

## REVIEW

# Accelerating drug discovery for Alzheimer's disease: best practices for preclinical animal studies

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### Abstract

Animal models have contributed significantly to our understanding of the underlying biological mechanisms of Alzheimer's disease (AD). As a result, over 300 interventions have been investigated and reported to mitigate pathological phenotypes or improve behavior in AD animal models or both. To date, however, very few of these findings have resulted in target validation in humans or successful translation to disease-modifying therapies. Challenges in translating preclinical studies to clinical trials include the inability of animal models to recapitulate the human disease, variations in breeding and colony maintenance, lack of standards in design, conduct and analysis of animal trials, and publication bias due to under-reporting of negative results in the scientific literature. The quality of animal model research on novel therapeutics can be improved by bringing the rigor of human clinical trials to animal studies. Research communities in several disease areas have developed recommendations for the conduct and reporting of preclinical studies in order to increase their validity, reproducibility, and predictive value. To address these issues in the AD community, the Alzheimer's Drug Discovery Foundation partnered with Charles River Discovery Services (Morrisville, NC, USA) and Cerebricon Ltd. (Kuopio, Finland) to convene an expert advisory panel of academic, industry, and government scientists to make recommendations on best practices for animal studies testing investigational AD therapies. The panel produced recommendations regarding the measurement, analysis, and reporting of relevant AD targets, the choice of animal model, quality control measures for breeding and colony maintenance, and preclinical animal study design. Major considerations to incorporate into preclinical study design include *a priori* hypotheses, pharmacokinetics-pharmacodynamics studies prior to proof-of-concept testing, biomarker measurements, sample size determination, and power analysis. The panel also recommended distinguishing between pilot 'exploratory' animal studies and more extensive 'therapeutic' studies to guide interpretation. Finally, the panel proposed infrastructure and resource development, such as the establishment of a public data repository in which both positive animal studies and negative ones could be reported. By promoting best practices, these recommendations can improve the methodological quality and predictive value of AD animal studies and make the translation to human clinical trials more efficient and reliable.

### Animal models of Alzheimer's disease: modeling targets, not disease

Animal models of Alzheimer's disease (AD) pathogenesis range from *Caenorhabditis elegans* to aged non-human

primates, but by far the most widely used are rodent models. Most animal models used for drug discovery over-express proteins with familial AD mutations (Table 1). While these models develop certain characteristics of AD-like pathology, they do not recapitulate the entirety of the human disease. Furthermore, it is unclear to what extent the pathogenic pathways in rodents mirror those in human AD. Other challenges in translation include mouse/human species differences (for example, differences in cerebrovascular anatomy, neuronal network complexity, connectivity and disease susceptibility, white/

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**Table 1. Animal models for use in Alzheimer's disease preclinical studies**

Model	Description	Outcome	Plaques	Neurofibrillary tangles	Neuron loss	Synaptic defects	Memory defects	Notes	Reference
<b>Single-transgenic</b>									
APP familial mutation models	Mutations at beta-secretase cleavage site (aa 670/71)	Enhanced cleavage by beta-secretase; overall more Aβ (all forms)	Yes: 9-12 months	No	No	Yes	Yes	Pathology includes mostly dense cored plaques and some tau hyperphosphorylation with age. Synaptic and memory defects generally precede amyloid deposits. Moderate oxidative stress can be detected.	[36]
Tg2576 (APP Swedish)	Mutations at gamma-secretase cleavage site (aa 717)	Enhanced cleavage by gamma-secretase; increased Aβ 42:40 ratio	Yes: 9-12 months	No	No	Yes	Yes	These models demonstrate higher levels of diffuse amyloid deposits.	[37,38]
PDAPP, APP London V717W (APP Indiana)	Mutations within Aβ sequence (aa 692/3/4)	Enhanced Aβ aggregation	Yes: 9-12 months	No	No	Yes	Yes	These models demonstrate pronounced cerebral amyloid angiopathy.	[39,40]
TgAPPair, APPDutch	Multiple APP familial mutations	Enhanced amyloid pathology over single mutation	Yes: variable	No	No	Yes	Yes	Example models include TgCRND8 and J20 mouse models.	[41-45]
APPArcticSwe/Tg-SwDI/hAPPsw/Ind/Arctic	Point mutations in human MAPT (FTD mutations; no tau mutations linked to AD)	Increased tau phosphorylation/aggregation	No	Yes (>6 months)	Yes	Yes	Yes	Significant lower motor neuron loss, limb paralysis, and prominent brainstem and spinal cord pathology in some strains may impede behavioral testing. Inducible promoter models (Tg4510) and hTau models show more forebrain pathology and are better for cognitive behavior analysis.	[46-49]
<b>Multi-transgenic</b>									
APP/PS	Double-transgenic (APP FAD mutant overexpression, PS FAD mutant expression, or knock-in)	Accelerated phenotype and pathology but minimal neurodegeneration	Yes: 3-6 months	No	No	Yes	Yes	Significant hippocampal neuron loss is seen in some subtypes (for example, APP(swe-hon)/PS1).	[50-52]
APP/Tau	Double-transgenic (APP FAD mutant overexpression and tau FTD mutant overexpression)	Accelerated phenotype and pathology but minimal neurodegeneration	Yes: 9 months	Yes	Yes	Yes	Yes	These models demonstrate increased amyloid deposition compared with Tg2576, but there are reports of high death rate and difficulty breeding.	[53-55]
APP/PS/Tau	Triple-transgenic; FAD APP and FTD tau transgenes in PS1 FAD knock-in	Accelerated phenotype and pathology, including NFTs	Yes: 3-6 months	Yes	Yes	Yes	Yes	This model demonstrates early intraneuronal deposits and plaques preceding tangles.	[56]
APP/NOS2 <sup>-/-</sup>	APP transgenic (Swedish alone or combined with other APP mutations) on a NOS2 knockout background	Increased tau pathology (hyperphosphorylation, redistribution, aggregation) and neuronal degeneration	Yes: 3-6 months	Some	Yes	Yes	Yes	Increased caspase-3 activation is seen along with higher levels of insoluble Aβ compared with single APP transgenic mice (only in APP(swe) not APP(SweD)) line; cerebral amyloid angiopathy, and neurovascular changes.	[32,34]

*Continued overleaf*

**Table 1. Continued**

Model	Description	Outcome	Plaques	Neurofibrillary tangles	Neuron loss	Synaptic defects	Memory defects	Notes	Reference
<b>Non-transgenic models</b>									
Aged rodent models (mice, rats, dogs, and non-human primates)	Old age >18-20 months		Yes; dogs and non-human primates	No	No	Yes	Yes	These models show cognitive deficits, brain hypometabolism, cholinergic deficits, altered calcium homeostasis, oxidative stress, and neophobia.	[57-60]
SAMP8	Spontaneously mutated inbred strain; senescence-accelerated prone mice	Shortened lifespan and accelerated aging phenotype. Elevated levels of endogenous (murine) APP and Aβ	No	No	No	Yes	Yes (>2 months)	Some tau hyperphosphorylation is seen along with decreased spine density and synaptic proteins. Increased gliosis and systematic oxidative stress are seen.	[61-63]
Acute Aβ injection	Direct injection of Aβ into the brain via cannulas	Acute local Aβ elevation	No	No	No	Yes	Yes	The Aβ type/preparation method is crucial. Types synthetic and natural (from culture or brain). Preparation methods water, ammonium bicarbonate, HFIP, and DMSO. Aβ conformations monomers, oligomers (ADDLs), or fibrils. Standardized protocols for this model are needed.	[64,65]
Induced ischemia	Occlusion of cerebral artery	Oxygen deprivation	No	No	Yes	Yes	Yes	Many models/techniques are available to induce ischemia. Infarct size can be variable.	[66]
Toxin-induced lesions	Direct injection of toxin (for example, STZ, IgG-192 saporin, 6-OH, and MPTP)	Neuronal degeneration/dysfunction in specific brain regions	No	No	Yes	Yes	Depends on neuronal populations that are affected	STZ model - In addition to cognitive decline, impairment of cholinergic transmission, oxidative stress, and astrogliosis are seen. IgG-192 saporin model - cholinergic dysfunction is seen. MPTP and 6-OH model - dopaminergic cell loss and motor phenotypes are seen.	[67,68]

This partial list of available strains serves to highlight the classes of models used in preclinical studies. For an extensive list of available models, please visit <http://www.alzforum.org> [69]. Aβ, amyloid-beta; ADDL, amyloid-beta-derived diffusile ligand; APP, amyloid precursor protein; DMSO, dimethyl sulphoxide; FAD, familial Alzheimer's disease; FTD, frontotemporal dementia; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; MAPT, microtubule-associated protein tau; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NFT, neurofibrillary tangle; NOS2, nitric oxide synthase 2; PS, presenilin; siRNA, small interfering RNA; STZ, streptozotocin.

gray matter ratios, cellular redox conditions, and dynamics of drug/target interactions [1]). Nonetheless, rodent models offer a means for testing pharmacodynamic properties of candidate molecules on drug targets that may be involved in AD pathogenesis.

This target-driven approach in animal models has already translated to therapeutic studies in humans. In the amyloid-beta ( $A\beta$ ) immunotherapy trial of bapineuzumab, for example, the immunotherapy cleared plaques in both mice and humans [2,3]. Gamma-secretase inhibitors developed at Eli Lilly and Company (Indianapolis, IN, USA) and Bristol-Myers Squibb Company (Princeton, NJ, USA) (semagacestat and BMS-708163, respectively) showed good target-focused preclinical animal data, reducing  $A\beta$  levels in mice and in the spinal fluid of human patients in a phase 2 study [4,5]. Demonstration of positive effects on cognitive outcomes from treatment with bapineuzumab of patients with AD is in the final stages of clinical testing. The phase 3 clinical trial of semagacestat was terminated prematurely because of lack of efficacy as well as serious side effects [6], whereas clinical testing of BMS-708163 is in progress. Thus, while these examples provide reassurance that well-executed preclinical studies can translate to human patients with regard to pathological targets, they also highlight our limited understanding between causative pathways and clinical decline of cognitive function in AD and our inability to accurately model all aspects of the disease in animals.

Therefore, animal models appear more useful as models of specific disease targets and pathways than of the complete human disease. To optimize their use in that manner, our advisory panel recommended choosing models for preclinical studies that exhibit significant and well-characterized pathology relevant to the disease process of interest (that is, amyloid plaques, tau pathology, neuronal loss, oxidative stress/inflammatory changes, and so on). In addition, models that do not rely solely on mutated human genes to induce pathology are currently underused and can be quite informative. These include aged rodents, pharmacologically and surgically induced models, and other non-transgenic models (Table 1). Since there is no one model for AD, hypothesis testing in multiple models is preferable in order to provide better preclinical validation. In the following sections, we present the panel's recommendations and guidelines for the design, execution, and interpretation of preclinical studies. The objective of this panel was to improve the predictive value of animal models for clinical benefit.

### **Know your model**

Many transgenic lines show high variability in the extent and time course of expression of disease phenotypes.

Table 2 illustrates common factors affecting phenotype variability, including environmental factors, age, sex, genetic background, litter, transgene copy number, and health status. Not all of these variables can be avoided, but measures can be taken so that phenotype changes due to such factors can be properly noted and potentially corrected [7-9].

### **Important points to keep in mind**

- Maintain good communication among laboratory members to track deviations from expected phenotypes. Keep careful records to track whether a change in phenotype occurs.
- Identify issues with breeding, such as longer litter intervals, smaller litter sizes, and fewer pregnancies. Identifying such problems early will help keep production on track.
- Screen gene copy numbers and transgene expression level regularly. Document and report.
- Freeze embryos early during characterization of the transgenic line in case phenotypic drift necessitates re-derivation of colony.
- Consider your genetic background: Mice may be healthier and more viable on a hybrid background, but genetic drift must be controlled to avoid confounding variables. Keep in mind that certain inbred strains are more prone to characteristics like blindness, hearing loss, and aggression.
- If working with an outside breeder or contract research organization, ask to see historical data on the colony. These data should include rearing conditions such as light cycle, housing type, diet, and health status as well as breeding schemes to assess genetic management of the strain background(s) in the colony.

### **Improving rigor in study design**

Many animal studies are flawed by methodological weaknesses that compromise study validity and reproducibility. In fact, it was reported that the majority of published effects in amyotrophic lateral sclerosis mice, for example, were likely measurements of noise in the sample population as opposed to actual drug effects [10]. By paying careful attention to study design before starting experiments, investigators can save time and money as well as minimize the probability of false-positive or false-negative results. Table 3 outlines key study design considerations. In addition, performance on behavioral assays can be highly sensitive to protocol design. For the Morris water maze, for example, variables that can affect performance include water tank size, number and kinds of visual cues, training protocol, how long animals are acclimated to the test room before testing, and strain differences (that can be differentially affected by genetic alterations or the aging process or both). It is important

**Table 2. Major factors affecting phenotypic variability in mice**

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Environmental
Housing system (type of caging/enrichment)
Housing density (number of mice per cage)
Handler/Investigator
Light cycle
Temperature and humidity
Noise and vibration
Diet
Health status
Biological
Age
Sex
Body condition
Genetic drift
Genetic background (mixed versus inbred)
Type of background strain
Transgene copy number
Transgene expression level

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to consider and control for these variables in experimental design and use multiple overlapping tests to substantiate behavioral changes.

#### **Develop and employ translatable biomarkers for animal preclinical studies**

Biomarkers have been instrumental in revolutionizing the way we think about human AD and have allowed us to improve clinical trial design and assess target engagement and response to treatments. Animal preclinical studies can also benefit immensely from the use of biomarkers to assess target engagement of investigative treatments, monitor biological responses to treatment in real-time, characterize the translatability of AD models, and determine the translatability of a novel therapeutic if the same biomarker can be used in a human clinical trial. Although more validation is needed, biomarker methods under development in rodents include imaging – magnetic resonance imaging (MRI), magnetic resonance spectroscopy, functional MRI, arterial spin labeling MRI, fluoro-2-deoxy-D-glucose-positron emission tomography (FDG-PET), PET amyloid imaging, PET tau imaging, single-photon emission computed tomography/computed tomography, and others – and biochemical assays on biological fluids such as plasma and cerebrospinal fluid [11-13]. It is important to be aware of the limitations of these biomarkers in rodents, however. For example, functional imaging in mice can be affected by the requirement for either anesthesia or restraint stress. Drawing cerebral spinal fluid from mice is difficult but doable, although it is important to avoid blood contamination [14]. Rat models are becoming more popular and may have

advantages in these types of biomarker studies. In any case, whenever possible, biomarker measurements should be incorporated into the study design.

#### **Timing of treatment**

Treatment timing should depend on whether the therapeutic goal is disease prevention, therapeutic intervention (that is, slowing/reversal of established pathology), or symptomatic relief. Tissue should be collected from a proper cohort of animals at the time when treatment is initiated to determine whether the treatment reduced pre-existing pathology in the brain or simply slowed its age-associated accumulation. The degree to which disease stages in mouse models correlate with those in humans is currently unclear. Amyloid mice which do not show tangles or neuronal loss may be representative of presymptomatic or early-stage AD, although this idea is not universally accepted [15]. Where a longitudinal assessment is possible (that is, using peripheral biomarkers, imaging, and certain behavioral responses), taking repeated measures of the same animal can be especially informative and add statistical power. Treatment should be timed on the basis of the optimal stage of pathology development in the animal, which will allow acceptable signal-to-background ratio and dynamic range for experimental treatments. Optimally, demonstration of assay validation should be a prerequisite to embarking on therapeutic studies. Because pathology can vary widely with animal age, control and treatment groups should be age-matched to the greatest extent possible (that is, within days of one another). Pathology and biochemical readouts can also vary widely among animals within a genetically engineered line. The variability in pathology with age and in outcome measures must be assessed in order to power the animal studies properly.

#### **Pharmacokinetics/Pharmacodynamics, ADME-Toxicology**

Studies should include pharmacokinetics (PK) and pharmacodynamics (PD) assessments to determine whether the compound exposure is sufficient and whether it is interacting with the target of interest. Depending on whether a study is exploratory or therapeutic (see 'Exploratory versus therapeutic studies'), the degree to which absorption, distribution, metabolism, excretion, and toxicity (ADMET) are profiled should be considered as part of the prospective study design. In therapeutic studies, it is critical (a) to demonstrate that the test compound has the capacity to reach its target with sufficient concentration and stability to be relevant to prior *in vitro* studies and (b) to guide the dosing concentrations and frequency to optimize the chance of achieving therapeutic effects. More information about these types of studies can be found in the Alzheimer's Drug Discovery

**Table 3. Key considerations for preclinical animal studies**

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Clearly delineate an *a priori* hypothesis for the study and include primary and secondary outcomes

Prespecify a specific measure to assess the primary and secondary outcomes.

Attempt to employ translatable biomarkers.

Consider issues of sex, timing of treatment, and age of animals.

Determine inclusion and exclusion criteria.

Demonstrate that the therapeutic compound reaches its intended target in a sufficient concentration to ensure that the hypothesis is being tested.

Carefully design a statistical analysis plan prior to initiation of the study

Perform power analysis and sample size estimates prior to initiation of the study and take into account previously measured variability in the outcome measures.

Include randomization methods for treatment groups and blinding procedures for those doing assessments.

Include procedures for dealing with dropouts and deaths of animals in statistical analyses.

Reduce publication bias

Report both positive results and negative ones in peer-reviewed journals or other open-access format.

Report details of strain, housing, diet, dropout events and in-trial exclusions so that variables can be assessed.

As in clinical trials, report the flow of animals through the treatment plan of the study.

Indicate potential conflicts of interest and whether investigators are third-party or primary investigators invested in the hypothesis.

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Foundation/Institute for the Study of Aging/Alzheimer's Research Forum Drug Development Tutorial [16]. It is important to note that genetically engineered models may not always be the most cost-effective and translatable models for measuring PK/PD. Wild-type mice are often preferable for use in these studies, but correspondence with genetic background strains in the transgenic studies should be considered.

#### Statistical analysis plan and methods

Statistical methods should be chosen before a study is begun, with the anticipated direction of change (one-sided or two-sided) in mind. Statistical considerations should be clearly stated in the Methods section of all data reporting. Assessment of endpoint variability in a large sample size is necessary and should be considered in the choice of statistical tests, as the type of variability (normal distribution versus skewed) dictates a parametric versus non-parametric statistical analysis of the data. Guidance or consultation of a statistician should be enlisted in the design of the study once the endpoint variability has been characterized.

#### Proper quantification

Both the area and magnitude of pathology should be quantified and reported. Adequate tissue sampling is critical for accurate estimation of pathological burden. For imaging, typically at least six or seven fields per section and six or seven sections per mouse (sampled across multiple affected brain regions) should be measured. The use of unbiased stereology and the optical fractionator method is critical to determining an accurate and statistically reliable neuronal count in brain sections

[17]. Staining and field sampling methods should always be stated in the Methods section, and sampling should be guided by statistical considerations of the variability in the endpoint being interrogated. Analysis and quantification of pathology should be conducted by an individual who is blind to the treatment condition.

#### Sample size

Animal studies are frequently underpowered. This was reported to be the single most important factor in influencing spurious research results with animal models [10]. Minimum sample size depends on the expected magnitude of the biological effect, the inherent variability of the target being measured (for example, cerebral spinal fluid A $\beta$  is much more variable than hippocampal A $\beta$ ), variability in behavioral measures or other outcomes, and other factors such as variations in survival within the particular cohort of animals. It is critical to be aware of the natural variability within and among animals in outcome measures in non-treated animals in order to determine the number of animals required for proper statistical powering of therapeutic effects. The sample size needed to achieve significant differences given the variability of disease outcomes in most AD mouse models has been estimated to be on the order of 20 to 30 per group, rarely achieved in most published mouse studies.

#### Exclusion criteria

Animals whose physiological condition appears to be compromised by factors unrelated to the normal progression of the disease should be excluded from the study. A statistical analysis plan should be developed to

address dropouts and death. Exclusion criteria should be established prior to the study and not on a *post hoc* basis. Records should be kept of which animals were excluded and why, and such information should be reported explicitly in the Methods section of data reporting.

#### **Balancing and randomization**

Sex-matching and age-matching are critical in study design as both of these factors significantly affect pathological expression. For example, A $\beta$  plaque loads can increase exponentially during the first stages of plaque deposition, and spurious drug effects may be seen in animals analyzed at this stage unless control and treatment groups are age-matched to within days of one another. Mice should be separated into groups by sex, age, and litter and then randomly assigned to either control or treatment groups. In addition, wild-type or young controls or both should be included in study design as a reference point.

#### **Blinding**

Individuals conducting the experiments and those analyzing the results should be blinded to treatment. In the event that a test compound has a readily obvious phenotypic impact on the treated animals, these potentially unblinded observations should be noted by the animal handler but kept segregated to the degree possible from the analyst until the experiment is unblinded. If this is not possible, a full re-design of the experiment may be required. For example, a compound that results in reduced feeding activity (and the phenotypic observation of reduced rate of weight gain) may have an impact on A $\beta$  levels for reasons unrelated to its therapeutic target.

#### **Reporting**

Investigators should report full details of target assay methods and detailed information on the animal model used, including genetic background, copy number, exclusion criteria, and statistical analyses. For behavioral assays, training as well as testing phases should be reported. When possible, scatter-plots should be shown rather than, or in addition to, bar graphs.

Publication bias fueled by a decreased ability or desire to publish negative results represents a huge problem for the field [18]. To increase efficiency, decrease redundant efforts, and learn from others' experiences, it is crucial that negative results be reported. Forums for discussing the quality of negative results, and results that differ from laboratory to laboratory, would aid in the interpretation of negative studies.

#### **Exploratory versus therapeutic studies**

Many investigators, particularly in academic settings, lack the infrastructure and budget to perform the

extensive preclinical studies incorporating all of the design, methodological, and statistical considerations recommended here. In addition, comprehensive analyses are not always warranted when the compound or target is being assessed in early stages. As a result, we propose to distinguish between exploratory and therapeutic studies (Table 4).

#### **Exploratory studies**

Exploratory studies should demonstrate that a particular molecular target is involved in a disease process. While exploratory studies do not require the extensive lead optimization, PK/PD, and toxicity analyses undertaken in therapeutic studies, they nonetheless should provide sufficient data to inform the decision of whether to proceed to a therapeutic animal study. Exploratory studies should contain a tolerability/toxicity assay to verify that selected doses are not causing an adverse effect. Multiple doses below the toxicity/tolerability range should be incorporated into an exploratory study, as doses approaching tolerability limits can frequently impact phenotypic outcomes unrelated to the therapeutic target being investigated. Furthermore, terminal blood and brain tissue samples should be collected for possible PK verification later, as the half-life of the test compound may or may not have been consistent with the timing of the putative therapeutic readout.

#### **Therapeutic studies**

Therapeutic studies should be compound-focused and include a full PK/PD and ADMET profile to ensure appropriate dosing and timing of outcomes with respect to exposure of the compound. Toxicity considerations are particularly critical in this context to minimize potential off-target phenotypic impacts on outcome measures. The design, conduct, analysis, and reporting of a therapeutic animal study should be analogous in rigor to those required for human clinical trials.

#### **Future directions**

Below, we list some overall recommendations and challenges that we hope will significantly advance the field by making animal studies more consistent and predictive of future clinical outcomes.

#### **Improve access, characterization, and standardization of existing Alzheimer's disease mouse and rat models**

The field should identify a few models in which key disease phenotypes are well replicated and characterize these models fully with regard to major targets and how they are affected by major biological and experimental variables (for example, age, gender, and housing conditions). Government funding of preclinical animal cores could improve availability and standardization of models.

**Table 4. Exploratory versus therapeutic preclinical studies**

Goal	Exploratory studies: mechanism/target-focused	Therapeutic studies: compound-focused
Study design	Efficacy data should be assessed through multiple outcome measures.  Both exploratory and therapeutic studies should be randomized, placebo-controlled, and blinded, with a dose response.  <i>In vivo</i> model considerations for both types of studies include pathogenic stage, age, length of treatment required, and exclusion criteria.	Efficacy results should be demonstrated in more than one model.
ADME	Studies should include initial physicochemical property considerations and terminal blood and brain tissue sampling for assurance of target exposure and possible pharmacokinetics verification.	Studies should include ADME profiling, full pharmacokinetics/ pharmacodynamics analysis and distribution/exposure of parent compound and metabolites.
Toxicity	Defined toxicity assessment is not needed, but a simple drug tolerability assay should be included.	Toxicology should be assessed in the model being studied, with treatment conducted at levels reliably below adverse event doses.
Statistics plan	While statistical considerations need not be as stringent, prospective power analysis should take into account variability in the model itself and in outcome measure readouts.	Prospective study design should include sample size power analyses, statistical evaluation plan, primary and secondary outcome measures, blinding, and randomization.

ADME, absorption, distribution, metabolism, and excretion.

In addition, intellectual property creates obstacles to model access. This is a major impediment that needs to be addressed by the scientific and business communities.

#### **Develop more animal models to non-traditional targets and make more use of available non-transgenic models**

New animal models that better recapitulate the full complement of human AD pathology and novel non-amyloid targets are needed (Box 1). Aged rodent and non-transgenic models should also be better used as described above.

#### **Standardize commonly used protocols**

To be better able to compare and pool research results, it is important to improve quality control measures across laboratories. Efforts to standardize biomarker protocols have been widely successful [19]. Standardizing protocols for common assays, such as A $\beta$ /tau extraction, behavioral assays, and measures of neuroprotection and neurodegeneration, in preclinical studies could rapidly advance the interpretation of the testing of novel treatments.

#### **Develop new and higher throughput methods for measuring disease-related outcomes**

Research efforts and funding should be targeted toward better characterizing and developing new methods for targets and outcome measures that are higher throughput for drug discovery. For example, for oxidative stress and inflammation, more emphasis should be placed on the development of pharmacological and metabolic imaging, gene expression, and proteomic screens that provide a broader view of redox and inflammatory changes and allow incorporation of both pro- and anti-oxidant or pro- and anti-inflammatory species. For behavioral tests, the field would benefit from new translatable behavioral tests that are sensitive to early changes and progression in cognitive function across lifespan and those that lend

themselves to repeated testing for optimal within-subject experimental design. Standardization of behavioral methodologies should be attempted to the highest possible degree in order to improve the ability for comparisons of results from multiple laboratories.

#### **Focus on novel targets and outcome measures**

More emphasis should be placed on non-amyloid disease processes and pathways (Box 1), including those related to neuroprotection, synaptic plasticity, oxidative stress, inflammation, vascular targets, mitochondria, and energy use. Assays are available to measure these alternative outcomes.

#### **Standardize review of animal studies in grant applications and scientific publications**

Our advisory panel recommended developing a study design checklist of items for consideration by funding agencies and scientific journals (Table 3). A rating system on study design quality of published papers and a centralized website that lists study results with a forum for comments and feedback would help to improve the selection of compounds for promotion into clinical trials. Furthermore, it would increase the incentive among academic researchers to improve study design.

#### **Establish a public data repository for animal studies**

A public data repository for both positive data and negative data from animal studies would help to improve research efficiency and disseminate negative data. Given that it is often difficult to distinguish a true negative result from a poorly designed study, the critical challenge of such a resource would be quality control. The considerations listed above will be important in providing this sort of distinction and will enable analyses of studies with various strengths and weaknesses in design. Such a repository could help to identify translatable biomarkers



### **Box 1. Thinking beyond amyloid: diversifying across disease processes**

While many reasons, including clinical study design, may have contributed to the high-profile clinical trial failures with anti-amyloid treatments to date, the AD field would benefit from diversifying its research portfolio to include non-amyloid disease pathways. Understudied disease processes include neuronal function, vascular changes, oxidative stress and inflammation, mitochondria function, and lipid metabolism. Here, we list methods that can be used to assess some of these processes and that should be more fully exploited.

#### **Neuronal function**

As Alzheimer's disease (AD) progresses, loss of synapses, shrinkage of dendrites, and neuronal death occur. It is these pathological changes that are most closely associated with the cognitive decline seen in the human disease. They can be modeled to some degree in a number of different animal models and are amenable to experimental monitoring as outlined below.

- Measure synaptic density by immunohistochemistry for the synaptic markers, such as synaptophysin. Non-homogeneity of synaptic markers in tissue surrounding plaques can present challenges in analysis. Also, PSD-95, AMPA-R, immediate early genes, and others are better markers for synaptic function than synaptophysin.
- Assess dendritic branching, neuronal structure volume, and total neuron numbers with careful stereology [22].
- Use T2-weighted magnetic resonance imaging (MRI) to indirectly assess neurodegeneration *in vivo* by measuring structure volumes, and use 1H magnetic resonance spectroscopy (1H MRS) to quantify N-acetyl-aspartate levels [23].
- Use electrophysiology to measure long-term potentiation in hippocampal slices.
- Employ behavioral studies to assess neuronal function. The Morris water maze, a spatial memory test that can be very sensitive to hippocampal function, is most commonly employed, but alternatives are available that may detect more subtle changes or may be more readily translatable – including attentional set shifting, delayed non-match-to-sample, recognition memory (novel object recognition), discrimination and reversal learning, contextual fear conditioning, and olfaction-based assays – or both [24-26]. Multiple behavioral tests may be needed to fully capture potential therapeutic effects. The use of multiple tests also helps control for factors, such as motivation and overall health, that may influence performance.

#### **Vascular targets**

Vascular pathology in human AD has received little attention, despite increasing evidence that vascular and neuronal dysfunction are closely intertwined and mutually exacerbating in the human disease. For example, cerebral amyloid angiopathy is seen in over 75% of patients with AD and can lead to vessel rupture, microbleeds, and hemorrhagic stroke [27,28]. Other vascular changes include reduced cerebral blood flow, degeneration of vascular endothelium, basement membrane and smooth muscle, and pathological changes in the neurovascular unit associated with astrocytes, pericytes, and microglia [29]. Attention to vascular targets is further warranted by evidence that amyloid-beta (A $\beta$ ) immunotherapy exacerbates cerebral amyloid angiopathy and microhemorrhages in both mouse AD models and human AD [30]. Experimental methods for monitoring vascular pathology include the following:

- Detect microhemorrhages with Prussian blue and double-stain for vascular A $\beta$ . T2-weighted MRI can also be used.
- Assess blood vessel area and patency (quantify using stains, such as tomato lectin, that bind to endothelial cells, together with imaging analysis). Counterstain for components of healthy blood vessels (for example, smooth muscle actin).
- Quantify retinal hemodynamics, which may be translatable as a biomarker in humans [31].
- Measure blood flow directly by arterial spin labeling and dynamic susceptibility contrast MRI and indirectly by FDG-PET or SPECT imaging. Blood volume can be sensitively measured by monocrySTALLINE iron oxide nanoparticle-enhanced MRI.
- Assess the structural integrity of the neurovascular unit by glial fibrillary acetic protein staining and counting total numbers of astrocytic end-feet and the number in contact with blood vessels.
- Assay neurovascular unit function by immunocytochemistry, quantitative polymerase chain reaction, or Western blot measurement of aquaporin 4 or potassium channels (Kir4.1, BK calcium-dependent potassium channel) that are enriched in astrocytic end-feet. It is important to do immunohistochemistry in addition to biochemical measurements as channel distribution can be altered without changes in total levels.

#### **Oxidative stress/Inflammation**

Oxidative stress and inflammation are known to be associated with AD and are relevant targets for drug development, in particular for sporadic AD. However, detecting reliable changes in AD models can be quite difficult.

High oxidative stress is not seen in the most commonly used amyloid precursor protein transgenic (Tg2576) but can be seen in more recent models in which redox pathways have been genetically manipulated [32-34]. These models also show more aspects of AD pathology. Different animal models vary in their upregulation of specific inflammatory profiles; tau models, in particular, show high levels of inflammation in association with neurodegeneration. The time points in which these pathways are assessed is critical since oxidative and inflammatory processes that are toxic at one time point may be protective at others; their levels and effects may also vary among brain regions.

*Continued overleaf*

### Box 1. Continued

Because reactive species are labile and cannot be measured directly, oxidative stress must be measured via surrogate markers:

- Chronic oxidative stress can be detected by monitoring lipid peroxidation or oxidized proteins (by carbonyl assay or by high-performance liquid chromatography) or by quantifying changes in genes and proteins known to increase or decrease with cellular redox balance [35].
- Commonly used markers also include oxidized DNA and anti-oxidant proteins (such as glutathione, which is known to be directly affected by A $\beta$ ).
- Free iron, copper, and zinc can be measured. Free iron is a linear indicator of disease progression in many neurodegeneration models and is an indicator of free radical generation around A $\beta$ .
- Many of these assays have low sensitivity and so likely will detect only robust drug effects. The more sensitive assays require mass spectrometry or high-performance liquid chromatography or 31P and/or 1H MRS.
- Emerging technologies include two-photon imaging with novel probes for reactive oxygen species, which could be used to separately monitor changes occurring in the parenchyma and vasculature.

Inflammation can be assessed by the following:

- Measuring invasion of peripheral cells or microglial activation or both. The number and morphology of microglia in tissue sections can be quantified.
- *In vivo* imaging of microglial activation markers (for example, PK11195 positron emission tomography imaging, autoradiography, or tissue sample scintillation counting). Not all of these markers are well characterized in terms of when during the inflammatory process microglia express them.
- Testing for blood-brain barrier leakage, which can be detected by steady-state gadolinium-enhanced T1-weighted MRI or classical intravenous bovine serum albumin and/or Evans blue brain parenchyma quantification. Related cerebral edema can be quantified by diffusion or absolute T2-weighted MRI or both.

FDG-PET, fluoro-2-deoxy-D positron emission tomography; SPECT, single-photon emission computed tomography.

in animal models that can also be used in human clinical trials to better predict outcomes.

### Conclusions

Our advisory panel produced recommendations in regard to the measurement, analysis, and reporting of relevant targets in AD animal models. These recommendations stressed the need for quality control measures in breeding and colony maintenance to manage phenotypic variability and outlined key issues related to preclinical animal study design. Distinguishing between exploratory and therapeutic animal studies will aid in defining the scope of the study and the interpretation of results and hopefully will bring some of the rigor of industry preclinical testing to the academic space.

Whereas A $\beta$  likely plays a key role in the development of AD, amyloid deposition alone does not represent the entirety of the disease process or the totality of targets worth investigating for therapeutic intervention. To propel innovation, we should broaden our focus to additional disease-relevant pathways and processes (such as tauopathy, neuroprotection, synaptic plasticity, oxidative stress, inflammation, vascular changes, and mitochondrial dysfunction). Furthermore, non-transgenic models of disease are often underused. Aging is the greatest risk factor for AD; hence, aged rodent models that show cognitive impairment may provide a useful

choice for testing investigational therapies targeting mechanisms of neuroprotection, learning, and memory. In addition, models that demonstrate clear neurodegeneration and cell death (such as tau transgenic models and pharmacologically induced models) may be better for testing neuroprotective therapies in general. There is no standard model or set protocol for testing investigational treatments in AD, and it is critical to tailor the choice of model, experimental plan, and outcome measures specifically for the therapy's target or proposed mechanism of action. Animal models of disease will never be able to predict all possible outcomes in humans.

While these recommendations are specifically geared toward AD, they echo many of the sentiments raised in other recent consensus efforts for related diseases such as stroke, vascular cognitive impairment, and amyotrophic lateral sclerosis [10,20,21]. These efforts, as a whole, highlight common challenges in animal model selection, study design, interpretation, and reporting which go beyond individual disease states. While each therapeutic area will undoubtedly have its own unique issues, there is much to be learned from shared barriers in translational research. Hopefully, these collective efforts will raise the bar for preclinical studies and will aid in designing animal studies to optimize their interpretability, improve their predictive value, and drive innovation, ultimately improving our efficiency in bringing effective treatments to patients.

## Abbreviations

A $\beta$ , amyloid-beta; AD, Alzheimer's disease; ADMET, absorption, distribution, metabolism, excretion, and toxicity; MRI, magnetic resonance imaging; PD, pharmacodynamics; PET, positron emission tomography; PK, pharmacokinetics.

## Competing interests

GSB is an employee of and holds shares in Elan Pharmaceuticals, Inc. (South San Francisco, CA, USA), which holds many patents in animal models of AD. BAH, IM, PS, and JY are full-time employees of Charles River Laboratories International, Inc. (Wilmington, MA, USA) or its affiliates and may own shares in the company. FL is a full-time employee of Abbott Laboratories (Abbott Park, IL, USA). DRR is a full-time employee of Pfizer Inc (New York, NY, USA). KS-L is a full-time employee of Genentech, Inc. (South San Francisco, CA, USA). CAC has applied for patent protection concerning a mouse model of AD discussed in the manuscript. All other authors declare that they have no competing interests.

## Acknowledgments

We would like to convey our appreciation to key members of the Alzheimer's Drug Discovery Foundation staff for their support in this effort. In particular, we thank Filomena Machleder, Adam Liebling, and Hannah Elkin. We are also grateful to the Charles River CHARTER (Commitment to Humane Animal Research Through Excellence and Responsibility) program for providing funding.

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Published: 28 September 2011

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doi:10.1186/alzrt90

**Cite this article as:** Shineman DW, et al.: Accelerating drug discovery for Alzheimer's disease: best practices for preclinical animal studies. *Alzheimer's Research & Therapy* 2011, **3**:28.