System) were obtained dissolved in DMSO at a concentration of 10 mM; 30 μl of each compound was further diluted in water to a final concentration of 100 μM and then mixed with instant *Drosophila* medium (Carolina Biological) for the primary screen. Controls were treated with DMSO only. The 7 compounds obtained from Sigma-Aldrich were dissolved in their optimal solvents suggested by the manufacturer's MSDS sheets (water for citalopram, dinitolmide, duloxetine, glycopyrrolate, and sulfanilate zinc; chloroform for β-sitosterol; and ethanol for praziquantel) to make stock solutions; 30 μl of each compound stock solution was further diluted in water at appropriate concentrations (Table 1) and then mixed with instant *Drosophila* medium (Carolina Biological) for dose–response analyses. Control flies were fed the appropriate concentration of solvent alone. Newly eclosed flies were raised on drug-embedded or solvent-embedded food for total of 3 d for GFAP level quantification and seizure quantification, 10 d for caspase activation analysis, and 15 d for TUNEL and oxidative stress analyses. Flies were transferred to new food every 3 d.

Behavioral analysis. Flies were collected under CO_2 anesthesia at 1 d after eclosion and kept at 5 animals per vial for 1 d, without further anesthesia, before analysis. Drug-fed flies were kept on drug-embedded food for 3 d before analysis. For testing, vials were mechanically stimulated on a VWR mini-vortexer for 10 s at maximum speed (Ganetzky and Wu, 1982; Wang et al., 2011). The seizure frequency was calculated by dividing the number of flies with seizures by the total number of flies tested. Statistical significance was evaluated using the χ^2 test.

Immunohistochemistry, immunofluorescence, and TUNEL analysis. Adult flies were fixed in formalin and embedded in paraffin; 4 μm serial frontal sections were prepared through the entire fly brain and placed on a single glass slide. Mouse brains and human brain tissue were fixed in 4% PFA, embedded in paraffin, and sectioned at a thickness of 6 μm.

Table 1. Compounds identified in the chemical screen[a]

Drug	Dose-responsive	Caspase activation (% of control)	Optimal concentration (μm)	Description
β-Sitosterol	Yes	37	500	Plant sterol
Citalopram	Yes	59	50	Selective serotonin reuptake inhibitor
Dinitolmide	No			
Duloxetine	Yes	60	25	Serotonin-norepinephrine reuptake inhibitor
Glycopyrrolate	Yes	62	50	Anticholinergic
Praziquantel	No			
Sulfanilate zinc	No			

[a]Dose–response analysis (0, 10, 25, 50, 100, 250, and 500 μm) for suppression of activated caspase in Alexander disease model flies showed that β-sitosterol, citalopram, duloxetine, and glycopyrrolate produce a dose-dependent reduction in the number of cleaved PARP-positive cells, whereas dinitolmide, praziquantel, and sulfanilate zinc do not. Higher drug doses did not show additional efficacy for any of the compounds. Control flies (0 μM) were fed solvent (water for citalopram, dinitolmide, duloxetine, glycopyrrolate, and sulfanilate zinc; chloroform for β-sitosterol; ethanol for praziquantel)-embedded food. Flies were 10-d-old and were treated with drug for 10 d. $n \geq 6$ per concentration. Genotype: *repo-GAL4, UAS-GFAP^{R79H}, UAS-CD8-PARP-Venus /+*.

For immunostaining, paraffin slides were processed through xylenes, ethanol, and into water. Antigen retrieval by boiling in sodium citrate, pH 6.0, was performed before blocking in PBS containing 0.3% Triton X-100 and 2% milk for 1 h. The following primary antibodies were used at the specified dilutions: PARP E51 (rabbit, 1:50,000, Abcam, ab32064), Repo (mouse, 1:5, DSHB), elav (mouse, 1:5, DSHB), β-galactosidase (mouse, 1:500, Promega, Z3781), mAChR M35 (mouse, 1:100, E. van der Zee), mAChR M_1 (rabbit, 1:200, Frontier Institute), NRF2 (rat, 1:50, Cell Signaling Technology), and GFAP (mouse N206/8, 1:1000, University of California-Davis/National Institutes of Health NeuroMab Facility; rabbit, 1:10,000, Dako). For immunohistochemistry, biotin-conjugated secondary antibodies (1:200, Southern Biotechnology) and avidin-biotin-peroxidase complex (Vectastain Elite, Vector Laboratories) staining was performed using DAB (Vector Laboratories) as a chromagen. For double labeling, secondary antibodies coupled to Alexa-488 or Alexa-555 (1:200, Invitrogen) were used.

Quantification of the number of cleaved poly (ADP-ribose) polymerase 1 (PARP)-positive cells was performed by counting the positive cells in serial frontal sections (4 μm) of the entire brains from at least 6 animals per genotype and treatment. Quantification of the number of β-galactosidase-positive cells was performed by counting the positive cells in the optic lobe from at least 6 animals per genotype.

Apoptotic cell death was visualized using TUNEL according to the manufacturer's instructions (TdT FragEL DNA fragmentation kit, Calbiochem), with an additional avidin-biotin-peroxidase amplification step. The number of TUNEL-positive cells was counted by examining serial frontal sections (4 μm) of the entire brains from at least 6 animals per genotype and treatment. Fluorescent TUNEL was performed using Alexa-488-conjugated streptavidin (Invitrogen).

Statistical analysis was performed using two-tailed *t* test for two groups and one-way ANOVA with Tukey's multiple-comparison test for three or more groups. Each data point represents the mean ± SEM.

Western blotting. Single adult *Drosophila* heads were homogenized in 1× Laemmli buffer (Sigma). For murine samples, corpus callosum was homogenized in Tris-saline buffer (50 mM Tris, 150 mM NaCl, pH 7.4, with protease inhibitor mixture; Thermo Scientific) and centrifuged at 300 × *g* for 15 min at 4°C. The supernatant was used for Western blotting. For human brains, white matter from frontal cortex was homogenized in Tris-saline buffer (50 mM Tris, 150 mM NaCl, pH 7.4, with protease inhibitor mixture; Thermo Scientific) and centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used for Western blotting. Samples were boiled for 5 min at 100°C and subjected to SDS-PAGE using 10% or 4%–12% gels (Lonza). Proteins were transferred to nitrocellulose membranes (Bio-Rad), blocked in 2% milk in PBS with 0.05% Tween 20, and immunoblotted using the following primary antibodies: GFAP (rabbit, 1:10^7, Dako, Z0334), actin JLA20 (mouse, 1:4000, DSHB), mAChR M_1 (rabbit, 1:4000, Frontier Institute), and GAPDH (rabbit, 1:100,000, Abcam). The appropriate anti-mouse or anti-rabbit HRP-conjugated secondary antibody (1:50,000, Southern Biotechnology) was applied, and signal was detected with West Femto chemiluminescent substrate (Thermo Scientific). All blots were repeated at least three times, and representative blots are presented in the figures.

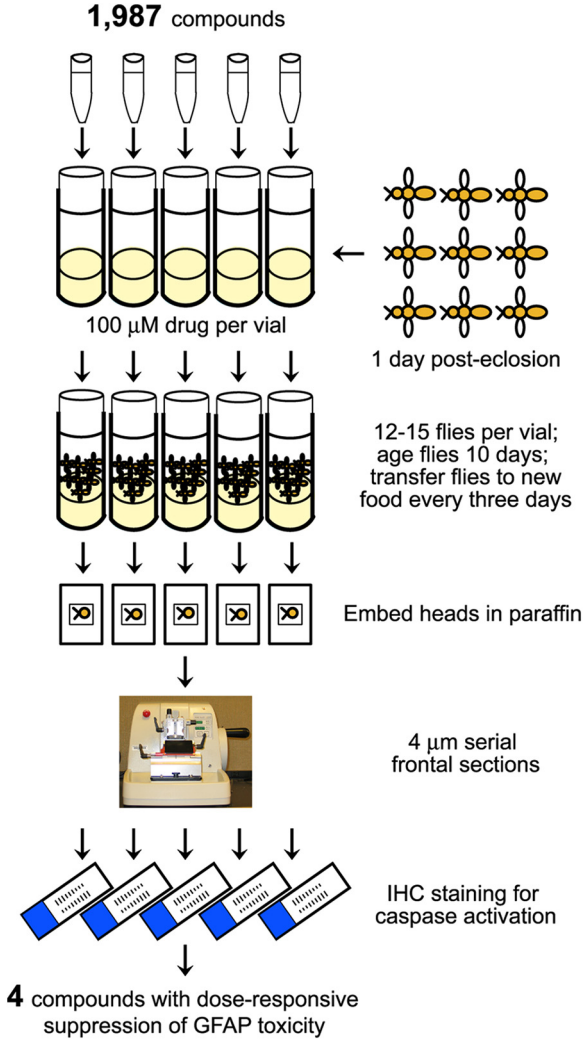

Figure 1. Screen design and results. Each compound from the Spectrum Collection was mixed with instant *Drosophila* medium individually at a final concentration of 100 μM. One-day-old Alexander disease model flies carrying a transgenic caspase reporter (*repo-GAL4, UAS-GFAP^{R79H}, UAS-CD8-PARP-Venus /+*) were raised on drug-embedded food for a total of 10 d, with transfer to a vial with fresh drug-embedded food every 3 d. Caspase activation was then monitored by determining the number of cleaved PARP-positive cells in the brain using immunohistochemical (IHC) detection on sections from paraffin-embedded material.

Confocal microscopy. All the fluorescent images were taken as *Z*-stacks on a Leica SP8 × confocal microscope at Harvard NeuroDiscovery Center Enhanced Neuroimaging Core facility or an Olympus FV1000 confocal microscope at Harvard Neurobiology Imaging Facility. All the images were visualized in 2D projections of *Z*-stacks. Control and experimental samples were imaged with the same laser setting and the same *Z*-stack thickness.

Real-time PCR. RNA was isolated from *Drosophila* head homogenates using QIAzol (QIAGEN) and reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed and monitored using SYBR Green PCR Master Mix (Applied Biosystems) in a StepOnePlus Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. *Drosophila* ribosomal protein *RpL32* was used as a control. Statistical analysis was performed using one-way ANOVA with Tukey's multiple-comparison test. Each data point represents the mean ± SEM.

Primers are as follows: *mAChR-A*, CATCG AGTAGTTGTCCCGCATC; GAGCAGGAG GAGCCTTCTGATT; *Gαq*, GCCGCGAGCT TAAACTGCT; CCAGCTTGATGTACCCAC GC; and *RpL32*, GACCATCCGCCCAGCAT AC; CGGCGACGCACTCTGTT.

Statistical analysis. Values are presented as mean ± SEM. GraphPad Prism 5 software was used for all statistical analysis. Chi-square test was used for seizure behavioral analysis. For others, unpaired *t* test was used for two groups and one-way ANOVA with Tukey's multiple-comparison test for three or more groups. A minimum of 6 animals per genotype and treatment was used to for caspase activation, cell death (TUNEL), and oxidative stress reporter assays. A minimum of 100 animals per genotype and treatment was used for seizure assays. All Western blots and RT-PCR determinations were repeated at least three times, and a representative blot was shown. The optimal concentrations of candidate compounds was determined by dose–response analysis.

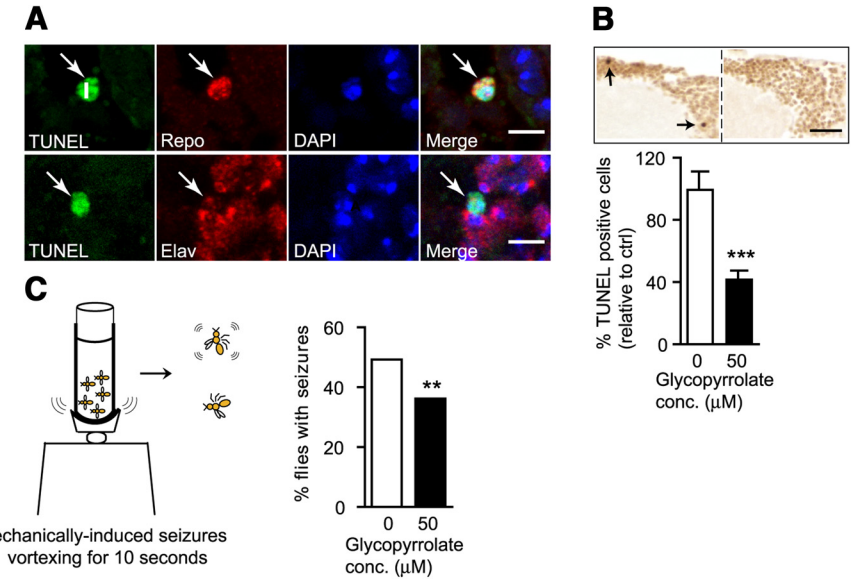

Figure 2. Glycopyrrolate reduces GFAP toxicity in Alexander disease model flies. ***A***, Double-label immunofluorescence reveals both glial (top panel, arrows) and neuronal (bottom panel, arrows) cell death in Alexander disease model flies. Apoptotic cells are labeled by TUNEL. Repo is a glial cell marker, and elav is a neuronal cell marker. DAPI labels nuclei. Scale bar, 3 μm. Genotype: *repo-GAL4, UAS-GFAP*$^{R79H/+}$. Flies were 15-d-old. ***B***, Glycopyrrolate significantly reduces the total number of TUNEL-positive cells at 50 μM. ***$p < 0.001$ (two-tailed *t* test). $n \geq 6$ per concentration. Flies were 15-d-old and were treated with drug for 15 d. Genotype: *repo-GAL4, UAS-GFAP*$^{R79H/+}$. Control flies were treated with solvent (water) only. Arrows indicate TUNEL-positive cells. Scale bar, 20 μm. ***C***, Glycopyrrolate significantly reduces the percentage of flies with seizures at 50 μM. **$p < 0.01$ (χ^2 test). $n >$ 100 per concentration. Flies were 3-d-old and were treated with drug for 3 d. Genotype: *repo-GAL4, UAS-GFAP*$^{R79H/+}$. Control (ctrl) flies were treated with solvent (water) only. The schematic represents the method for seizure induction.

Results

Chemical screen identifies compounds ameliorating GFAP toxicity in Alexander disease model flies

To identify therapeutic compounds for Alexander disease, we performed a chemical screen of 1987 compounds from the Spectrum Collection (MicroScource Discovery System) in our *Drosophila* model of Alexander disease. To monitor GFAP toxicity under screening conditions, we used a transgenic caspase reporter, *UAS-CD8-PARP-Venus*, which consists of the extracellular and transmembrane domain of mouse CD8 fused to 40 amino acids from human PARP, including the caspase cleavage site, and Venus, a yellow fluorescent protein variant. Caspase activation can be detected using an antibody specific for the cleaved form of human PARP (Williams et al., 2006). Alexander disease model flies carrying the caspase reporter (*repo-GAL4, UAS-GFAP*^{R79H}, *UAS-CD8-PARP-Venus/+*) were raised on drug-embedded *Drosophila* culture media at a final compound concentration of 100 μM for a total of 10 d. Flies were transferred to a new vial with fresh drug-embedded medium every 3 d (Fig. 1). Because available ELISA assays displayed insufficient sensitivity and specificity in our model, immunohistochemistry on sections taken through entire fly brains was used to monitor caspase activation (the number of cleaved PARP-positive cells) in the glia of drug-treated flies. Drugs that led to ≥75% reduction in caspase activation were selected as initial hits. Suppression of activated caspase was confirmed by repeat analysis on the first set of drug-fed flies and then by feeding compounds to a second group of animals. Seven compounds were confirmed in the second round of drug feeding (Table 1). These seven compounds were purchased from Sigma-Aldrich, and a dose–response analysis was performed. Four compounds (β-sitosterol, citalopram, duloxetine, and glycopyrrolate) were confirmed to reduce caspase activation in GFAP^{R79H} transgenic flies. Three compounds did not show statistically significant rescue of toxicity or did not do so in a dose-dependent manner (Table 1).

Among the four compounds that showed a dose-dependent reduction in caspase activation, glycopyrrolate is an antagonist targeting the mAChR. mAChRs are expressed in astrocytes and can modulate important cellular functions, including Ca^{2+} homeostasis and proliferation (Guizzetti et al., 1996; Porter and McCarthy, 1997; Navarrete et al., 2012). To investigate further the actions of glycopyrrolate on toxicity in our Alexander disease model, we examined cell death and seizures. We observed both glial and neuronal cell death in our Alexander disease model flies (Fig. 2*A*). We found that glycopyrrolate significantly reduced the total number of TUNEL-positive cells and the percentage of flies with seizures (Fig. 2*B*,*C*). To ensure that glycopyrrolate did not simply reduce transgenic expression of GFAP^{R79H}, we performed Western blots on drug-fed flies. We found no reduction in GFAP levels in the presence of therapeutic levels of glycopyrrolate (Fig. 3*J*).

mAChR signaling controls GFAP toxicity *in vivo*

To confirm the role of mAChR signaling in promoting toxicity in our Alexander disease model, we tested two other mAChR antagonists, atropine and scopolamine. Consistent with the beneficial effects of glycopyrrolate, both atropine and scopolamine rescued caspase activation in a dose-dependent manner in GFAP^{R79H} transgenic flies, with optimal concentrations of 100 and 50 μM, respectively (Fig. 3*A*,*B*). To determine whether activating mAChR signaling would increase GFAP^{R79H} toxicity, we fed flies pilocarpine, an mAChR agonist. We found a significant, dose-dependent increase in caspase activation in Alexander disease model flies, with an optimal concentration of 500 μM (Fig. 3*C*). Importantly, pilocarpine did not activate caspase in control flies without human GFAP^{R79H} expression (data not shown). Furthermore, atropine and scopolamine significantly reduced, whereas pilocarpine increased, the number of TUNEL-positive cells and the percentage of flies with seizures in the brains of Alexander disease model flies (Fig. 3*D*–*I*). Pilocarpine did not induce cell death, as monitored by TUNEL staining, in control

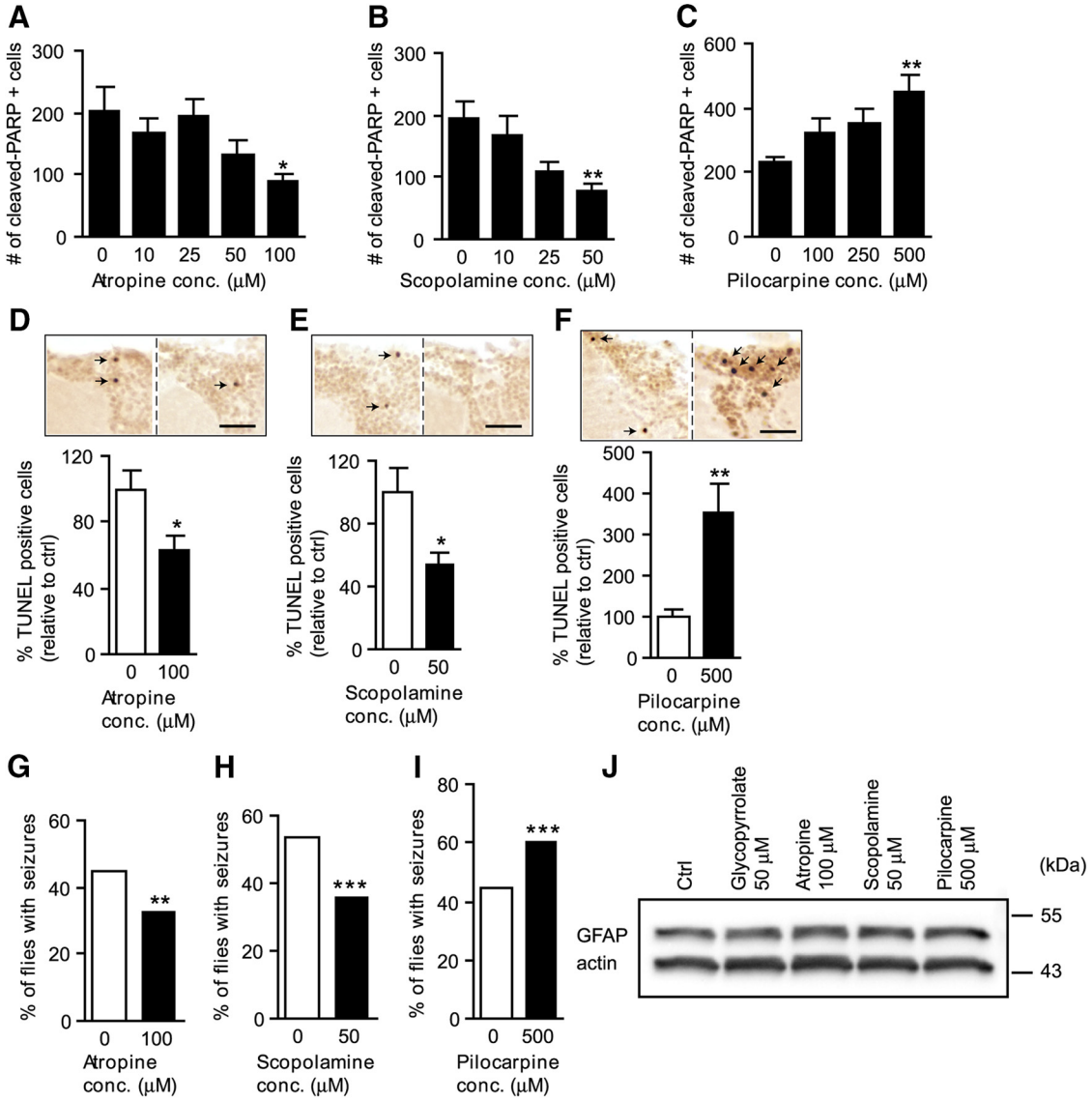

Figure 3. Compounds inhibiting mAChR reduce GFAP toxicity in Alexander disease model flies. ***A–C***, Dose–response analysis of three compounds targeting mAChR. mAChR antagonists, atropine (***A***) and scopolamine (***B***) reduce the number of cleaved PARP-positive cells in a dose-dependent manner, whereas the agonist, pilocarpine (***C***), increases the number of cleaved PARP-positive cells in a dose-dependent manner. $^{*}p < 0.05$ (one-way ANOVA with Tukey's multiple-comparison test). $^{**}p < 0.01$ (one-way ANOVA with Tukey's multiple-comparison test). $n \geq 6$ per concentration. Flies were 10-d-old and were treated with drugs for 10 d. Genotype: *repo-GAL4*, *UAS-GFAP*^{R79H}, *UAS-CD8-PARP-Venus/+*. ***D–F***, Cell death analysis in mAChR compound-fed flies. Atropine (***D***) and scopolamine (***E***) significantly reduce, whereas pilocarpine (***F***) increases, the number of TUNEL-positive cells in Alexander disease model flies. $^{*}p < 0.05$ (two-tailed *t* test). $^{**}p < 0.01$ (two-tailed *t* test). $n \geq 6$ per concentration. Flies were 15-d-old and were treated with drugs for 15 d. Genotype: *repo-GAL4*, *UAS-GFAP*$^{R79H/+}$. Ctrl: solvent (water)-fed flies. Arrows indicate TUNEL-positive cells. Scale bar, 20 μm. ***G–I***, Seizure analysis in mAChR compound-fed flies. Atropine (***G***) and scopolamine (***H***) reduce, whereas pilocarpine (***I***) increases, the percentage of flies with seizures. $^{**}p < 0.01$ (χ^2 test). $^{***}p < 0.001$ (χ^2 test). $n > 100$ per concentration. Flies were 3-d-old and were treated with drugs for 3 d. Genotype: *repo-GAL4*, *UAS-GFAP*$^{R79H/+}$. ***J***, Western blot shows equivalent GFAP protein levels in control (ctrl) and drug-fed flies. Flies were 3-d-old and were treated with drug for 3 d. The blot was reprobed with an antibody for actin to illustrate equivalent protein loading. Genotype: *repo-GAL4*, *UAS-GFAP*$^{R79H/+}$. Control (Ctrl) flies were treated with solvent (water) only.

flies without human GFAP^{R79H} expression (data not shown). As for glycopyrrolate, modulation in GFAP toxicity by other compounds targeting mAChR was not due to alterations in GFAP levels (Fig. 3*J*). Together, these data strongly suggest that mAChR signaling, as probed with well-characterized small molecules, plays a key role in modulating GFAP toxicity *in vivo*.

We have previously described a critical role for oxidative stress in mediating toxicity in Alexander disease model flies (Wang et al., 2011). To explore the mechanism by which compounds targeting mAChR signaling modified GFAP toxicity, we used an oxidative stress reporter, *GstD1-lacZ* (Sykiotis and Bohmann, 2008), whose activation can be detected by immunostaining for β-galactosidase (Fig. 4*A*). In Alexander disease model flies carrying the GstD1 oxidative stress reporter, we observed that atropine and scopolamine significantly decreased the reporter activation, whereas pilocarpine markedly increased the reporter activation, suggesting that mAChR signaling influences GFAP toxicity via oxidative stress (Fig. 4*B–D*).

Genetic inhibition of mAChR signaling reduces GFAP toxicity

The *Drosophila* genome contains one well-characterized mAChR, mAChR-A, which has a high degree of amino acid similarity and a pharmacological profile similar to mammalian mAChRs (Onai et al., 1989; Shapiro et al., 1989; Millar et al., 1995). To probe the role of mAChR signaling genetically in our Alexander disease

model, we reduced the expression of mAChR-A in GFAP^{R79H} transgenic flies using two independent transgenic RNAi lines. We observed a significant reduction of the number of TUNEL-positive cells with reduced mAChR-A levels (Fig. 5*A*). The rescue of GFAP toxicity was not due to lower levels of GFAP protein (Fig. 5*D*). To confirm the expected reduction in transcript levels by RNAi targeting *mAChR-A*, we performed RT-PCR and observed significantly reduced *mAChR-A* mRNA levels compared with controls (Fig. 5*E*). Consistent with rescue of cell death, the number of flies with seizures was also markedly reduced by reducing the expression of *mAChR-A* (Fig. 5*B*). Mechanistically, reduction of *mAChR-A* expression also decreased the activation of oxidative stress reporter in Alexander disease model flies, consistent with an effect of oxidative stress downstream of mAChR signaling (Fig. 5*C*).

mAChRs are G-protein-coupled receptors. In mammals, there are five subtypes of mAChRs ($M_{1–5}$) and they preferentially couple to different G-proteins and signal through various effectors (Felder, 1995; Nathanson, 2000; Wess et al., 2007). In *Drosophila*, mAChR-A has been shown to mediate Ca^{2+} release through Gαq-Plcβ signaling in neurons (Agrawal et al., 2013). To test whether mAChR-A also signals through Gαq to modulate GFAP toxicity in glia in our Alexander disease model, we reduced the expression of *Gαq* using two independent transgenic RNAi lines and observed significant decreases in the number of TUNEL-positive cells in GFAP^{R79H} transgenic flies, but no change in GFAP protein levels (Fig. 5*F*,*I*). The reduction of *Gαq* mRNA transcript levels by two RNAi lines was significant as measured by RT-PCR (Fig. 5*J*). Consistent with rescue of cell death, the percentage of flies with seizures was also markedly reduced by the reduction of *Gαq* in Alexander disease model flies (Fig. 5*G*). Reduction of *Gαq* expression also decreased the activation of oxidative stress reporter in Alexander disease model flies (Fig. 5*H*). Together, these data support the hypothesis that Gαq is a downstream mediator for mAChR-A signaling in modulation of GFAP toxicity and acts via oxidative stress.

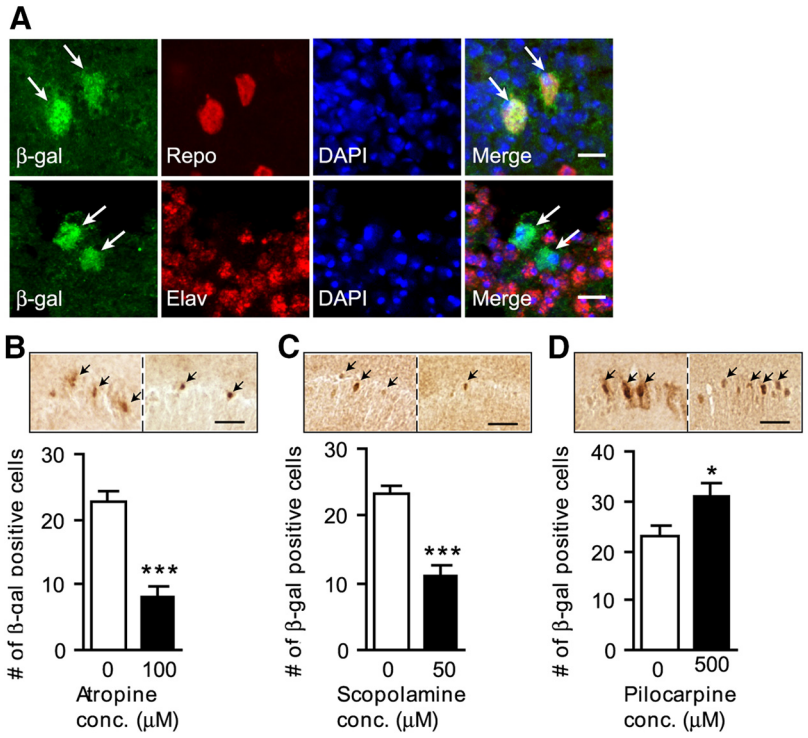

Figure 4. mAChR compounds modulate oxidative stress in Alexander disease model flies. ***A***, Double-label immunofluorescence shows activation of the oxidative stress reporter, *GstD1-lacZ*, in glial cells (arrows) of Alexander disease model flies. Repo marks glia, and elav marks neurons. DAPI labels nuclei. Scale bar, 3 μm. Genotype: *GstD1-lacZ/+*; *repo-GAL4, UAS-GFAP^{R79H}/+*. Flies were 15-d-old. ***B–D***, Oxidative stress analysis in mAChR compound-fed flies. Atropine (***B***) and scopolamine (***C***) reduce, whereas pilocarpine (***D***) increases, the number of β-galactosidase-positive cells in Alexander disease model flies. *$p < 0.05$ (two-tailed *t* test). ***$p < 0.001$ (two-tailed *t* test). $n \geq 6$ per concentration. Flies were 15-d-old and were treated with drugs for 15 d. Genotype: *GstD1-lacZ/+*; *repo-GAL4, UAS-GFAP$^{R79H/+}$*. Arrows indicate β-galactosidase-positive cells. Scale bar, 20 μm.

Increased mAChR expression in Alexander disease model mice and in patients

Because reduction of mAChR signaling was protective in the *Drosophila* model of Alexander disease, to increase the translational relevance of our findings, we next investigated a potential role for mAChRs in mammalian systems. We began by assessing expression of mAChRs using a well-characterized and widely used pan-mAChR antibody, M35 (van der Zee and Luiten, 1999), in Alexander disease model mice (*Gfap$^{R236H/+}$*). These mice have been engineered by homologous recombination to express the equivalent of the common and deleterious human R239H mutant form of GFAP and develop clinical and pathological features reminiscent of human Alexander disease (Hagemann et al., 2006). We observed significantly increased immunoreactivity for M35 in the astrocytes of 6-month-old *Gfap$^{R236H/+}$* mice compared with wild-type littermate controls (Fig. 6*A*, arrows). To investigate the subtype of muscarinic cholinergic receptor upregulated in Alexander disease model mice, we performed immunostaining using a previously validated M_1-subtype specific antiserum (Narushima et al., 2007; Yamasaki et al., 2010; Anisuzzaman et al., 2013). M_1 receptors are the predominant mAChR subtype expressed in cerebral cortex and hippocampus. We observed increased expression of M_1 receptors in the astrocytes of 6-month-old *Gfap$^{R236H/+}$* mice (Fig. 6*B*, arrows). Consistent with immunostaining results, a significant increase of M_1 receptor expression in the corpus callosum was detected by Western blotting in 6-month-old *Gfap$^{R236H/+}$* mice compared with wild-type littermate controls (Fig. 6*C*). With both antibodies, we observed some plasma membrane staining along with prominent punctate cytoplasmic staining, as has been described previously (Anisuzzaman et al., 2013; Uwada et al., 2014).

To extend our studies to the human disease itself, we next investigated M_1 receptor expression in the postmortem brain tissue from Alexander disease patients. We observed increased expression of M_1 receptors in the astrocytes of Alexander disease patients compared with controls using both double-label immunofluorescence (Fig. 7*A*, arrows) and Western blotting (Fig. 7*B*).

Finally, to test the effect of muscarinic antagonism in our mammalian system, we treated Alexander disease model mice with pirenzepine (Ishibashi et al., 2014), an M_1 antagonist, for 10 d. We investigated the effect of pharmacological M_1 inhibition on NRF2, an oxidative stress-responsive transcription factor induced in mouse models of Alexander disease (Hagemann et al.,

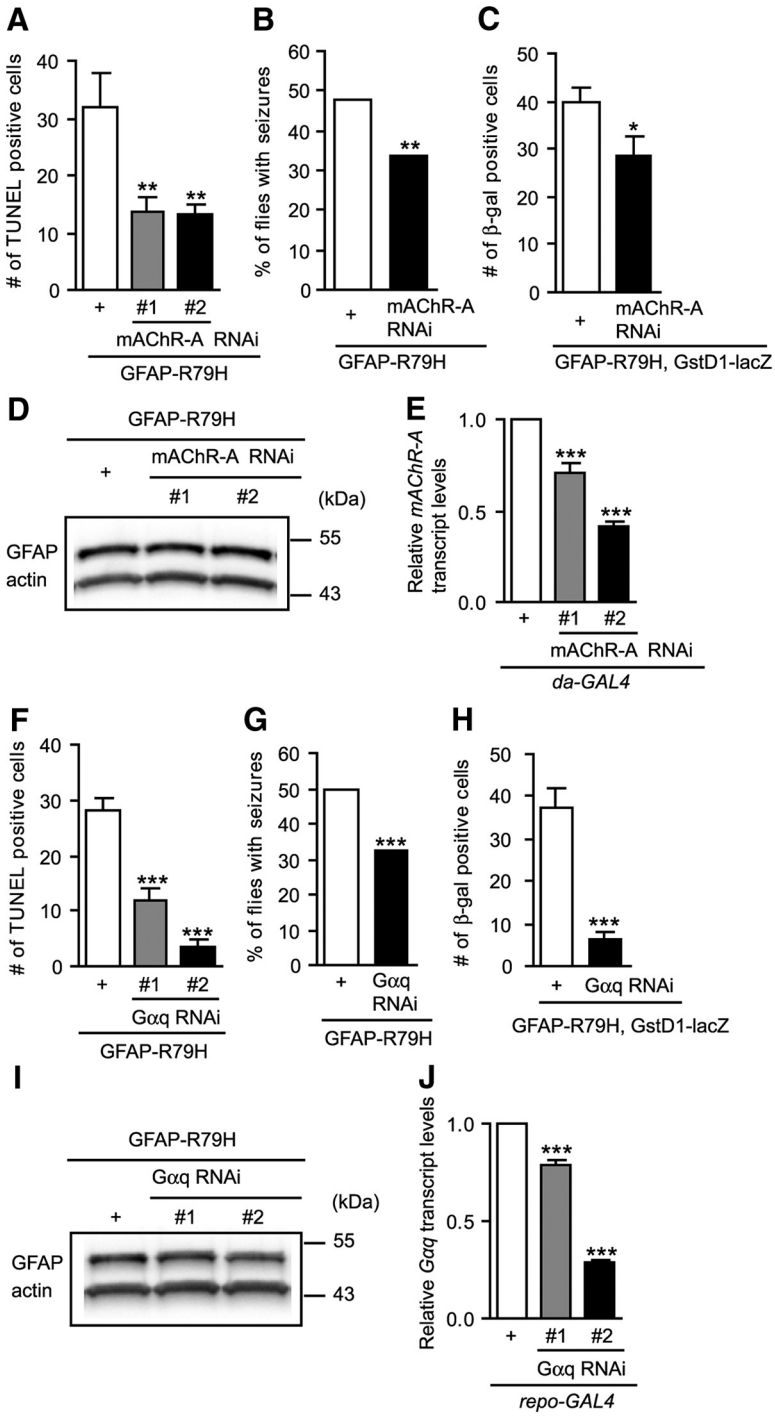

Figure 5. Genetic inhibition of mAChR pathway reduces GFAP toxicity in Alexander disease model flies. In all panels, GFAP-R79H indicates *repo-GAL4*, *UAS-GFAP$^{R79H/+}$*. mAChR-A RNAi indicates *UAS-mAChR-A RNAi* (***A–E***), and Gαq RNAi indicates *UAS-Gαq RNAi* (***F–J***). ***A***, ***F***, Reducing expression of mAChR-A or Gαq with transgenic RNAi significantly reduces the number of TUNEL-positive cells. $^{**}p < 0.01$. $^{***}p < 0.001$. $n \geq 6$ per genotype. Control (+): *repo-GAL4*, *UAS-GFAP$^{R79H/+}$*. ***B***, ***G***, Reducing expression of mAChR-A or Gαq significantly decreases the percentage of flies with seizures. $^{**}p < 0.01$. $^{***}p < 0.001$. $n > 100$ per genotype. Control (+): *repo-GAL4*, *UAS-GFAP$^{R79H/+}$*. ***C***, ***H***, Decreased expression of mAChR-A or Gαq reduces the number of β-galactosidase-positive cells in Alexander disease model flies. $^{*}p < 0.05$. $^{***}p < 0.001$. $n \geq 6$ per genotype. Control (+): *GstD1-lacZ/+*; *repo-GAL4*, *UAS-GFAP$^{R79H/+}$*. ***D***, ***I***, Western blots demonstrate equivalent GFAP levels in GFAP^{R79H} transgenic flies alone and in combination with mAChR-A RNAi or Gαq RNAi lines. The blots were reprobed with an antibody to actin to illustrate equivalent protein loading. Control (+): *repo-GAL4*, *UAS-GFAP$^{R79H/+}$*. ***E***, ***J***, RT-PCR shows reduced *mAChR-A* and *Gαq* mRNA levels in transgenic RNAi lines, using *da-GAL4* or *repo-GAL4* as drivers. *Rpl32* was used as a control. $^{***}p < 0.001$. $n = 3$ biological replicates. Control (+): *da-GAL4/+* (***E***) and *repo-GAL4/+* (***J***). ***A***, ***C***, ***F***, ***H***, Flies were 20-d-old. ***B***, ***D***, ***E***, ***G***, ***I***, ***J***, Flies were 1-d-old. Statistical tests: ***A***, ***E***, ***F***, ***J***, one-way ANOVA with Tukey's multiple-comparison test; ***B***, ***G***, χ^2 test; ***C***, ***H***, two-tailed *t* test.

2005, 2009). With double-label immunofluorescence, we observed a significant reduction in the number of NRF2-positive astrocytes in pirenzepine-treated compared with saline-treated Alexander disease model mice (Fig. 8).

Discussion

Here we describe an unbiased screen of 1987 compounds in a *Drosophila* model of Alexander disease, a unique and clinically severe glial-based neurological disorder for which there is currently no effective treatment. A notable feature of our screening strategy was immunohistochemical detection of caspase activity on brain sections as a marker of cellular toxicity. Prior chemical screens of similar scale have been reported in *Drosophila* but have typically relied on simple behavioral or reporter assays, often in more easily manipulated embryonic or larval stages of development (Chang et al., 2008; Gasque et al., 2013; Markstein et al., 2014). Because *Drosophila* is now more commonly being used to model complex, age-dependent diseases (Feany and Bender, 2000; Wittmann et al., 2001; Colodner and Feany, 2010; Wang et al., 2011), the need to screen for alterations in phenotypes best assayed with the precision of histological analysis may increasingly emerge. We demonstrate here that such histological screening is feasible on the scale of thousands of compounds.

A potential caveat to screening based on histology is the possibility that compounds may be identified that rescue the histological phenotype, but not improve overall health or behavior of the animal. Our findings, that a signaling pathway identified first through histological screening significantly alters seizure frequency in our GFAP^{R79H} transgenic fly model of Alexander disease, suggest that efficacious treatments can emerge from a primary histological screen. Our prior experience with genetic modifiers in the Alexander disease model (Wang et al., 2011) and other models of human disease further supports a strong correlation between tissue neuropathology, behavior, and lifespan (Feany and Bender, 2000; Wittmann et al., 2001; Colodner and Feany, 2010).

As in previous moderate throughput screens, we attempted to enrich for clinically relevant hits by screening the Spectrum Compound library, which contains many FDA-approved drugs and natural products. So-called "repurposing" of drugs is a strategy used to decrease the cost and risk of drug discovery. Because exist-

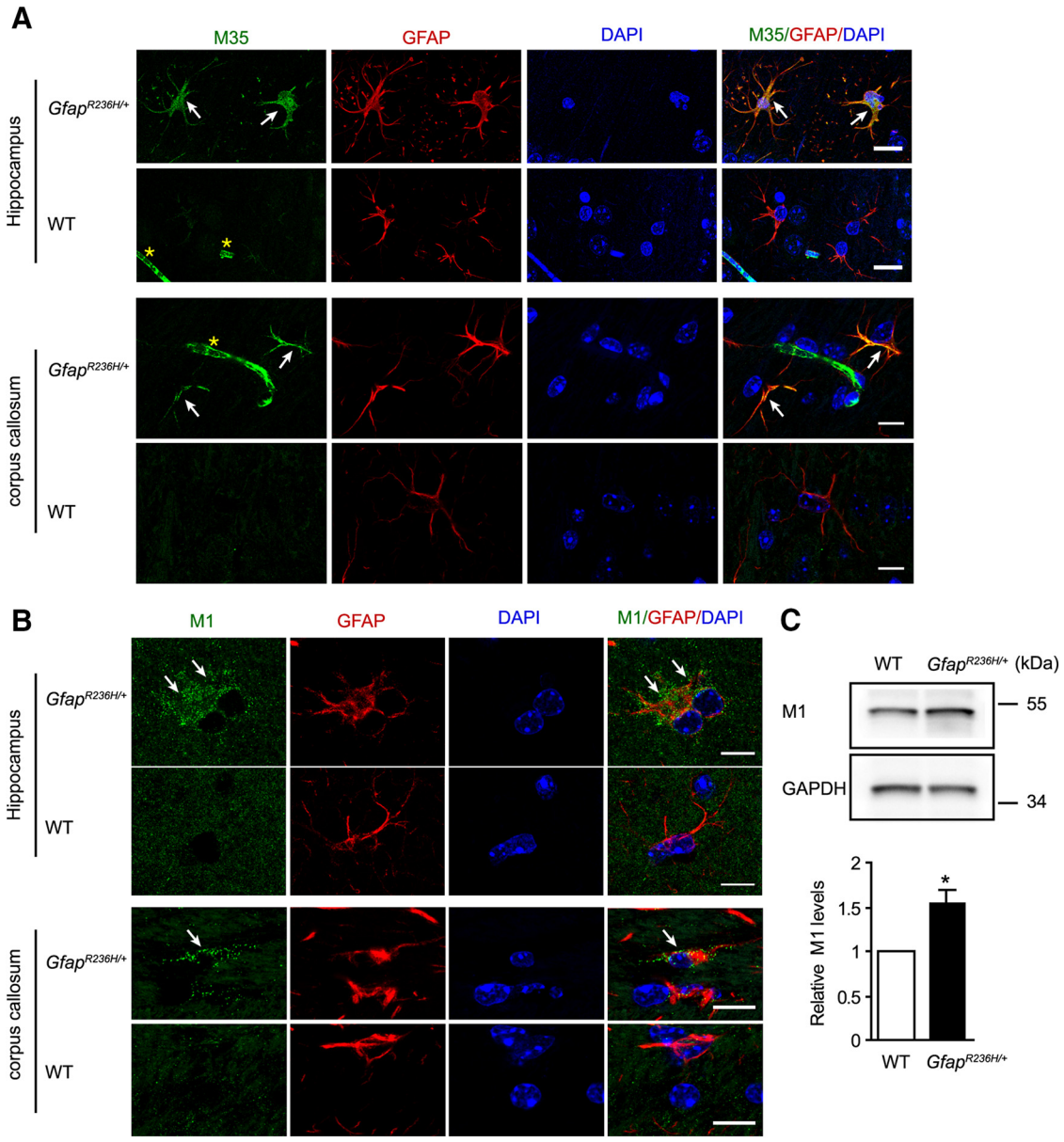

Figure 6. Increased mAChRs in astrocytes of Alexander disease model mice. ***A***, Double-label immunofluorescence reveals increased immunostaining with pan-mAChR antibody, M35, in the astrocytes of 6-month-old $GFAP^{R239H/+}$ mice (arrows). GFAP is an astrocytic marker. Asterisks show blood vessels. DAPI labels nuclei. Scale bar, 20 μm. ***B***, Double-label immunofluorescence reveals upregulation of the M_1 receptor in astrocytes of 6-month-old $GFAP^{R239H/+}$ mice (arrows). DAPI labels nuclei. Scale bar, 10 μm. ***C***, Western blot demonstrates increased expression of M_1 receptor in the corpus callosum of 6-month-old $GFAP^{R236H/+}$ mice. The blot was reprobed with an antibody to GAPDH to illustrate equivalent protein loading. $n = 4$ biological replicates. $^*p < 0.05$ (two-tailed Student's *t* test).

ing drugs have known pharmacokinetic and safety profiles, they can be evaluated rapidly in Phase II clinical trials, thus saving significant development costs (Chong and Sullivan, 2007). In addition, many of the compounds in the Spectrum Compound library are known to cross the blood–brain barrier efficiently, a critical roadblock in the conventional drug discovery pipeline. We indeed identified two pathways, muscarinic cholinergic signaling and serotonin reuptake, for which there are effective, FDA-approved drugs with documented penetration into the CNS.

To validate cholinergic signaling as a therapeutic target in Alexander disease, we extended our screening results, taking advantage of both the strengths of *Drosophila* genetics and the increased translational relevance of mammalian systems. We provide strong support for a signaling cascade involving Gαq and oxidative stress downstream of mAChR-A signaling in mediating GFAP toxicity using a genetic approach in flies, although lack of suitable antibody reagents limited our ability to assess receptor levels directly in the *Drosophila* system. In a complementary approach in a mouse model of Alexander disease and in patient brain tissue, we documented increased expression of mAChRs, consistent with a protective effect of downregulating mAChR expression genetically in flies and pharmacological blockade of M_1 receptors in mice. The mechanism by which mAChR expression is altered in Alexander disease is not clear. However, upregulation of mAChRs has been observed in the context of inflammation (Español et al., 2014), and previous studies have demonstrated a robust stress response in the brains of Alexander disease model mice (Hagemann et al., 2005, 2006, 2009), including upregulation of multiple inducible cytokines and their receptors (Hagemann et al., 2005).

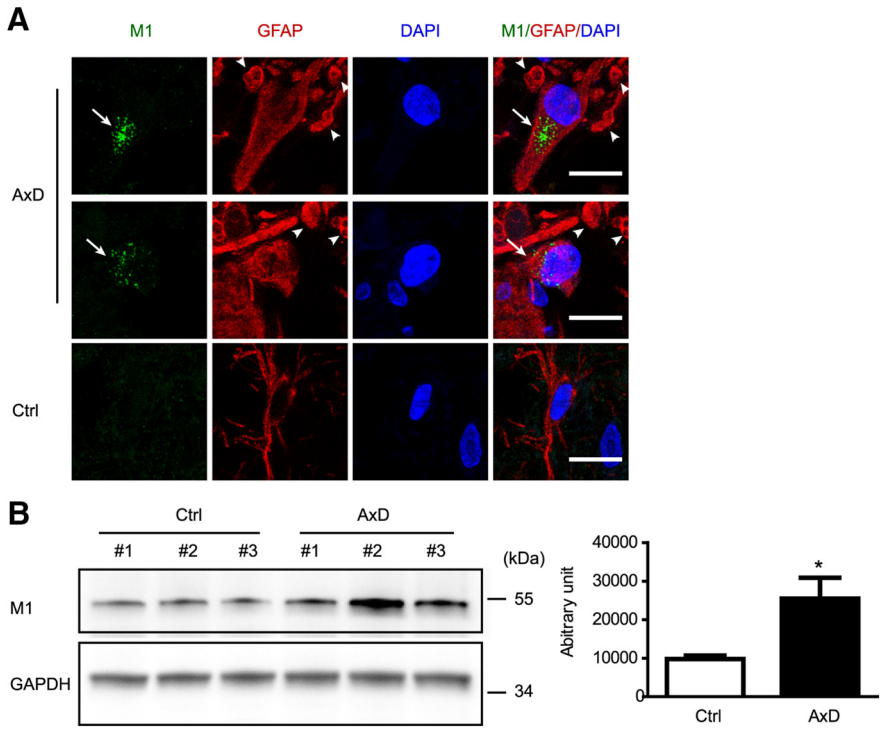

Figure 7. Increased expression of M_1 receptor in Alexander disease patients. ***A***, Double-label immunofluorescence reveals upregulation of M_1 receptor in the astrocytes of an Alexander disease patient (top and middle panels, arrows). Arrowheads indicate Rosenthal fibers. GFAP marks astrocytes. DAPI labels nuclei. Scale bar, 10 μm. ***B***, Western blot shows significantly increased expression of M_1 receptors in the white matter of Alexander disease patients. The blot was reprobed with an antibody to GAPDH to illustrate equivalent protein loading. $^*p < 0.05$ (two-tailed Student's *t* test).

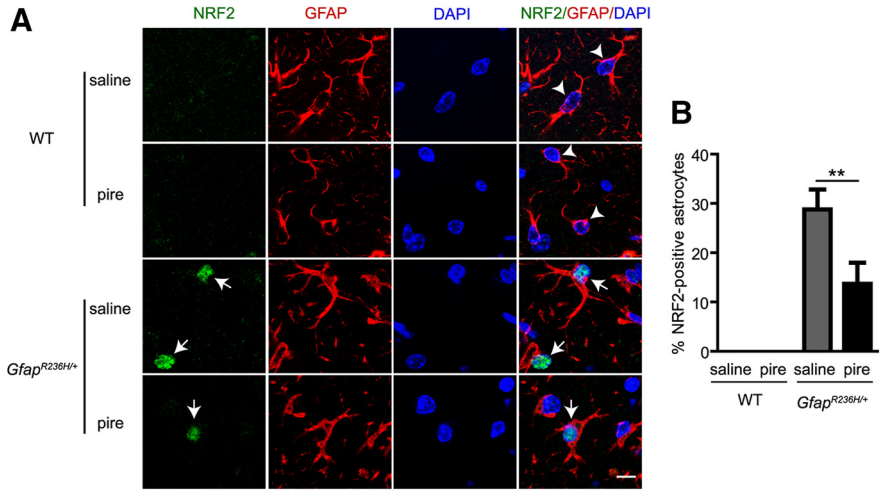

Figure 8. Pirenzepine treatment reduces NRF2 in Alexander disease model mice. ***A***, Double-label immunofluorescence reveals expression of NRF2 in astrocytes of Alexander disease model mice (bottom two rows), but not in age-matched wild-type control mice (top two rows). GFAP marks astrocytes. DAPI labels nuclei. Arrows indicate NRF2-positive astrocytes. Arrowheads indicate NRF2-negative astrocytes. Scale bar, 10 μm. ***B***, Quantification in the hippocampal CA1 region shows a significant reduction of the percentage of NRF2-positive astrocytes in pirenzepine (pire)-treated Alexander disease model mice compared with saline-treated Alexander disease mice. $^{**}p < 0.01$ (one-way ANOVA with Tukey's multiple-comparison test). Fifty astrocytes per animal and 5 or 6 animals per genotype/treatment were counted. Mice were 8 weeks old and were treated with 20 mg/kg pirenzepine or saline for 10 d.

We chose to focus on muscarinic cholinergic signaling in our studies because astrocytes are known to express mAChRs (Guizzetti et al., 1996; Porter and McCarthy, 1997), and activation of these receptors controls a variety of important cellular processes. Of particular note, a number of studies have linked activation of mAChRs to increases in astrocyte calcium levels (Takata et al., 2011; Chen et al., 2012; Navarrete et al., 2012). Intracellular calcium in turn controls a variety of cellular processes, including exocytosis (Jourdain et al., 2007; Liu et al., 2011; Zorec et al., 2012; Kawamata et al., 2014). Because non–cell-autonomous neuronal death is a feature of astrocyte gliopathy in Alexander disease patients, in our *Drosophila* model of Alexander disease, and in other neurological disorders as well (Ilieva et al., 2009), inhibition of release of neurotoxic substances is an intriguing potential mechanism for the protective effect of mAChR blockade in our experiments. Alternatively, as G-protein-coupled receptors, muscarinic receptors regulate a number of other signaling cascades, including tyrosine and MAP kinase pathways (Felder, 1995; Nathanson, 2000; Wess et al., 2007), deregulation of which may lead to glial dysfunction and secondary neuronal toxicity. A recently published study suggests that targeting muscarinic cholinergic signaling may be a particularly attractive therapeutic approach in Alexander disease, a disorder with prominent white matter pathology in many patients. Deshmukh et al. (2013) demonstrated that benztropine, an FDA-approved anticholinergic compound currently in clinical use for Parkinson's disease, enhances remyelination in mouse models of experimental demyelination. In these studies, benztropine acted by antagonizing M1 and/or M3 receptors on oligodendrocyte precursor cells and promoting their differentiation. Thus, modulation of cholinergic signaling may have beneficial effects on multiple cell types affected directly or indirectly by mutant GFAP expression in astrocytes.

In conclusion, we have conducted an unbiased chemical screen using a *Drosophila* model of Alexander disease previously developed in our laboratory (Wang et al., 2011). We identified four compounds with prior approved use in humans, which showed dose-dependent efficacy in our model. We further performed genetic and additional pharmacological studies to validate muscarinic cholinergic signaling in the control of GFAP toxicity. Importantly, we confirmed key findings from our *Drosophila* model in Alexander disease model mice and postmortem patient tissue. Our results highlight the utility of *Drosophila* for unbiased chemical screens in drug development, even in the context of complex, aging-dependent phenotypes in the adult brain. In addition, we identify promising compounds and target pathways, including glial muscarinic cholinergic signaling, for a devastating and currently untreatable nervous system disorder.

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