Neurotherapeutics (2015) 12:126–131
DOI 10.1007/s13311-014-0335-5

REVIEW

A Systems Approach to Drug Discovery in Alzheimer's Disease

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Published online: 22 January 2015

Abstract In the articles included in this volume, one feels a strong frustration among the writers with the slow course of therapeutics development for Alzheimer's disease and with the clinical failure of targeted therapeutic agents despite substantial progress in our understanding of the biology and biochemistry of the disease.

Keywords Master regulator · Interactome · Human neurons

To date, approaches to Alzheimer disease (AD) therapeutics have followed 2 main avenues. The first addresses neurotransmitter deficits in the early phases of the disease. This approach has led to the handful of AD drugs currently on the market that provide short-term symptomatic improvement but do not alter the course of the disease. The second approach has been directed at decreasing the burden of beta-amyloid (Aβ) in the brain by active or passive immunization or by inhibiting activity of β- or γ-secretases. To date, none of the approaches in this second class has been successful, although trials on β- or γ-secretase (BACE) inhibitors are ongoing.

The lack of overt success has been even more frustrating because, in transgenic (Aβ-overproducing) mouse models of AD, Aβ-lowering approaches, as well as treatments with small molecules and biologics, have been successful in blocking memory loss, restoring memory function, reversing dendritic spine alterations, and reversing inhibition of long-term potentiation [1–3]. Indeed, as animal models only partially recapitulate the human AD phenotype and because human brain tissue is available only postmortem, translation of preclinical findings to the clinics has been fraught with difficulties.

For instance, the characteristic intraneuronal neurofibrillary tangles and extensive neuronal loss observed in human AD are absent in mouse models of the disease. This dichotomy suggests that mouse models replicate only presymptomatic AD stages, while clinical manifestations appear only after neuronal loss becomes irreversible. This hypothesis is directly addressed by ongoing trials, where individuals with genetic mutations that predispose to early AD onset are being treated with Aβ-targeted therapies to assess whether presymptomatic treatment may block an otherwise certain disease progression. Beyond Aβ-targeted therapies, novel approaches are being developed based on inhibition of tau phosphorylation and aggregation, and on blockade of synaptic loss, leveraging "candidate" target approaches derived from human and animal data. Unfortunately, it has so far been impossible to discern whether "candidate genes" represent causal determinants of the disease process or are the downstream result of them.

In this review, we will discuss the potential of adapting integrative systems biology approaches that have already proven extremely valuable in understanding multiple cancer related phenotypes to human neurodegenerative diseases.

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Developing Human to Mouse to Human Approaches

A key limitation of AD studies is that the mechanisms of disease initiation and progression, as well as their modulation using small molecule inhibitors, cannot be assessed in a human host. As a result, *in vitro* and *in vivo* models of AD have flourished. Yet, many of these models recapitulate only specific facets of the disease and, as a result, translation of findings from cell lines and transgenic mouse models to humans has been disappointing at best.

To address this impasse and to avoid findings that are idiosyncratic to a specific biological model but fail to generalize to the human context, we propose a cross-species approach that has been highly successful in the study of human malignancies [4]. Specifically, we propose assembling accurate, genome-wide regulatory models for both mouse models of AD and for their human counterpart to assess systematically the subset of the regulatory logic of AD neurons that is conserved or divergent between the two species. The rationale for this strategy is that investigation of therapeutic targets within conserved regulatory circuits will lead to successful translation from mouse models to human studies, while those embedded within a divergent cellular circuitry will prove to be idiosyncratic and will not be successfully translated.

Over the last 10 years there has been a veritable explosion of methodologies for the systematic reverse engineering of mammalian regulatory and signal transduction networks (henceforth *interactomes* for short) that are cell context specific and that have been thoroughly experimentally validated (see [5, 6] for a review). Murine interactomes including neuron-specific interactomes [8, 9], have been obtained by harvesting cells from mouse models representing diverse genetic backgrounds, tissues, and/or environmental stimuli, including small molecule perturbation *in vivo* [7]. For instance, to assemble an accurate murine interactome for cross-species analysis of prostate cancer (PC) progression, 13 distinct transgenic PC models were perturbed *in vivo* with 13 small molecule compounds and dimethyl sulfoxide to generate a set of 364 gene expression profiles from murine PC tissues and normal controls. These data were used to assemble a genome-wide transcriptional regulatory model using the ARACNe algorithm [10, 11], where each transcription factor (TF) was associated with its PC-specific transcriptional targets (i.e., the *TF regulon*). The corresponding human PC interactome was produced by ARACNe analysis of a set of gene expression profiles from ~200 patient-derived PC samples, representing the full spectrum of disease progression. Comparison of the human and murine interactomes, using a novel algorithm, revealed that 70% of the regulatory programs in PC are highly conserved between these 2 species, including those of 2 synergistic master regulators (MRs) of progression to aggressive disease (forkhead box protein M1 and centromere protein F), inferred by the Master Regulator Inference algorithm (MARINa) [7, 12–15], and experimentally validated both in mouse and in human tissue. However, the analysis also showed that 30% of the programs are not conserved, including those representing a few PC-related genes that would thus be unlikely to produce patient-relevant results if studied or targeted in a murine context.

We propose that application of this approach to Alzheimer's disease will allow direct assessment of whether specific functional drivers and genetic determinants of human AD neurodegeneration, discovered by interrogating human neuronal interactomes with AD patient-derived signatures, are conserved in a mouse model of AD. This would allow their successful elucidation and targeting in a mouse context and the subsequent translation of relevant findings, including targeted therapeutics, back to a human context, implementing a *bona fide* human → mouse → human approach.

Creating the Assembly Manual of the Alzheimer's Cell

The mechanisms that regulate AD initiation and progression have been investigated largely without the benefit of an accurate and comprehensive map of the molecular interactions that underlie normal physiologic neuronal activity and, ultimately, its pathological dysregulation prior to neuronal demise in AD. Existing regulatory and pathway models are inaccurate, sparse, and, most importantly, not representative of neuron- and AD-specific biology. In particular, canonical pathways, which are generally represented as universal rather than cell-specific models, are largely inadequate for the task of modeling the mechanism of disease initiation and progress. For instance, they fail to represent the cross-species diversity and intraspecies redundancy of signaling pathways, which are critical reasons why therapies do not translate effectively from the bench to the clinic or from model organisms to human biology.

Over the last few years, we have pioneered computational and experimental methods for the accurate dissection of tissue- and cell-specific molecular interaction networks, including those controlling transcriptional(protein–DNA)[10, 11, 16, 17], post-transcriptional(RNA–RNA and protein–RNA) [18–21], signal transduction and other post-translational processes protein-protein interactions (PPI and metabolic) [22–26], and drug interactions [27–30]. These methods and datasets allow for the reconstruction of the regulatory and signaling logic of specific cell types by combining specific knowledge about regulatory mechanisms (e.g. transcription factors regulating expression, etc.) and information theoretic [11, 25], Bayesian [31, 32], and other machine learning methodologies [33] that can be used to infer causality and remove indirect interactions. Following reverse engineering analysis with these algorithms, each TF signaling protein

and microRNA represented in an interactome is causally associated with a regulon containing dozens to hundreds of highly accurate and cell-context-specific targets and substrates. For instance, predictions by the ARACNe, MINDy, PrePPI algorithms have been typically validated at a very high rate (~70%) by chromatin immunoprecipitation, gene expression profiling following silencing, coimmunoprecipitation and other relevant assays [12, 19, 24, 25, 34, 35]. Other systems approaches have also been applied successfully in AD [36–39], and our focus here is to maintain clarity rather than review the entire literature. Each approach has a series of advantages and disadvantages. The approach outlined here is unbiased, not dependent on a large number of transcriptomes to detect changes between control and diseased tissues, and predicts alterations in transcription factors and transcriptional regulators that themselves might not be altered in the transcriptome.

Critically, these regulons provide the computational equivalent of a highly accurate gene reporter assay to measure the activity of the corresponding protein or other regulatory gene product. The MARINa algorithm was developed to infer the regulators that are causally responsible for implementing a phenotype-specific gene expression signature initially from multiple gene expression profiles [12, 14], and, more recently, even from a single gene expression profile [7, 40]. This is accomplished by measuring the enrichment of over- and underexpressed genes in positively regulated and repressed targets in the regulon of every possible regulator protein represented in the interactome, thus providing an accurate and extremely robust predictor of the differential activity of a regulator. This has allowed the discovery of key functional drivers of tumorigenesis and drug sensitivity, including single regulators [13, 15, 41–43], synergistic regulator pairs [4, 12, 14], and additive mechanisms that could not have been elucidated using traditional methods. Indeed, virtually none of the regulators that were experimentally validated were significantly differentially expressed at the RNA level and yet they were confirmed as individual or synergistic phenotypic drivers following identification by regulatory network analysis, thus elucidating novel mechanisms of disease initiation/progression [4, 12, 13, 41–46], chemosensitivity [15, 28], and normal physiologic regulation [14]. Lately, we have successfully extended these methodologies to the study of neurological, neurodevelopmental, and stem cell phenotypes [35, 42, 47], and, more recently, to neurodegenerative phenotypes, resulting in a series of manuscripts, currently in review, where we report on the elucidation and experimental validation of novel genes mediating neurotoxicity in ALS [48], as well as biomarkers of AD progression [9].

More recently, we have shown that MARINa-inferred MRs represent tight regulatory bottlenecks that integrate a large spectrum of germline variants and somatic alterations in upstream pathways [40]. These bottlenecks allow genetic variants and alterations patterns that are substantially distinct in different patients to converge on activating or inactivating key genetic programs that represent the hallmarks of the disease. Indeed, by traversing pathways upstream of MARINa-inferred MRs, using the MINDy algorithm, we identified the vast majority of established germline variants and genetic alterations that causally associated with glioma and breast cancer tumorigenesis, as well as AD predisposition. These alterations were not statistically significant by genome wide association studies. In addition, novel alterations predicted by the methodology for the mesenchymal subtype of high-grade glioma were experimentally validated as the most frequent causal determinants of the disease subtype [40]. Critically, this analysis could be performed on an individual sample basis, thus completely avoiding mechanism heterogeneity as a confounding factor that would negatively affect discovery when averaging on patients representing distinct mechanisms (e.g., all AD-affected individuals). In AD, the method identified 14 predisposition variants, including known ones, such as *APOE* and *TYROBP*, among others. Surprisingly, 3 of 14 predisposition alleles identified by the algorithm were in the integrin pathway, including *ITGB2*, *ITGBL1*, and *ITGAM*, which has been functionally linked to aberrant tau and Aβ activity but never previously linked to AD in terms of risk predisposition alleles.

Finally, these methodologies have proven extremely valuable in elucidating context specific mechanisms of action of small molecules, as well as their activity in cells both individually and in combination [28, 49], thus paving the road to the mechanism-based identification of both single agent and combination therapy. While some are still in review, the results included in these manuscripts suggest that the regulatory network analysis strategies developed for cancer are eminently applicable to neurodegenerative diseases.

Development of a Quantitative Systems Pharmacology Approach for the Identification of Molecular Targets and Associated Small Molecule Modulators

There are several criticisms of traditional high-throughput screening (HTS) assays to identify small molecules that may be useful in AD. First, HTS approaches are limited to use in *in vitro* models of a disease, often using a reporter that is only indirectly linked to the human relevant phenotype. Second, results obtained using *in vitro* disease models rarely translate to an *in vivo* context. The latter is due to a variety of reasons: from the 3-dimensional *versus* 2-dimensional nature of the culture, to the presence of critical signals from other cellular niches in the neural microenvironment, to the presence of local paracrine and distal endocrine signals as well as other factors.

To address these limitations, a radically different approach has been proposed. In this approach, one first identifies candidate MRs of AD pathogenesis from AD patient gene signatures. This can be accomplished by interrogating a human

neuron-specific interactome, using the MARINa algorithm as discussed in the previous section, with signatures of disease progression using gene expression profiles from normal and demented AD patients. We have tested this approach for its feasibility by generating a human neuronal interactome using transcriptional data generated from laser-dissected control, nondemented and AD human neurons from multiple regions of postmortem brains (GSE5281 and GSE9770) [50–52]. This interactome was then queried using gene expression profiles from AD and control tissues. Alterations in YY1, p300, and ZMYM3 were predicted by this approach and then validated by immunohistochemistry in AD brains and in Aβ-treated rat primary hippocampal neurons. These represent novel biomarkers and potential therapeutic targets for the disease. A number of other predicted MRs are currently under study and a paper is currently under review [9].

Candidate MRs will need further validation—first *in vitro*, by RNA interference or clustered regularly interspaced short palindromic repeats-based silencing in neurons, and then *in vivo*, following cross-species analysis to identify an appropriate model—to assess whether they truly control the predicted genetic programs that are dysregulated in AD. Validated MRs will then be evaluated as candidate functional targets of both single compounds and compound combinations, using a nonredundant library of US Food and Drug Administration-approved drugs and probe/toolkit compounds. A powerful new screening tool is a novel technology for Pooled Library Amplification for Transcriptome Expression (PLATE-Seq), recently developed in the Sims lab at Columbia University, to perform multiplexed, barcoded RNA-Seq of 96 samples as a single library, thus achieving a cost of $20–30 per sample at a depth of 2M reads. In PLATE-Seq, well-specific barcodes are introduced during reverse transcription. Thus, complementary DNA libraries from individual wells can be pooled immediately after reverse transcription, such that all subsequent bead- and column-based isolations steps, second strand synthesis, and polymerase chain reaction can take place on a single, pooled sample. This approach dramatically simplifies generation of large-scale gene expression signatures following perturbation with relatively large libraries of US Food and Drug Administration-approved and experimental compounds. In cellular systems, for which an ARACNe-based interactome is available, PLATE-Seq libraries can be used to assess compound effectors and targets with no fidelity loss compared with 30M read RNA-Seq owing to the integration of expression values over each protein's regulon. Taken together with the low coverage requirements of our algorithms, the reduction in reagent cost and hands-on time afforded by PLATE-Seq allows generation of genome-wide expression signature on an unprecedented scale.

Such an approach can be used to screen cells following perturbation with compound libraries, thus helping elucidate the compound effectors and substrates using the same MARINa algorithm and the same regulatory models used for AD MR inference. Importantly, as this analysis can be aimed at identifying modulators of specific protein activities, rather than AD-relevant endpoint phenotypes, it is well suited to *in vitro* analysis as compound substrates and targets are generally well conserved *in vitro* and *in vivo*. This approach can thus be used to prioritize all library compounds that are likely to reverse the specific pattern of aberrant MR activation and inactivation observed in demented AD patients, as well as to determine their full activity and mechanism of action, thus identifying potential toxicity issues. A novel algorithm (SynGen) was developed to assess the potential synergy of 2 compounds in implementing a specific target phenotype using such single-compound RNA-Seq signatures. The analysis was tested against a public database as part of the dialogue for reverse engineering assessments and methods (DREAM) challenge on drug synergy, and was effective in predicting 60% of the experimentally assessed synergistic compound combinations at an 8% false discovery rate [28].

We propose that such a hybrid experimental/computational methodology will allow identification of optimal compounds and compound combinations that will help revert the MR signatures detected in AD patients toward a more normal physiologic state. Top single agents and compound combinations emerging from the analysis would be tested in increasingly specific models and finally in the murine models of AD assessed to be most human-relevant, based on cross-species network analysis. Specifically, these analyses will test whether the specific mechanisms targeted by compounds or compound combinations are a) conserved in the mouse and b) equivalently targeted by the small molecule in the mouse.

Approaches to Validation

Validation of MRs can be performed by established techniques, using a) phenotypic assays *in vitro* and *in vivo,* and b) molecular assays based on PlateSeq or Fluidigm multiplexed quantitative polymerase chain reaction, to determine whether RNA interference or clustered regularly interspaced short palindromic repeats-based modulation of individual MRs and predicted synergistic MR pairs will induce the predicted regulatory outcome and phenotypic end points. Such efforts will produce a set of validated MRs that will be used to screen for specific small molecule compound activity. Compounds will thus be prioritized based on their ability to abrogate the aberrant activity of validate MRs based on analysis of their key effectors following perturbation of neuronal cells, as assessed by analysis of PlateSeq-based RNA-Seq profiles of perturbed cells.

Small molecule modulators (either as single compound or synergistic compound pairs) can be prioritized through these quantitative model-based approaches and then be experimentally validated. This is accomplished by the establishment of robust and reproducible correlative profiles between

compound-induced changes in AD-specific expression signatures (using the validated MRs) *versus* functional changes in our available phenotypic assays using *in vitro* mouse and human model as well as *in vivo* mouse models. For *in vitro* models, models include dissociated mouse primary neurons or embryonic stem cell-derived pyramidal neurons derived from the Tg2576 mouse model of AD. HTS assays exist for cell-associated production of sAPPβ [BACE1 cleavage product of amyloid precursor protein (APP)] in neuronal cells, Aβ-induced redistribution of intracellular tau in embryonic stem cell-derived neurons, and apolipoprotein E secretion from human astrocytes. Additional assays are also available for follow-up validation, including Aβ-induced synaptic dysfunction using multiple measures of electrophysiology, biochemistry, and morphology, in primary neurons as well as *ex vivo* brain slice models. AD-relevant phenotypic assays in human induced pluripotent stem cells (hiPS)-derived neurons for Aβ-triggered neuronal death and synaptic deficits are also being developed. Validation end points will be reversal of MR activity in these cellular assays, as assessed by the MARINa analysis of RNA-Seq profiles following compound perturbation, as well as surrogate phenotypic readouts, such as dendritic spine density, caspase activation or tau phosphorylation. Small molecule modulators that are validated using these *in vitro* models can then be tested in well-characterized animal models to determine AD-relevant preclinical efficacy. Potential model systems include transgenic and nontransgenic mouse models of AD, including the APP/PS1 mouse model of amyloid elevation [53, 54], the hTau/Tau knockout transgenic mouse line in which the mouse tau gene is replaced by the nonmutated human tau gene [55, 56], and mice exposed to Aβ and tau oligomers through cannulas implanted onto dorsal hippocampi as nontransgenic models of AD [57]. These animals can be treated with a given compound/s as indicated by the experiments on *in vitro* models, and then undergo long-term potentiation assessment of synaptic function and behavioral testing with a battery of cognitive tasks, including fear conditioning, radial arm water maze, and the Morris water maze [57]. The successful completion of this step will establish the compound–target and target–disease networks that are functioning through the diverse mechanisms, including the one target–one compound paradigm, as well as polypharmacology.

These approaches have the potential to rapidly improve the route to AD drug discovery. The ability to screen on the reversal of effects on MRs before going into time-consuming animal models makes the approach both effective and economically sensible.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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