A translational continuum of model systems for evaluating treatment strategies in Alzheimer’s disease: isradipine as a candidate drug

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SUMMARY

A growing body of evidence supports the ‘calcium hypothesis’ of Alzheimer’s disease (AD), which postulates that a variety of insults might disrupt the homeostatic regulation of neuronal calcium (Ca2+) in the brain, resulting in the progressive symptoms that typify the disease. However, despite ongoing efforts to develop new methods for testing therapeutic compounds that might be beneficial in AD, no single bioassay permits both rapid screening and in vivo validation of candidate drugs that target specific components of the Ca2+ regulatory machinery. To address this issue, we have integrated four distinct model systems that provide complementary information about a trial compound: the human neuroblastoma MC65 line, which provides an in vitro model of amyloid toxicity; a transgenic Drosophila model, which develops age-dependent pathologies associated with AD; the 3×TgAD transgenic mouse, which recapitulates many of the neuropathological features that typify AD; and the embryonic nervous system of Manduca, which provides a novel in vivo assay for the acute effects of amyloid peptides on neuronal motility. To demonstrate the value of this ‘translational suite’ of bioassays, we focused on a set of clinically approved dihydropyridines (DHPs), a class of well-defined inhibitors of L-type calcium channels that have been suggested to be neuroprotective in AD. Among the DHPs tested in this study, we found that isradipine reduced the neurotoxic consequences of β-amyloid accumulation in all four model systems without inducing deleterious side effects. Our results provide new evidence in support of the Ca2+ hypothesis of AD, and indicate that isradipine represents a promising drug for translation into clinical trials. In addition, these studies also demonstrate that this continuum of bioassays (representing different levels of complexity) provides an effective means of evaluating other candidate compounds that target specific components of the Ca2+ regulatory machinery and that therefore might be beneficial in the treatment of AD.

INTRODUCTION

As originally proposed by Khachaturian and colleagues (Khachaturian, 1987), the ‘Ca2+ hypothesis of Alzheimer’s disease (AD)’ was based on observations that age-dependent increases in cellular Ca2+ are exacerbated in AD, which in turn might perturb multiple aspects of neuronal metabolism and activity (Peterson et al., 1985; Gibson and Peterson, 1987; Toescu et al., 2004). Both genetic and environmental insults that disrupt Ca2+ homeostasis in the brain can accelerate the accumulation of neurotoxic amyloid (Aβ) peptides and hyperphosphorylated tau, and pathological aggregates of these proteins can themselves alter neuronal Ca2+ levels via a variety of signaling pathways (reviewed in Green and LaFerla, 2008; Yu et al., 2009; Berridge, 2010). Among the voltage-gated channels that regulate Ca2+ influx, L-type calcium channels (LTCCs) represent a particularly attractive target for treating Ca2+ imbalances associated with AD. Several LTCCs (including the Ca2.1.2 and Ca2.1.3 subtypes) are expressed at high levels by most CNS neurons, and they are upregulated in the brains of AD patients and in mouse models of the disease (Coon et al., 1999; Yang et al., 2009; Willis et al., 2010). Other studies have suggested that elevated LTCC activity might contribute substantially to memory loss and neurodegeneration associated with dementia (Missiaen et al., 2000; Kelly et al., 2006; Thibault et al., 2007), and in vitro studies have shown that Aβ peptides can perturb LTCC activity, either directly or indirectly (Ueda et al., 1997; Scragg et al., 2005; Anekonda et al., 2011). In addition, a number of dihydropyridines (DHPs) targeting specific LTCC subtypes are currently used to treat hypertension and related conditions, making them attractive candidate drugs that could be ‘repurposed’ to treat the mild-to-moderate stages of AD.

However, several cautionary issues have dampened enthusiasm for the potential value of DHPs in treating dementia. Although some epidemiological studies suggest that LTCC antagonists can prevent AD (Farette et al., 1998) or slow its rate of progression (Tollefson, 1990; Fritze and Walden, 1995), large clinical trials with the LTCC antagonist nimodipine failed to show any beneficial effect in AD patients (Morich et al., 1996; Lopez-Arrieta and Birks, 2002). Recent studies have also raised the concern that some DHPs (including nimodipine and nifedipine) can modulate other signaling pathways besides those involving LTCCs (Jeremy et al., 1986; Ding and Vaziri, 2000; Dong et al., 2010). In particular, nimodipine was shown to stimulate the secretion of Aβ both in vitro and in 3×TgAD mice.
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(a murine model of AD), via a mechanism that was independent of its Ca\textsuperscript{2+}-blocking effects (Facchinetti et al., 2006). By comparison, several recent studies have suggested that the DHP isradipine might represent a superior compound for improving the neuropathological symptoms associated with AD. Isradipine is a well-tolerated anti-hypertensive drug that is currently in use in patient trials for Parkinson’s disease (Chan et al., 2007), and clinically relevant doses have been shown to antagonize hypoxia-induced hippocampal damage and memory impairments in rats (Barhwal et al., 2009). However, isradipine has not yet been tested as a therapeutic drug for treating AD.

A continuing challenge to the development of drugs for treating AD has been the lack of rapid, reliable bioassays that can predict the potential benefits and limitations of candidate compounds. In vitro assays using immortalized or primary cell cultures permit high-throughput screening, but they are not good predictors of how trial compounds might affect the intact nervous system. Invertebrate models of AD can be used for efficient in vivo bioassays of selected compounds and genetic screens for novel drug targets, but the utility of these systems is contingent on the conserved nature of proteins targeted by a trial compound, and they do not provide information about the pharmacodynamics of drugs in higher organisms. Murine models (including transgenic mouse models of AD) provide more suitable whole-animal assays, but the duration and expense of these experiments restricts their value for screening large numbers of potential compounds. To address this issue, we have developed a coordinated set of four distinct bioassays that each have a different level of complexity (Fig. 1) and can be used in combination to test the safety and effectiveness of candidate drugs at the molecular, cellular and organismal level. A stably transfected human neuroblastoma line that expresses pathogenic forms of Aβ (MC65) provides a rapid in vitro assay for the neurotoxic effects of intracellular Aβ accumulation (a ‘high-throughput’ assay; completed in 24-72 hours). A Drosophila transgenic model of AD, in which overexpression of human amyloid precursor protein (APP) in the fly brain induces the pathological sequelae associated with AD, provides the basis for both genetic tests of interacting proteins and acute pharmacological assays of selected compounds (a ‘medium-throughput’ assay; requiring 12-14 days to screen selected subsets of compounds). An acute embryonic culture assay (using the lepidopteran Manduca sexta) provides an acute in vivo assay for the neurotoxic effects of amyloid peptides on neuronal motility and synaptic growth (also a ‘medium-throughput’ assay; requiring 6-7

Fig. 1. An integrated suite of model systems for testing candidate drugs to treat AD. (A) The MC65 human neuroblastoma cell line is suitable for high-throughput screening of candidate compounds. Upon Tet removal, this stably transfected cell line expresses the C-terminal fragment of APP (C99), resulting in the progressive accumulation of neurotoxic Aβ peptides as intracellular aggregates (arrowheads) and cell death (arrows). Aβ immunoreactivity is shown green; phalloidin staining in magenta. (B) Transgenic Drosophila lines can be induced to express human APP\textsubscript{695} in specific cell types (using the GAL4-UAS system), resulting in age-dependent accumulations of amyloid fragments, progressive neurodegeneration and accelerated mortality (a medium-throughput assay). (C) An embryonic preparation of the hawkmoth M. sexta provides a second medium-throughput in vivo bioassay for testing the acute effects of exogenous Aβ\textsubscript{1-42} on neuronal migration, outgrowth and synaptogenesis. (D) The triple transgenic mouse AD model (3xTgAD) expresses genes encoding human PS1\textsubscript{M146V}, APP\textsubscript{swen} and tau\textsubscript{P30L}, resulting in age-dependent accumulation of Aβ and phosphorylated aggregates in the brain that resemble the pathology found in human AD (a low-throughput assay). Each of these model systems provides distinct advantages and disadvantages for testing potential compounds that might protect against Aβ-related neurotoxicity. By coordinating these four assays, we propose to establish an efficient paradigm for identifying promising drugs that can be advanced to clinical trials. Scale bar: 20 μm (A); 0.5 mm (B); 1 cm (C); 0.5 cm (D).
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RESULTS

DHPs are protective in an in vitro assay of Aβ toxicity (MC65 cells)

MC65 cells are derived from a human neuroblastoma cell line (SK-N-MC) that has been stably transfected with an APP-derived construct, consisting of the N-terminal 17 residues of APP (corresponding to the signal peptide) fused to the C-terminal fragment of APP generated by β-secretase cleavage (C99) (Gossen and Bujard, 1992; Sopher et al., 1994). Upon withdrawal of tetracycline (Tet), expression of this construct is detectable in the cells within 3-4 hours; the signal peptide is efficiently cleaved, and the resultant C99 protein is processed by endogenous γ-secretase activity to yield monomeric fragments that co-migrate with Aβ in SDS/urea/Bicine gels, and as 8-kDa apparent dimeric species in SDS/Tricine gels (Woltjer et al., 2003; Wolterj et al., 2005b). The progressive accumulation of Aβ results in a detectable increase in apoptotic death within the cultures by 12 hours, culminating in complete death of the cultures by 72 hours. In previous work, Wolterj and colleagues showed that Aβ-induced cell death in these assays is associated with oxidative stress, and that treatment with antioxidants can reduce the accumulation of Aβ aggregates and preserve cell viability (Wolterj et al., 2005a). These and other studies have demonstrated that the MC65 cell line is a viable in vitro model for evaluating the role of potential signaling mechanisms associated with Aβ-associated cytotoxicity, and provide the basis for a high-throughput screen of candidate drugs that could potentially ameliorate these neurotoxic responses.

To establish conditions for testing the protective role of DHPs in this assay, we monitored the levels of C99 and Aβ peptides produced by MC65 cells following Tet removal. As shown in Fig. 2A, the predominant protein species initially expressed by the cells had the predicted size of the C99 construct (10 kDa), followed by the time-dependent accumulation of an 8-kDa species (consistent with the predicted size of Aβ dimers), and subsequently by the appearance of higher-molecular-weight aggregates that were recognized by antibodies against Aβ. By contrast, little or no secreted Aβ could be detected in the surrounding medium over this period (data not shown). Neither monomeric nor oligomeric Aβ was detectable in cells cultured in Tet(+) conditions, although low levels of endogenous APP and its derivatives are present in MC65 cells grown both with and without Tet (Wolterj et al., 2007). The accumulation of Aβ in the MC65 cultures following Tet removal also corresponded with a progressive loss in cell viability (Fig. 2B), which was first detectable between 1.5-2 days and culminated in complete culture death by 3 days. By contrast, cells maintained in the presence of Tet remained viable throughout this period, which is consistent with other published studies using this assay (Sopher et al., 1994; Wolterj et al., 2003; Maezawa et al., 2004). For the current analysis, cell viability was monitored using MTT assays that were carried out in triplicate or quadruplicate (repeated at least four times), and statistical differences among treatment groups were determined with Student’s t-tests and one- or two-way ANOVA.

To assess whether the accumulation of Aβ in the MC65 cells modulated their expression of LTCCs, we used quantitative real-time PCR (qRT-PCR) to measure the expression levels of Ca1.2 (α1C) and Ca1.3 (α1D), the predominant LTCC α-subunits that are...
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expressed in neurons (Striessnig et al., 2006; Sinnegger-Brauns et al., 2009). As shown in Fig. 2C, we found that the expression of Ca$_{1,2}$ was significantly upregulated by 48 hours after Tet removal compared with Tet(+) cultures, which showed no increase in Ca$_{1,2}$ expression over basal levels. By comparison, Ca$_{1,3}$ expression was not detectable in MC65 cells (data not shown). When we examined the intracellular free Ca$^{2+}$ levels ([Ca$^{2+}$]$_{\text{int}}$) in MC65 cultures by Fluo-4 imaging, we detected a progressive increase in [Ca$^{2+}$]$_{\text{int}}$ following Tet removal that was significantly elevated by 72 hours (Fig. 2D), whereas [Ca$^{2+}$]$_{\text{int}}$ levels in Tet(+) cultures remained unchanged. These results are consistent with reports that LTCCs are upregulated in the brains of AD patients (Coon et al., 1999), and with previous studies showing that both APP and Aβ can promote the expression of LTCCs in cultured neurons and neuroblastoma cells (Chiou, 2006; Yang et al., 2009). They also support the hypothesis that perturbations in Ca$^{2+}$ homeostasis might play an important role in Aβ-induced cytotoxicity (Yu et al., 2009; Wu et al., 2010).

To test whether LTCC antagonists could prevent the rise in [Ca$^{2+}$]$_{\text{int}}$ following the onset of Aβ accumulation, we treated MC65 cells with several clinically approved DHPs at the time of Tet removal. As shown in Fig. 2D, both nimodipine (10 μM) and isradipine (50 nM) completely blocked the increase in Ca$^{2+}$ that was normally observed in Tet(−) cultures, although these treatments did not alter the production of Aβ by the MC65 cells (Anekonda et al., 2011). We next examined the effects of four different DHPs on the viability of MC65 cultures after 3 days of treatment, a stage when cultures grown in the absence of Tet are typically dead (Fig. 2B). Initially, we tested each drug over a broad range of physiologically relevant concentrations (1-100 μM) to determine tolerated dosages that also provided some improvement in cell survival. On the basis of the effective concentrations identified in these preliminary trials, we subsequently tested the ability of each DHP to protect against Aβ-associated toxicity. As shown in Fig. 3, low concentrations of verapamil, diltiazem, nimodipine and isradipine produced only minimal levels of nonspecific cytotoxicity in MC65 cells grown in the presence of Tet (magenta curves), although all of the compounds produced dose-dependent reductions in cell survival at higher concentrations in these control cultures. All four DHPs also significantly improved survival rates in Tet(−) cultures (expressing Aβ), to varying degrees. Although verapamil provided the most robust protective effect (75% survival at 15 μM), it also caused a dramatic loss in viability at higher doses (50% survival at 65 μM and 0% survival at 100 μM). Likewise, nimodipine (the subject of recent trials in AD patients) provided intermediate protection, supporting ~60% survival at 10 μM, but also had considerable deleterious effects at higher concentrations. Diltiazem was the least effective in protecting the cultures from the neurotoxic effects of Aβ expression, providing only 40% survival at 55 μM. By contrast, treatment with isradipine resulted in substantial improvements in viability over a broad dosage range, resulting in ~70% survival at 1 μM and 50% survival at 15 μM. Equally important, isradipine was substantially less toxic at higher doses than the other DHPs: at concentrations up to 100 μM, isradipine continued to protect against Aβ-associated toxicity without causing a substantial increase in cell death (Anekonda et al., 2011) (and data not shown). These experiments showed that clinically approved DHPs provide a protective effect against Aβ-induced neurotoxicity in this cell culture assay, and that isradipine was well tolerated over a range of concentrations. In the context of integrating our bioassays to screen for compounds that might be therapeutic in AD, these in vitro studies (using MC65 cells for a rapid initial screen) provided the justification for testing isradipine in our more time-consuming in vivo assays, as described below.

**Isradipine is protective in a genetic model of Aβ neurotoxicity (Drosophila)**

Recent studies by several groups have used _Drosophila_ as a genetic system for investigating the mechanisms of Aβ neurotoxicity associated with AD (reviewed in Lu and Vogel, 2009; Wentzell and Kretzschmar, 2010). The sole APP ortholog expressed in insects is APPL (Amyloid precursor protein-like protein), which contains the same types of protein interaction domains found in APP (Luo et al., 1999; Toroja et al., 1999; Swanson et al., 2005). Most of the major classes of APP-interacting proteins are also conserved in _Drosophila_, including homologs of the α-, β- and γ-secretase families (Ye and Fortini, 1999; Guo et al., 2003; LaVoie and Selkoe, 2003; Carmine-Simmen et al., 2009), and transgenic flies expressing either human APP or Aβ$_{1-42}$ exhibit age-dependent amyloid deposits and neuropathological damage, accompanied by progressive behavioral deficits and premature death (Fossfreig et al., 1998; Finelli et al., 2004; Greeve et al., 2004; Iijima et al., 2004; Leyssen et al., 2005). Intriguingly, overexpression of _Drosophila_ Aβ$_{1-42}$ (the predicted Aβ-like fragment derived from APPL) also results in the accumulation of amyloid-like deposits, behavioral deficits and neurodegeneration (Carmine-Simmen et al., 2009), suggesting that important pathogenic features of human APP might be conserved in APPL. In addition, _Drosophila_ expresses an ortholog of the mammalian Ca$_{1,3}$ (α1d) subunit (DmCa1D) that is sensitive to DHPs (Gielow et al., 1995; Zheng et al., 1995; Ren et al., 1998; Radysh et al., 2006; Clark et al., 2008). On the basis of the outcome of the MC65 assays described above, we therefore used our
transgenic fly model of AD to test whether chronic treatment with selected DHPs could modulate the premature lethality that typically occurs in this model (Greeve et al., 2004).

For these experiments, we used the GAL4-UAS system (Brand and Perrimon, 1993) to drive the expression of human APP695 (the most predominant APP isoform in neuronal tissue) in Drosophila. As an initial test of this model, we showed that expression of APP695 specifically in the eye resulted in the accumulation of Aβ peptides, amyloid deposits and age-dependent degeneration (Fig. 4A-C), consistent with previous work showing that Drosophila possesses all of the secretases required for cleaving Aβ peptides from APP (Fossgreen et al., 1998; Greeve et al., 2004; Carmine-Simmen et al., 2009). Our transgenic lines expressing APP695 therefore provide a convenient whole-animal model for testing drugs that might ameliorate the neurotoxic effects of amyloid accumulation.

To evaluate the protective effects of oral DHP treatment, we took advantage of our established developmental lethality assay to test how the expression of APP695 in all cells affected the survival of the flies to adults. An advantage of this assay is that it can be initiated using a predefined number of mating pairs in a single genetic cross, which generates both control and experimental animals (expressing APP695) that will be raised under identical conditions (in the same vials of medium) and that can be readily distinguished by genetic markers. Subsequently, the effects of a drug treatment on survival rates of control versus APP695 flies can be unambiguously scored and analyzed using simple statistical methods.

In addition, the entire procedure takes 12-14 days (as opposed to 60-90 days required for adult neurodegeneration assays) (Ye and Fortini, 1999; Guo et al., 2003; Carmine-Simmen et al., 2009), and it avoids the more laborious histological procedures required for analyzing retinal degeneration, as illustrated in Fig. 4A-C. This developmental lethality assay thus provides an efficient ‘medium-throughput’ screen for testing subsets of drugs that are selected from high-throughput screens (such as the MC65 cell assay) and might be neuroprotective against amyloid toxicity in vivo.

In the current study, female flies carrying the Actin-GAL4 construct were crossed with male flies containing UAS-APP695 (to induce the expression of human APP695 in all cells), and the females were then allowed to lay eggs on control medium or medium containing different concentrations of isradipine or verapamil; a minimum of ten males and ten females were used to initiate each trial. The adult flies were then removed and their progeny were allowed to feed ad libitum throughout larval life. Drugs were diluted to their final concentrations in Drosophila media containing blue dye (Sargent-Welch), which provided a convenient marker for monitoring overall feeding rates by control and APP695-expressing flies in each vial. Upon emergence of the newly eclosed progeny, we compared the number of APP695-expressing flies with siblings that lacked the Actin-GAL4 promoter construct (and therefore did not express APP695) but shared the same genetic background. As described in the Methods, survival rates of APP695-expressing progeny were calculated as a percentage of surviving control progeny that were obtained from the same genetic cross (see Greeve et al., 2004). On the basis of the genotype of the parents, both APP695-expressing and non-expressing control flies should theoretically be produced in equal numbers; however, owing to the enhanced lethality induced by APP695 expression, only about 6.5% of these flies survived under normal conditions, providing a highly sensitive assay of any ameliorative effect of the drugs administered during the larval feeding stage. We performed the following number of independent tests per condition: 20 control tests (0 µM isradipine); 10 tests with 25 µM isradipine; 12 tests with 250 µM isradipine; 10 tests with 10 µM verapamil; 8 tests with 100 µM verapamil; and 3 test with 500 µM verapamil.

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![Fig. 4. Treatment with dietary isradipine protects against the neurotoxic effects of APP/Aβ overexpression in transgenic Drosophila.](Image)

(A-C) Retinal degeneration in transgenic flies caused by the induced expression of human APP695 (using the GMR-GAL4 driver line). (A-C) Horizontal sections of fly brains from similar levels. (A) A 25-day-old control fly (containing only the GMR driver line) shows no signs of degeneration. me, medulla; la, lamina; re, retina. (B) A 1-day-old transgenic fly expressing APP695 (via GMR-GAL4) already shows the formation of a few, small vacuoles, indicative of degeneration (arrowheads). (C) A 25-day-old fly expressing APP695 exhibits severe retinal degeneration.

(D-E) Effects of dietary verapamil on the mortality of flies expressing human APP695 and their wild-type sibling controls (raised in the same vial). (D) Survival rates of control flies (not expressing APP695) were significantly reduced when raised on medium containing 10-100 µM verapamil; higher concentrations caused 100% lethality within the population (see Results). (E) Treatment with verapamil induced no significant improvement (ns) in viability of APP695-expressing flies at any of the concentrations tested. Survival rates were determined by comparing the number of viable APP695 flies in each assay with their non-expressing siblings; values were calculated as a ratio of surviving flies of each genotype versus their predicted survival rate (50%). Five independent experiments were performed for flies treated with 10 µM verapamil, and six experiments for flies treated with 100 µM. (F-G) Effects of dietary isradipine on control and APP695-expressing flies. (F) Dietary isradipine caused no significant change in the survival rates of sibling control flies at any of the concentrations tested (ns). (G) Treatment with isradipine significantly increased the survival rate of APP-expressing flies in a concentration-dependent manner. 20 independent experiments were performed for the untreated group, 10 experiments for flies treated with 25 µM isradipine and 12 experiments for flies treated with 250 µM isradipine. Error bars indicate means ± s.e.m. *P<0.05; **P<0.01 (Student’s t-test).
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Isradipine is protective in an in vivo assay of neuronal motility (Manduca)

Recent studies have also shown that neurotoxic forms of Aβ can perturb multiple aspects of neuronal motility, including neuronal migration, outgrowth and dendritic remodeling (Shrestha et al., 2006; Evans et al., 2008; Crews and Masliah, 2010). In previous work, Copenhaver and colleagues used embryonic cultures of M. sexta (tobacco hornworm) to show that APPL is strongly upregulated in motile neurons (Swanson et al., 2005; Copenhaver, 2007), which is consistent with the upregulation of APP in regions of neuronal growth and synaptic remodeling in the mammalian brain (Loffler and Huber, 1992; Akaaboune et al., 2000; Lahiri et al., 2002). In addition, the relatively large size and stereotyped organization of the Manduca nervous system permits pharmacological treatments to be applied directly to identified sets of motile neurons, an approach that is not feasible in smaller preparations such as Drosophila. A variety of methods have also been developed for imaging and quantifying the effects of an experimental manipulation of multiple aspects of neuronal motility and synaptic growth in this system (Copenhaver, 2007; Coate et al., 2009). We tested whether the larger Manduca model could be used as a second ‘medium-throughput’ assay for drugs that ameliorate Aβ-dependent defects in neuronal motility.

For these experiments, we focused on identified neurons within the enteric nervous system (ENS) of Manduca, a region that permits acute manipulations of neuronal motility and synaptic growth in cultured embryos (Fig. 5A,B) (Copenhaver, 2007). Because the neurons of the ENS occupy the most superficial layer of the visceral musculature, they can be directly treated with exogenous compounds while undergoing normal synaptic growth (Fig. 5C) (Horgan and Copenhaver, 1998; Wright et al., 1999; Coate et al., 2009). Embryos were opened in defined saline at 70% of development, a stage when the neurons have completed their migration into the ENS but have not established their terminal arbors on the visceral musculature of the foregut (Fig. 5D). Graded concentrations of soluble Aβ1-42 (prepared as described in the Methods) or a variety of control proteins were then applied directly to the neurons, and the preparations were allowed to develop for an additional 24 hours (spanning the period of synaptic differentiation). Embryos were then rapidly immunostained as whole-mount preparations with antibodies against Fasciclin II (Fas II; Fig. 5B) and cyclic GMP (Fig. 5C) to visualize the neurons and their terminal synaptic arbors on the surrounding musculature, which were then quantified from camera lucida images (for details, see Wright et al., 1998). At least 15 preparations were used for each condition, and each experiment was repeated at least three times. Because the entire protocol can be completed in 6-7 days (including statistical analysis of camera lucida-based measurements), this preparation represents a second ‘medium-throughput’ assay that complements the Drosophila assay described above, and is therefore suitable for testing selected subsets of compounds identified in the high-throughput MC65 assay.

As illustrated in Fig. 5E, neurons in control preparations established elaborate synaptic arbors by completion of the experimental period. By contrast, treatment with 10 μM exogenous Aβ1-42 induced a profound reduction in terminal branch density (Fig. 5F). The effects of Aβ1-42 on synaptic growth were also concentration dependent (Fig. 5G, black histograms), resulting in significant reductions in synaptic branching compared with controls (P<0.01 at 5 μM; P<0.005 at 10 μM), accompanied by the accumulation of swollen terminals on many of the neurites (Fig. 5F, arrowheads). By contrast, treatment with fusion proteins containing the soluble extracellular domain of APPL (sAPPL) had no deleterious effects on synaptic growth (Fig. 5G, hatched histogram), nor did a variety of control proteins applied at similar concentrations (including BSA and AP; not shown). As reported for other pharmacological manipulations using this preparation (Horgan and Copenhaver, 1998; Coate et al., 2009), higher concentrations of Aβ1-42 were needed to elicit an abnormal response from the enteric neurons than are typically required for cells in vitro. This difference might reflect the robust nature of this intact preparation, as well as species differences in their sensitivity to pharmacological compounds. However, even at these relatively high concentrations, Aβ1-42 did not induce any increase in cell death over the course of these experiments. The dose-dependent nature

To monitor whether the drugs used in this study affected the viability of control flies, we counted the number of sibling control flies (not expressing APP695) that completed adult development, compared with untreated vials. As shown in Fig. 4D, we found that verapamil was not well tolerated: at 10 μM, we observed a 54% reduction in survival compared with the viability of vehicle-treated controls (\(P<0.023\)), and treatment with 100 μM induced a 73% reduction (\(**P<0.01\)). Treatment with 500 μM verapamil resulted in 100% lethality (none of the animals survived to adulthood), consistent with the adverse effects of higher verapamil concentrations in the MC65 cultures (see Fig. 3). Conversely, when we examined the effects of verapamil on the viability of APP695 flies (as a percentage of surviving flies in each treatment group), we observed no protective effect at either 10 μM (7% survival) or 100 μM (8% survival), respectively (Fig. 4E; ns, not significant). Of note is that none of the drug concentrations used in this analysis caused detectable changes in larval feeding behavior (monitored by the uptake of blue dye from the medium), indicating that the effects of verapamil on lethality were not simply due to an aversive response to the treated food.

By contrast, we found that isradipine provided far superior results. Treatment with either 25 μM or 250 μM isradipine had no significant effect on the viability of sibling control flies (Fig. 4F), in marked contrast to the toxic effects of verapamil. In addition, whereas treatment with 25 μM isradipine did not confer any protective effect to the APP695 flies, inclusion of 250 μM dietary isradipine significantly enhanced their survival rates, almost doubling the number of viable adults (Fig. 4G; \(^*P<0.05\)). As with verapamil, there were detectable aversive responses to isradipine in the medium. Because the effective concentrations of isradipine delivered to the brain by this method could not be readily quantified, the dosages used in this study should be judged for their relative effects on survival, rather than comparing their absolute potencies in other bioassays. Nevertheless, these results demonstrate that Drosophila can be used as a viable genetic model for testing candidate compounds that might protect against Aβ-associated neurotoxicity, and they provide additional evidence that isradipine is a potentially useful drug that could be beneficial in AD.
and specificity of the effects of \( \alpha_B1-42 \) in this assay indicate that it can be used to screen for compounds that ameliorate \( \alpha_B \)-induced perturbations of neuronal motility and synaptic growth in vivo.

Like *Drosophila, Manduca* also expresses evolutionarily conserved LTCCs that are sensitive to DHP treatment (Allen et al., 1992; Lohr et al., 2005; Burkert and Duch, 2006), although higher DHP concentrations are typically required to inhibit insect LTCCs than their mammalian counterparts. To test whether the disruption of synaptic growth by \( \alpha_B1-42 \) was dependent on \( \text{Ca}^{2+} \) influx, we cultured embryos in low-\( \text{Ca}^{2+} \) saline \((~0.4 \text{ mM external } \text{Ca}^{2+}\rangle\). In control preparations grown under these conditions, we observed only a slight enhancement in synaptic branching (Fig. 5G, first white histogram), which is consistent with previous evidence that embryos continue to develop normally under these conditions (Horgan and Copenhaver, 1998). When we treated preparations with 5 \( \mu \text{M} \) \( \alpha_B1-42 \) in low-\( \text{Ca}^{2+} \) saline (Fig. 5G, second white histogram), the neurotoxic effects observed in normal \([\text{Ca}^{2+}]_{\text{ext}}\rangle \) were largely prevented. These results indicate that the reduced synaptic growth caused by \( \alpha_B1-42 \) was \( \text{Ca}^{2+} \)-dependent.

To determine the involvement of LTCC activity in this response, we tested whether treatment with isradipine could prevent the deleterious effects of amyloid peptides. When we treated preparations with 10 \( \mu \text{M} \) isradipine alone in normal \([\text{Ca}^{2+}]_{\text{ext}}\rangle \), we observed a slight increase in synaptic sprouting compared with untreated controls (Fig. 5G, first light gray histogram), which is consistent with the growth-promoting effects seen in cultures incubated in low-\( \text{Ca}^{2+} \) saline. Most notably, when we simultaneously treated preparations with both an intermediate concentration of \( \alpha_B1-42 \) (5 \( \mu \text{M} \)) plus 10 \( \mu \text{M} \) isradipine, the inhibitory effect of \( \alpha_B \) on terminal branch formation was largely reversed (Fig. 5G, second light gray histogram). Although the extent of synaptic growth in these preparations was still slightly reduced compared with untreated controls, it was significantly better than in preparations treated with \( \alpha_B1-42 \) alone (a ~20% improvement; \( P<0.05 \)). These data are consistent with the protective effects of isradipine in our MC65 cell and *Drosophila* assays, and they demonstrate that the *Manduca* culture preparation can be used as a novel assay for evaluating drugs that ameliorate the deleterious effects of amyloid peptides on neuronal motility in vivo.

Lastly, we also tested whether verapamil could protect against \( \alpha_B1-42 \) toxicity in this assay. As shown in Fig. 5G (first dark gray histogram), treatment with 10 \( \mu \text{M} \) verapamil alone induced only a minor decrease in synaptic growth, in contrast to the significant effects that the drug had at this concentration in the *Drosophila* assay; possibly, this difference reflects the relatively short duration of the *Manduca* culture preparation (6-7 days) compared with the developmental lethality assay (12-14 days). Simultaneous treatment with both verapamil (10 \( \mu \text{M} \)) and \( \alpha_B1-42 \) (5 \( \mu \text{M} \)) resulted in a partial protection of normal outgrowth (Fig. 5G, second dark gray histogram), similar to the protective effects of isradipine, although this response was not significantly different from preparations treated with \( \alpha_B1-42 \) alone (\( P>0.09 \)). These results further highlight the value of using all three simpler bioassays (MC65 cells, *Drosophila* and *Manduca*) to select drugs that are both safe and effective in different biological contexts.

**Isradipine is protective in a transgenic mouse model of AD**

On the basis of the promising results obtained with isradipine in our other preparations, we proceeded with an initial trial of its neuroprotective effects in our mouse model of AD (the 3\( \times \)TgAD transgenic line) (Oddo et al., 2003b; Laslo et al., 2004). The...
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3×TgAD mouse model, which harbors transgenes for human PS1M146V, AβP1-40, and tauP301L, develops age-dependent and region-specific Aβ and tau aggregations in the cortex and hippocampus by 8-9 months, in a pattern that mimics the pathology seen in human AD (Oddo et al., 2003a; Oddo et al., 2003b). These mice also develop age-related short- and long-term spatial cognitive deficits (Oddo et al., 2003b; Billings et al., 2005), providing the basis for both histological and behavioral evaluations of drugs that might ameliorate the deleterious symptoms associated with AD. However, owing to the cost and duration of this preparation, it is only suitable for 'low-throughput' screens of selected compounds.

For this pilot study, a small cohort of female 3×TgAD mice was selected at 17 months (well after the initial appearance of AD pathology at 8-9 months) and implanted with carrier-bound isradipine pellets (3 µg/g/day, 60-day release; n=3) or vehicle control pellets (n=4). At the end of the treatment period, mice were euthanized, blood was collected, and the brains quickly removed for dissection and biochemical analysis, as previously described (Quinn et al., 2007). Using liquid-chromatography–tandem-mass-spectrometry (LC-MS/MS) to determine drug levels in the tissue samples (see Park et al., 2009), we found that isradipine was present at clinically relevant concentrations in both plasma (89 nM) and brain lysates (126 nM); no isradipine was detectable in the vehicle-treated controls. Although these plasma levels were somewhat higher than therapeutic plasma concentrations of isradipine used for the management of hypertension (13-26 nM) and also higher than the concentration needed to prevent Aβ-induced cytotoxicity in MC65 cells (48 nM; Fig. 2), weekly body weight measurements were not significantly different in vehicle-treated versus isradipine-treated animals (an indirect indicator of toxicity; Fig. 6A), indicating that the dosage used in this study was well tolerated. There was also no mortality or obvious signs of morbidity in any of the treated cohorts.

When we quantified the amount of Aβ present in the brain samples by ELISA, we found that the levels of insoluble Aβ1-42 were substantially reduced in the isradipine-treated animals, compared with vehicle-treated controls (Fig. 6B). Insoluble Aβ1-40 levels were also slightly reduced, but to a lesser degree. By contrast, isradipine had no apparent effect on soluble Aβ1-40 (Fig. 6C), whereas soluble Aβ1-42 was undetectable in our assays. When we immunostained brain sections with anti-pan-Aβ antibodies, we observed that the density of Aβ-positive plaques was also markedly reduced within both the cortex and hippocampal regions of isradipine-treated animals, compared with vehicle controls (Fig. 6D,E). Despite the dramatic differences seen in some samples, this reduction in plaque density did not achieve significance in either region (Fig. 6F; P<0.1), reflecting the relatively small number of animals that were included in this pilot study. Nevertheless, these results suggest that isradipine could be a safe and effective drug for mitigating the accumulation of amyloid load in the brain.

An examination of phosphorylated tau (tau-P) levels revealed similar trends. By western blot analysis, tau-P levels were reduced in isradipine-treated brain lysates, compared with vehicle-treated controls (Fig. 7A,B). A more dramatic difference was detected in immunostained preparations (Fig. 7C,D), which revealed a consistent reduction in tau-P immunoreactivity within the hippocampus, and more variably in the cortex. Although, once again, these differences did not achieve statistical significance in this initial trial (Fig. 7F), these results suggest that isradipine might provide significant beneficial effects against the neurotoxic forms of amyloid and tau that accumulate in AD, consistent with the beneficial effects seen in our other three assays.

**DISCUSSION**

Although the accumulation of Aβ plaques and neurofibrillary tangles are the hallmark features of AD, the molecular mechanisms...
linking these pathological features to neuronal damage remain controversial. Recent evidence has provided new support for the Ca\textsuperscript{2+} hypothesis of AD, which postulates that the sustained dysregulation of Ca\textsuperscript{2+} homeostasis in the brain plays a central role in the progressive neurodegeneration seen in AD patients, ultimately resulting in senile dementia and death. On the basis of early studies suggesting that normal age-dependent increases in cellular Ca\textsuperscript{2+} are exacerbated in AD, the Ca\textsuperscript{2+} hypothesis has evolved to include multiple factors that might perturb Ca\textsuperscript{2+} homeostasis within the brain (Peterson et al., 1985; Gibson and Peterson, 1987; Toescu et al., 2004). Both A\textsubscript{β} peptides and other proteolytic fragments derived from APP can interact with membrane channels that regulate Ca\textsuperscript{2+} influx, including NMDA channels, AMPA channels and voltage-gated Ca\textsuperscript{2+} channels (LaFerla, 2002). Globular clusters of amyloid peptides might also form a novel type of cation channel that permits unregulated Ca\textsuperscript{2+} entry (Lin et al., 2001; Kagan et al., 2004; Jang et al., 2010). Other studies have shown that oligomeric forms of A\textsubscript{β} can activate Ca\textsuperscript{2+}-dependent phosphatases, cysteine proteases and caspases, which in turn can accelerate A\textsubscript{β} production and also hyperactivate downstream signaling cascades that lead to neuronal dystrophy and death (Lebart and Benyamin, 2006; Hajieva et al., 2009; Wu et al., 2010; D’Amelio et al., 2011). Independent of A\textsubscript{β}, clinically relevant mutations in both APP and presenilins (associated with familial AD) have been linked to aberrant Ca\textsuperscript{2+} regulation in the endoplasmic reticulum (ER) and mitochondria (reviewed in LaFerla, 2002; Celsi et al., 2009; Pimplikar et al., 2010), possibly via perturbations that affect conventional store-operated Ca\textsuperscript{2+} channels, SERCA (endoplasmic reticulum Ca\textsuperscript{2+} ATPase) channels and CALHM1 (calcium homeostasis modulator 1) channels (Dreses-Werringloer et al., 2008; Green and LaFerla, 2008; Berridge, 2010). Thus, a complex interplay of reciprocal interactions between pathogenic proteins linked with AD and a variety of Ca\textsuperscript{2+}-dependent pathways might therefore exacerbate a degenerative cascade, leading to neuronal dystrophy and death (Demuro et al., 2010).

On the basis of the growing body of evidence that dysregulated Ca\textsuperscript{2+} homeostasis might play a prominent role in AD, drugs targeting different aspects of this process have been tested in both animal models and human patients, albeit with variable results. Treatment with memantine (an NMDA receptor antagonist) has yielded the most promising outcomes in clinical trials (Thomas and Grossberg, 2009; Galimberti and Scarpini, 2010), and it is currently the only approved drug for treating patients with moderate-to-severe symptoms of AD. Drugs targeting the Ca\textsuperscript{2+}-calmodulin-dependent phosphatase calcineurin have also been shown to reverse A\textsubscript{β}-associated deficits in learning and memory in the Tg2576 mouse model of AD (Dinlely et al., 2007), and they inhibit the generation of amyloid peptides in SH-SYSY cells (Cho et al., 2008). By contrast, pharmacological agents directed against other proteins involved in the regulation of intracellular Ca\textsuperscript{2+} have produced more variable results, including drugs that inhibit AMPA receptors, Ca\textsuperscript{2+} channels in the endoplasmic reticulum, and channels formed by A\textsubscript{β} itself (Yu et al., 2009; Supnet and Bezprozvanny, 2010). Despite growing evidence that the progressive disequilibrium of neuronal Ca\textsuperscript{2+} contributes to the pathology of AD, available treatments that can mitigate the deleterious effects of Ca\textsuperscript{2+} overload in the brain remain limited.

Given the extensive use of DHPs to treat hypertension and cerebrovascular disease (Taddei et al., 2003; Inzitari and Poggesi, 2005; Bangalore and Messerli, 2006), these drugs represent a promising class of clinically approved compounds that could be beneficial for patients suffering from AD. Besides substantial evidence that LTCCs are upregulated in the course of the disease (Coon et al., 1999; Yang et al., 2009; Willis et al., 2010), a variety of studies have suggested that amyloid peptides might interact with this class of channel, both directly and indirectly. In HEK293 cells overexpressing human LTCC α\textsubscript{1C} (Cav1.2) subunits, A\textsubscript{β} peptides promoted the insertion of α\textsubscript{1C} subunits into the plasma membrane and enhanced Ca\textsuperscript{2+} channel activity (Scragg et al., 2005). Likewise, in both rat primary cortical neurons and synaptosome preparations, treatment with A\textsubscript{β} peptides enhanced voltage-activated inward Ca\textsuperscript{2+} currents that resulted in neurotoxic responses, and these effects were attenuated by treatment with DHPs (Weiss et al., 1994; Fu et al., 2006). Conversely, depolarization-induced elevations in cytosolic Ca\textsuperscript{2+} in rat cortical neurons stimulated their production of A\textsubscript{β} \textsubscript{1-42} and cell death, and both of these effects were inhibited by nimodipine (Pierrot et al., 2004). Besides the effects of A\textsubscript{β} on evoked Ca\textsuperscript{2+} release, chronic exposure to amyloid peptides also results in sustained elevations in intracellular Ca\textsuperscript{2+} that are sensitive to DHP treatment. Adult neurons from the two different mouse models of AD (APPswe and 3×TgAD) exhibited a twofold increase in resting free Ca\textsuperscript{2+} when compared with wild-type mice, a phenomenon that was partially blocked by another DHP, nifedipine (Lopez et al., 2008). Thus, despite the failure of recent clinical trials to demonstrate a beneficial effect for nimodipine in AD, there is substantial evidence that other DHPs might represent attractive candidate drugs that could be used to treat patients with mild-to-

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**Fig. 7. Chronic treatment with isradipine inhibits the accumulation of tau-P in 3×TgAD mice.** (A) Western blot of tau-P levels (detected with the AT8 antibody) in brain lysates from vehicle-treated versus isradipine-treated AD mice. Lower gel shows β-tubulin levels. (B) Quantification of tau-P levels in western blots (normalized to β-tubulin) shows that isradipine treatments reduced overall tau-P levels, although this difference was not significant. (C) Representative brain section from a vehicle-treated AD mouse, immunostained for tau-P (using biotinylated AT8); (D) immunostained brain section from an isradipine-treated AD mouse; C,D illustrate the most dramatic differences seen between vehicle- and isradipine-treated animals. Arrowheads indicate hippocampus; arrow indicates cortex. Scale bar: 400 μm. (E) Quantification of tau-P burden, as detected by immunohistochemistry. Isradipine treatments caused a reduction in tau-P levels in the hippocampus but not the cortex.
moderate dementia. In particular, our pilot studies indicate that isradipine is both more potent and better tolerated than nimodipine in multiple assays of amyloid-induced neurotoxicity, suggesting that it represents a potentially useful candidate drug for advancement to clinical trials.

A primary goal of this study was to demonstrate whether our rapid, inexpensive bioassays in neuroblastoma and insect models could be used to identify promising compounds for subsequent analysis in mouse models of AD, with the ultimate goal of advancing new therapeutic drugs to clinical trials. The conceptual framework for this approach is to use the MC65 cell assay as an initial high-throughput screen of large numbers of candidate compounds, followed by subsequent in vivo testing of the most promising subset for this approach is to use the MC65 cell assay as an initial high-throughput screen of large numbers of candidate compounds, followed by subsequent in vivo testing of the most promising subset for this approach is to use the MC65 cell assay as an initial high-throughput screen of large numbers of candidate compounds, followed by subsequent in vivo testing of the most promising subset. Neuroassays are crucial for selecting promising compounds, whereby only those compounds that perform well on all three assays are then advanced to testing in the more laborious mouse AD model. The potential value of this coordinated bioassays approach was illustrated by the responses observed with verapamil: although this compound performed reasonably in the MC65 assay and provided partial protection in the Manduca assay, it proved to be toxic in the Drosophila assay. By contrast, isradipine was found to be both safe and effective in all three of these preparations, providing the rationale for focusing on this compound in our mouse AD model. In the current study, we selected our drug delivery system for the mouse assay (subcutaneously implanted time-release pellets) based on the well-characterized pharmacology of isradipine (Chan et al., 2007; Barhwal et al., 2009). However, this assay should be equally amenable for testing compounds requiring alternative delivery systems, as needed.

Thus, the advantage of our coordinated set of bioassays is that it provides an efficient means of evaluating the benefits and limitations of a compound at multiple levels of complexity (biochemical, cellular and whole organism) before proceeding with more in-depth studies using transgenic mice. A potential drawback to this strategy is that it can only be used to test drugs directed against evolutionarily conserved targets (such as the LTCCs targeted by DHPs), and therefore might be uninformative for drugs with selective activities in higher mammals. However, given the remarkable similarities between insect and human isoforms of proteins involved in APP-dependent signaling and Aβ-associated toxicity (Vazquez-Martinez et al., 2003; Jacobsen and Iverfeldt, 2009; van de Hoef et al., 2009), we postulate that many candidate drugs that target these pathways will have analogous effects in our simpler assays.

As with any set of bioassays, the preparations described in the current study also have potential limitations: differences in drug sensitivities among the preparations might produce conflicting results (a beneficial effect in one assay but a negative response in another). Likewise, because each preparation involves distinct strategies for delivering experimental compounds, the pharmacodynamic properties of a drug might successfully protect against Aβ neurotoxicity in some of our assays but not in others. Selecting only those compounds that are safe and effective in all three of our simpler assays (MC65 cells, Drosophila and Manduca) might therefore be overly stringent in some instances, producing ‘false negative’ results for drugs that might be beneficial in mammalian systems. Nevertheless, we propose that the complementary information provided by our different preparations can be used to identify the most promising compounds in an efficient manner, prior to advancing them to the more laborious analyses associated with mouse models of AD.

As recently demonstrated for other neurodegenerative diseases (Celotto and Palladino, 2005; Krettschmar, 2005; Gotz and Ittner, 2008), this model-systems approach can provide an effective method for characterizing the biological effects of ‘repurposed’ drugs (the focus of our current study), an approach that allowed us to incorporate the known pharmacodynamic properties of the compounds into our protocols for drug delivery. However, the methods developed in this study should also be readily adaptable for testing compounds that have been less well characterized, accompanied by an appropriate analysis of their solubility, stability and metabolic turnover. We propose that the integration of our four distinct preparations (targeting different aspects of Alzheimer’s-associated pathology) can now be used as a translational suite of bioassays for evaluating candidate drugs for their therapeutic value.

METHODS

Materials

Cell culture media, L-glutamine, trypsin/EDTA and normal horse serum were obtained from Invitrogen (Carlsbad, CA); fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA); Ca2+ channel blockers (verapamil, diltiazem, nimodipine and isradipine) were from Sigma-Aldrich (St Louis, MO). β-amyloid(1-42) was purchased from American Peptide (Sunnyvale, CA). The 4G8 monoclonal antibody against residues 1-17 of human Aβ1-42 was obtained from Covance Laboratories (Princeton, NJ). The 6E10 monoclonal antibody against residues 17-24 in human Aβ1-42 was from Biosource (Invitrogen, Carlsbad, CA). Biotinylated anti-AT8 against tau-P was from Pierce (Rockford, IL). Custom antibodies against Fas II and cGMP have been previously described (Wright et al., 1999). Fluorescent secondary antibodies were from Invitrogen; biotinylated secondary antibodies and ABC detection kits were from Vector Laboratories (Burlingame, CA). Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich.

Assay of Ca2+ channel blockers and amyloid toxicity in MC65 cells

MC65 cells, a human neuroblastoma cell line, were cultured as previously described (Woltjer et al., 2003; Woltjer et al., 2005a). Cells were maintained in a CO2 incubator (5% CO2) at 37°C in MEM Alpha growth medium with fetal bovine serum (10%), L-glutamine (2 mM) and Tet (1 μg/ml). Conditional expression of APP C-terminal fragments (CTFs) and subsequent generation of Aβ-immunoreactive aggregates was induced by Tet removal. Confluent cells were trypsinized, washed with phosphate-buffered saline (PBS), resuspended in OptiMEM and plated at 3.5×104 cells/cm2, with or without 1 μg/ml Tet. Expression and aggregation of Aβ was evaluated as described previously (Woltjer et al., 2007). MC65 cells (+Tet) were harvested at times between 0-3 days. Cell pellets were then lysed in SDS buffer and separated by electrophoresis in Tricine-SDS gels to evaluate Aβ levels by western blotting (detected with the 6E10 antibody against the N-terminal portion of Aβ).
some experiments, cells were fixed in 4% paraformaldehyde (in PBS) and immunostained with anti-βA antibodies (4G8), detected with Alexa-Fluor-488-conjugated anti-mouse secondary antibodies, and counterstained with rhodamine-phalloidin (1:200; Sigma).

To quantify the effects of βA expression and Ca\(^{2+}\) channel blockers on cell viability, replicate MC65 cultures were harvested every 6 hours over a period of 3 days. Cell survival was determined by the MTT assay (Promega), following our published methods (Woltjer et al., 2005b; Woltjer et al., 2007). Briefly, cells were plated in 48-well plates and, at the onset of each assay, Tet(+) medium was replaced with Tet(−) medium, with or without Ca\(^{2+}\) channel blockers (dissolved in ETOH). Viability of the cultures was subsequently analyzed by reduction of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], using background-corrected spectrophotometric determination of the solubilized formazan product (at 570 nm). Percent cell survival in each treatment group was quantified relative to survival rates in matched Tet(+) control cultures. All MTT assays were carried out in triplicate or quadruplicate wells for each condition, and all experiments were repeated at least four times. Statistical differences among treatment groups were determined with Student’s t-tests and one- or two-way ANOVA, using GraphPad Prism 5 software.

Analysis of cytosolic Ca\(^{2+}\) levels in MC65 cells
Cytosolic Ca\(^{2+}\) levels [Ca\(^{2+}\)]\(_{\text{cyt}}\) were determined as previously described (Anekonda and Adamus, 2008; Anekonda et al., 2011). Briefly, [Ca\(^{2+}\)]\(_{\text{cyt}}\) was quantified using the Fluo-4 NW Ca\(^{2+}\) Assay Kit (Invitrogen), following the manufacturer’s instructions. MC65 cells (15×10\(^2\) cells/well) were grown in black 96-well culture plates, with or without Tet. The cultures were then treated with the different DHPs (using the same batch of cells for each condition), followed by the addition of 100 μl Fluo-4 NW to each well (plus 2.5 mM probenecid; an inhibitor of anion transporters). After a 30-minute incubation at 37°C, [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels were measured as relative fluorescence units (RFUs) in live cells for 10 minutes at room temperature, using the Victor3 1420 Multilabel Counter (Perkin Elmer; excitation =485/20 nm; emission =535/20 nm). For each experiment, relative fluorescent intensities were calibrated against Tet(+) control cultures to eliminate potential errors arising from differences in cell density, growth conditions and dye-loading efficiency. This approach allowed direct comparisons of equivalent treatment groups in different experiments. Each experiment was repeated three to five times, and the experimental averages were evaluated for statistical significance using Bonferroni post-hoc tests to compare all other treatments with the Tet(+) control group.

For qRT-PCR, cells were grown in 100×20 mm Petri dishes (±Tet) for 24-72 hours, with or without each DHP. Total RNA was isolated from the cultures using RNAqueous kits (Ambion, Foster City, CA) and reverse transcribed using the SuperScript III First-strand Synthesis System (Invitrogen). Taqman gene expression assays were then performed, using commercial primer sets specific for Ca.1.2 (αCa) and Ca.1.3 (αCa), subunits, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Plates were analyzed on a StepOnePlus Real Time PCR system using the DDCT method (Livak and Schmittgen, 2001). All PCR reagents were from Applied Biosystems (Foster City, CA).

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**Assay of Ca\(^{2+}\) channel blockers and amyloid neurotoxicity in Drosophila**

The UAS-APP\(_{695}\) line of Drosophila melanogaster (Meigen) was kindly provided by Renato Paro (Fossgrep et al., 1998). The Actin-GAL4 line and GMR-GAL4 lines were obtained from the Bloomington Stock Center. Eye-specific expression of human APP\(_{695}\) was induced by crossing UAS-APP\(_{695}\) flies with the GMR-GAL4 driver line. As previously shown, induction of APP expression in the eye results in accumulation of neurotoxic βA, which in turn leads to the formation of amyloid deposits and age-dependent neurodegeneration in the retina (Greeve et al., 2004). For survival assays, ubiquitous expression of APP\(_{695}\) was induced using actin-GAL4. Flies were raised at 25°C on 0.5 g Instant Drosophila media (Sargent-Welch, Tonawanda, NY), containing different concentrations of isradipine (0, 25 or 250 μM) or verapamil (0, 10, 100 or 500 μM). Survival rates of APP\(_{695}\)-expressing progeny were calculated as a percentage of surviving control progeny (lacking the promoter constructs) that were obtained from the same genetic cross, and therefore were raised under identical conditions [as previously described (Greeve et al., 2004)]. We performed the following number of independent tests per condition: 20 control tests (0 μM isradipine); 10 tests with 25 μM isradipine; 12 tests with 250 μM isradipine; 10 tests with 10 μM verapamil; 8 tests with 100 μM verapamil; and 3 tests with 500 μM verapamil. A minimum of ten males and ten females were used to initiate each trial, and their progeny were subsequently counted. Statistical comparisons were calculated with two-tailed, equal variance Student’s t-tests to compare means. For plastic sections, fly heads were fixed in 5% glutaraldehyde (overnight at 4°C), washed in PBS and treated with 2% osmium tetroxide on ice. The preparations were then washed again, dehydrated by sequential rinses in 50, 70, 80, 95 and 3×100% ethanol for 10 minutes each, incubated in 100% ethanol:Epon 1:1 overnight, and embedded in Epon. Plastic sections (1 mm) were cut on a microtome and stained with 1% toluidine blue plus 1% borax.

**Assay of Ca\(^{2+}\) channel blockers and amyloid neurotoxicity in Manduca**

Staged embryos of M. sexta (Linnaeus; tobacco hornworm) were collected from an in-house breeding colony and used to test the effects of exogenous βA\(_{42}\) on neuronal motility, using the ENS as a convenient in vivo assay system (Copenhaver, 2007). This preparation permits direct manipulations of neuronal migration, axon elongation and synaptic growth in cultured embryos, and the effects of an experimental treatment can be quantified unambiguously in whole-mount preparations (Horgan and Copenhaver, 1998; Wright et al., 1998; Coate et al., 2008). Embryos were opened in defined culture saline at 50% development (at the onset of neuronal migration) or at 70% development (at the onset of synaptogenesis), and soluble βA\(_{42}\) peptides were directly applied to the ENS [prepared as described in Sokolov et al. (Sokolov et al., 2006)]. Briefly, lyophilized βA\(_{42}\) (American Peptide) was resuspended in hexafluoroisopropanol (HFIP) and diluted in ddH\(_2\)O. After evaporation of the HFIP, stock βA\(_{42}\) solutions (500 μM; in sterile defined saline) were incubated for 20-40 minutes to initiate oligomerization, then diluted to final concentrations of 0.1-100 μM before being introduced to the culture preparations. Soluble ectodomain fragments of Manduca APPL (sAPPL) were

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Isradipine is protective in AD models

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generated as fusion proteins [in-frame with placental alkaline phosphatase (AP)], using methods developed by Flanagan and colleagues (Flanagan and Cheng, 2000; Flanagan et al., 2000). The fusion proteins were purified from the medium of transfected HEK293 cells, quantified by western blot and AP activity, and applied directly to the developing ENS as described above. Bovine serum albumin (IgG-free BSA; Promega) and AP (produced from HEK293 cells transfected with vectors encoding AP alone) provided additional controls. Isradipine was prepared as a 0.5 mM stock solution and applied simultaneously with APb1-42. For embryo cultures in low external Ca2+, defined saline was prepared with no added CaCl2, which results in an extracellular Ca2+ concentration of ~0.4 mM Ca2+ (Horgan and Copenhaver, 1998).

Analysis of synaptic outgrowth in Manduca embryos
Treated embryos were allowed to develop for 24 hours at 28°C, then fixed and immunostained in whole-mount with antibodies against the neuronal markers Fas II and cGMP to reveal the complete distributions of the neurons, their axons and their terminal synaptic processes (Wright et al., 1998; Wright et al., 1999). The ABC-HRP method (Vector Laboratories, Burlingame, CA) was used to detect bound antibodies, with diaminobenzidine (DAB)-H2O2 plus NiCl as a substrate (Wright et al., 1998). The extent of synaptic branching was quantified in whole-mount preparations, using camera lucida and photomicrographic techniques [as described by Wright et al., (Wright et al., 1998)]. The effects of each treatment on neuronal viability were analyzed by comparing the number of pyknotic nuclei within the defined population of ENS neurons (Copenhaver and Taghert, 1989). For some preparations, primary antibodies were visualized with Alexa-Fluor-488-conjugated secondary antibodies (Invitrogen) and imaged with an LSM 7 Zeiss laser scanning confocal microscope in the Neuroscience Imaging Center at Oregon Health and Science University (OHSU). z-stack images were then flattened, pseudo-colored and uniformly adjusted for brightness and contrast as needed in Photoshop (Adobe Systems, San Jose, CA). Statistical analyses were performed using Student’s two-tailed t-test to compare means; at least 15 preparations were used for each condition, and each experiment was repeated at least three times.

Assay of Ca2+ channel blockers in a mouse model of AD
Breeding pairs of 3×TgAD mice (Mus musculus; Linnaeus) were raised in an in-house facility at the Portland VA Medical Center. Mice were maintained in a climate-controlled environment with a 12-hour light/12-hour dark cycle, and fedAIN-93M Purified Rodent Diet (Dyets, Bethlehem, PA). Diet and water were supplied ad libitum. Litters were weaned and group-housed (4-6/cage) until commencement of the experiments. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the institutional Animal Care and Use Committee of the Portland VA Medical Center.

Cohorts of female 3×TgAD mice were implanted at 17 months with either carrier-bound isradipine pellets (3 mg/g/day, 60-day release; Innovative Research of America) or placebo control pellets. Body weight was monitored weekly as an indirect measurement of toxicity. At the end of each treatment, mice were euthanized by CO2 inhalation and cervical dislocation. Plasma and liver samples were collected and frozen, and the brains were quickly removed and prepared for analysis, as previously described (Quinn et al., 2007). Briefly, the anterior 3 mm of the bilateral frontal cortex and a 1 mm section behind the frontal cortex (frontal slice) were dissected and frozen for analysis of Aβ and tau levels by ELISA and western blotting methods. Concurrently, the right hemisphere was immersion-fixed in 4% formaldehyde in PBS for histochemoanalysis.

Analysis of Aβ and tau-P in mouse brain
To determine expression levels of tau and Aβ peptides, frontal cortex tissue was homogenized in Tris-buffered saline (TBS) containing protease inhibitor cocktail (EMD Chemicals, Gibbstown, NJ), then centrifuged at 100,000 g for 30 minutes at 4°C. The supernatant from this step was designated the cytoplasmic fraction, and the pellet was re-homogenized in TBS plus protease inhibitors and 1% Triton X-100 (Roche, Indianapolis, IL), and centrifuged at 100,000 g for 30 minutes. The supernatant was collected and designated as the soluble membrane fraction, whereas the pellet from this step was collected, washed in TBS containing protease inhibitors and centrifuged at 100,000 g for 30 minutes. The pellet from this spin was re-homogenized in 70% formic acid, incubated for 1 hour at room temperature and centrifuged at 100,000 g for 30 minutes. The resulting supernatant was collected and designated as the insoluble fraction. Levels of tau, Aβ1-40 and Aβ1-42 were measured in the soluble membrane and insoluble fractions using commercial ELISA kits (Invitrogen, Camarillo, CA) according to the manufacturer’s instructions. For some experiments, isradipine levels were measured in the brain and plasma samples by LC-MS/MS, with the assistance of Dennis Koop in the OHSU Bioanalytical Shared Resource and Pharmacokinetics Core. Statistical differences between paired samples were calculated using Student’s t-tests.

For immunohistochemical analysis, 40 μM frozen coronal sections were cut on a freezing microtome. Sections were incubated with agitation in blocking buffer (in TBS, pH 8.0, with 2 mg/ml BSA, 2% horse serum, 0.5% Triton X-100) for 2 hours. They were then incubated overnight with primary antibodies against either Aβ or tau-P (diluted 1:1000 in blocking buffer). Sections were incubated for 2 hours with biotinylated secondary antibody (1:200; Vector Labs, Burlingame, CA) for 2 hours with an avidin-linked peroxidase complex (ABC; Vector Labs), then developed with DAB (Sigma) in PBS. Sections were washed, mounted in Permount (Fisher Scientific, Pittsburg, PA) and sealed under coverslips. Protein immunoreactivity was analyzed in at least three coronal sections from each mouse, representing anterior, middle and posterior regions of the hippocampus and cortex. Hippocampal and cortical areas were subsequently traced using a computerized stage and stereo investigator software (Image J; Wayne Rasband, NIH, USA). Both Aβ and APP levels were expressed as percentage of total area within the hippocampus or cortex that were occupied by these proteins (as detected by immunohistochemistry). Mean values for each parameter were calculated from at least three sections per animal, and statistical differences were calculated as described above.

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TRANSLATIONAL IMPACT

Clinical issue
Alzheimer’s disease (AD) is the leading cause of age-related dementia, affecting an estimated 27 million people worldwide. Primary symptoms include premature memory loss, cognitive decline and disoriented behavior, inevitably culminating in death. Although the underlying cause of AD remains controversial, a variety of studies support the hypothesis that neuronal calcium (Ca$$^{2+}$$) levels become dysregulated in AD. Neurotoxic β-amyloid (Aβ) peptides can alter Ca$$^{2+}$$ levels via several different mechanisms, including the activation of membrane Ca$$^{2+}$$ channels. Conversely, elevated Ca$$^{2+}$$ levels can accelerate Aβ aggregation. Therefore, drugs that maintain the homeostatic balance of Ca$$^{2+}$$ in the brain might provide a treatment strategy for AD, particularly during the initial stages of the disease. However, identifying compounds that prevent neuronal Ca$$^{2+}$$ overload without deleterious side effects has been hindered by the lack of appropriate bioassays: cell culture assays can be used for high-throughput screening but do not provide useful information about the effects of a drug in vivo, whereas mouse models of AD provide more clinically relevant information about specific drugs but cannot readily be used to evaluate large panels of related compounds.

Results
To overcome the limitations of currently available bioassays, the authors of this study coordinate four distinct models of amyloid toxicity that provide complementary information about a trial compound. They use this ‘translational suite’ of bioassays to investigate the therapeutic value of clinically approved dihydropyridines (DHPs), a class of drug that targets low-voltage-gated Ca$$^{2+}$$ channels expressed in the brain and other tissues. They first combine cell culture and invertebrate model systems to test a panel of related DHPs, and then advance the most promising drug to further analysis in the 3×TgAD mouse model of AD. Neurotoxic β-amyloid (Aβ) peptides MC65 cells, a stably transfected neuroblastoma line expressing neurotoxic Aβ, is used for a rapid initial screen of DHPs that are currently used to treat hypertension (verapamil, diltiazem, nimodipine and isradipine). A transgenic Drosophila model of AD, which exhibits age-dependent accumulation of neurotoxic Aβ, is used to test the protective effects of DHPs on survival. Embryonic preparations of the larger insect Manduca provide a novel assay to test whether DHPs can protect against the neurotoxic effects of Aβ on synaptic growth. Of the compounds tested, only isradipine provides a significant protective benefit and minimal toxicity in the MC65, Drosophila and Manduca assays.

Moreover, the sustained delivery of isradipine in the mouse model of AD protects against AD-associated pathologies that are induced by Aβ accumulation in the brain.

Implications and future directions
These results provide proof of principle for the strategy of using coordinated bioassays to evaluate candidate drugs that protect against amyloid-induced toxicity. By focusing on conserved molecular targets in all four assay systems, this approach can also be used to investigate other key signalling pathways that might be disrupted in AD, including the dysregulation of neuronal Ca$$^{2+}$$. Finally, these experiments suggest that isradipine is a more potent and less toxic alternative to nimodipine for treating the neurotoxic effects of amyloid peptides, justifying a more comprehensive evaluation of its effects in mouse models of AD as a prelude to clinical trials.

COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
P.F.C., D.K., R.L.W. and J.F.Q. developed and designed the original concept of coordinating the four bioassays used in this study. T.S.A., T.L.W., K.M.R. and R.L.W. conducted the experiments in MC65 cells. D.K. and D.M. conducted the experiments in Drosophila. P.F.C., J.M.R. and T.L.S. conducted the experiments in Manduca. T.L.W., T.S.A. and J.F.Q. conducted the experiments in the AD mouse model. P.F.C. performed the confocal analysis of MC65 cells and Manduca preparations. The manuscript was prepared and edited by all participating authors.

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