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Title: A systems-level framework for anti-epilepsy drug discovery

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Abstract

Modern anti-seizure drug development yielded benefits in terms of improved pharmacokinetics, safety and tolerability profiles, but offered no advances in efficacy compared to previous older generations of anti-seizure drugs. Despite significant advances in our understanding of the genetic bases to epilepsy, and a welcome renewed interest on the severe monogenic epilepsies, modern genetics has yet to directly inform more effective or disease-modifying anti-seizure drugs. Here, we describe a new approach to the identification of novel disease modifying anti-epilepsy drugs. The systems genetics approach aims to first identify pathophysiological mechanisms by integrating polygenic risk with cellular gene expression profiles and then to relate these molecular mechanisms to druggable targets using a gene regulatory (regulome) framework. The approach offers an exciting and flexible framework for future drug discovery in epilepsy, and is applicable to any disease for which appropriate cell-type and disease-context specific data exist.

Introduction

Drug discovery has historically relied on phenotypic screens, serendipitous pharmacological observations, often from pre-clinical disease models, and a “chemocentric” clustering of innovation around established drug targets. Thus in 2007, an analysis of the global set of relationships between protein targets and all US Food and Drug Administration (FDA) approved drugs revealed a strong clustering of structurally unique drugs around a relatively small number of protein targets¹. This analysis highlighted the overabundance of ‘follow-on’ drugs from the first approval around an established and proven target as a means of de-risking drug development pipelines. In the past decade more focus has been placed on novel target-based drug discovery, which draws from a better understanding of disease biology and selective targeting of pathomechanisms by various therapeutic modalities. However, despite a renewed focus on mechanistic target-based drug discovery, the majority of approved first-in-class drugs are still the result of either phenotypic screening or chemocentric efforts where compounds with known pharmacology provide the starting point for drug development.²

In the field of epilepsy these two approaches (chemocentric and phenotypic screening) produced a series of new anti-seizure drugs (ASDs) that whilst offering gains in pharmacokinetics and improvements in drug-drug interaction and safety/tolerability, offered no advances in efficacy compared to previous older generations of ASDs³. Conceptually modern ASD development did not depart from the established repertoire of synaptic proteins as targets and the field was left with a series of drugs that were all symptomatic treatments modulating neuronal excitability. Indeed, to date, none of the licensed ASDs have been proven to prevent or reverse the development of epilepsy and no ASD is unequivocally superior in efficacy to any other ASD. Consequently, the growing number of approved new ASDs with optimized

pharmacokinetic/safety properties but no evidence of superior efficacy or disease modification has become problematic to both US and European healthcare funders, who were seeking clear improvements in efficacy. With some notable exceptions, large Pharma's initial response was largely to divest their epilepsy R&D programs to focus on what was viewed as more tractable (i.e., non-CNS) and "less crowded" therapeutic areas, leaving a significant unmet clinical need for patients with epilepsy unaddressed. More recently however, potential facilitated drug licensing arrangements for rare disease by both the US FDA and the European Medicines Agency (EMA) has encouraged new R&D that aims to position novel ASDs for rare epilepsy or seizure types⁴. This rare disease strategy in epilepsy has followed two broad paths; precision medicine approaches that seek to target a specific gene defect underlying a monogenic epilepsy⁵ or the less precise use of rare severe epilepsies as the testing ground for novel AEDs which albeit still mostly relate to established concepts of excitability/inhibition in epilepsy or were identified using traditional phenotypic screens⁴. To date, the published examples of precision medicine in epilepsy have been mostly in subjects where the epilepsy results from gain-of-function (GOF) mutation in an ion channel subunit gene⁵, with few examples of precision medicine targeting the much larger list of monogenic epilepsies arising from loss-of-function (LOF) mutation in non-ion channel genes such as *LGII*, *STXBP1*, *PCDH19*, *TCF4*, *CDKL5* etc. Given that LOF mutation in non-ion channel genes accounts for the majority of the monogenic epilepsies, and that restoring gene function is pharmacologically more problematic than blocking function, the development of precision therapies for epilepsy broadly is likely to remain a significant challenge. This background highlights the need for new approaches to drug discovery in epilepsy that departs from the traditional focus on symptomatic therapies based on phenotypic screens, risk averse chemocentric reasoning or the traditional excitation/inhibition paradigm and instead incorporates the broader advances in our knowledge of the genetic and epigenetic underpinnings of epilepsy. This Review considers the prospects for the development of novel epilepsy disease modifying therapies (i.e., "anti-epilepsy" drugs) from such a systems-genetics perspective.

Disease modification

There is debate about the meaning of the term “disease-modifying” in epilepsy which is not unique to epilepsy. Recently, the EMA published guidelines on the clinical investigation of medicines for the treatment of Alzheimer’s disease (AD)⁶. This guidance acknowledges the limited attention paid to understanding the experimental paradigms required to establish disease-modification in AD but falls short of offering specific guidance to drug developers. For a progressive disease such as AD, the property of disease modification would probably require divergence in the slope of decline for the treated and untreated patient groups, but this is unlikely to be an informative strategy for a generally non-progressive disease such as adult epilepsy (although some childhood onset neurodevelopmental epilepsies may be amenable to this approach). Moreover, for epilepsy, there is the added complication that one could consider prevention of epilepsy following an epileptogenic insult as well as the amelioration of disease severity in established epilepsy as both being examples of disease modification. Whilst in some circumstances both antiepileptogenesis and amelioration of disease severity in chronic epilepsy may share mechanistic underpinnings, equally they may not, and therefore in this Review we consider disease modification in chronic epilepsy as potentially distinct from antiepileptogenesis. One proposal for demonstrating disease modification in chronic epilepsy (as opposed to antiepileptogenesis) is to seek to demonstrate an anti-seizure treatment effect that outlasts the treatment period. However, given the emergent evidence that a key aspect of drug resistance in epilepsy is intrinsic disease severity⁷, a disease-modifying agent may “shift” a patient from a severe ASD treatment unresponsive state to a less severe ASD treatment responsive state in such a way that the therapy is “disease modifying” even if the effect does not outlast the duration of exposure. An analogy may be drawn here with the treatment of severe asthma, whereby disease modifying agents shift the severity of the asthma such that it becomes responsive to symptomatic bronchodilator therapy but where the effectiveness of symptomatic therapy still wanes over time if the disease modifying agent is withdrawn. To encourage both

academic and industry drug developers to focus on epilepsy disease modification and given the uncertainties that pervade this discussion, we argue that “disease modification” should not be understood too narrowly, which in the current state of uncertainty could easily preclude certain lines of investigation or further discourage industry investment in disease modifying anti-epilepsy drug (AED) discovery.

In addition to the uncertainties surrounding the meaning of disease modification, it is also clear from the discussion above that the development of disease modifying drugs in epilepsy will require a new scientific approach. Whilst there has been much interest in the use of genetics to inform new drug target discovery generally⁸, as well as in epilepsy specifically⁹, germline genetic predictors of efficacy with clinically meaningful effects have yet to emerge from modern gene discovery¹⁰. Moreover, for epilepsy at least, the assumption that targeting a protein which is a known genetic cause of disease must *de facto* result in disease modifying effects is unlikely to hold true – many of the current licensed ASDs target ion-channel gene subunits which are known causes of monogenic epilepsy or genome-wide significant GWAS “hits” without any evidence that they are disease modifying. For this reason, in our own research, we have shifted our attention from single underlying gene defects to take a broader systems-wide perspective of the perturbed functional pathways that underpin and/or maintain the epilepsy disease state.

The signature reversal paradigm

Systems-genetic analyses provide powerful techniques for elucidating molecular processes and pathways underlying disease using the principle of convergence¹¹. A convergent framework postulates that multiple diverse biological perturbations (i.e., diverse genetic and epigenetic changes) carrying risk for a given disorder can converge mechanistically on common functional pathways. This common pathway can manifest at many levels, including gene and protein levels, but in our own research we have focused on identifying cellular gene expression signatures as

drivers of disease because this level of analysis provides a tractable translational opportunity which we discuss below.

To date, the published work undertaken in epilepsy and other brain phenotypes using the systems-genetic approach has mostly utilized bulk brain tissue gene expression data. However, because the expression of every gene in a bulk tissue dataset is essentially an average of the expression values of every cell sampled, cell-type specific differential gene expression cannot be deconvoluted from differences in cell loss or gain between conditions, confounding biological signal detection. Moreover, averages of measurements between conditions can follow trends that qualitatively differ from the measurements that actually describe each condition, a phenomenon known as Simpson's paradox¹². Whilst these limitations can now more readily be overcome by measuring transcriptional levels in individual cells (i.e., single cell RNA-sequencing, scRNA-seq) which we discuss below, until the recent technological developments in scRNA-seq the approach to extracting cell-type and state-specific functional gene expression signatures from bulk-tissue gene expression data was based around co-expression network analysis.

The co-expression network approach involves first constructing networks reflecting the functional and regulatory relationships between genes using genome-wide gene expression data. These co-expression networks exhibit functional modularity and cell-type specificity, allowing the extraction of cell-type and disease context specific gene expression profiles^{13,14,15,16}. Polygenic risk is then mapped onto network genes to identify networks with a putative causal relationship to disease¹⁷ – if the network is enriched for genetic risk to a disease then it is considered to have a proximal relationship to disease rather than consequential. This integrative framework captures both functional pathways and convergent heritability, and has increased power to detect functional pathways compared to traditional single variant or single gene genetic analyses because it combines the marginal effects of multiple susceptibility

variants across many distinct but functionally related genes. The integrative co-expression approach does not rely on prior hypotheses, but applies a data-driven framework to the discovery of often previously unconsidered disease pathways. Using integrative approaches, we have mapped novel convergent pathways for epilepsy^{13,15,18,16} as well as for human intelligence and neurodevelopmental diseases more broadly¹⁴. For example, interneurons are a key cell-type governing excitatory/inhibitory (E/I) balance in epilepsy (and cognition), and we have previously reported convergence of *de novo* deleterious mutations from patients with developmental epileptic encephalopathy (DEE) as well as common variants for common forms of epilepsy on a gene co-expression network which is highly expressed in interneurons and functionally enriched for synaptic processes¹⁵.

From a translational drug discovery perspective the representation of a disease state's pathophysiology in terms of a cell-type specific gene expression profile provides a starting point for novel therapeutic discovery¹⁹. At this systems-level, the "target" is the gene expression signature underpinning the disease state and therapeutic compounds are therefore judged not by their binding affinity to a particular protein, but by their ability to induce a transcriptional response (i.e., a gene expression profile) that is anti-correlated to the coordinated transcriptional program underpinning the disease (Figure.1). This is termed the "signature reversion paradigm"¹⁹ and is orthogonal to traditional Pharma concepts of drug discovery. In particular, a shift in a disease's gene expression signature toward health represents a biomarker of drug efficacy, such that drugs and targets identified by this route are, by definition, predicted to be disease modifying, and the shift in the expression signature represents an endophenotype of therapeutic response. Starting from this perspective, we have developed and applied methods to translate disease-related gene expression profiles to therapeutic discovery including the development and application of Bayesian methods to map the master genetic regulators of co-expression networks resulting in a patented novel drug target for epilepsy¹³, published proof-of-concept for the use of gene co-expression networks as biomarkers

(endophenotypes) for drug screening in epilepsy¹⁵, and developed and validated a gene-regulatory network (i.e., regulome, GRN) framework for predicting novel drug targets from disease-related gene expression data (Causal Reasoning Analytical Framework for Target discovery, CRAFT)¹⁶.

For our regulome-based drug target discovery tool CRAFT¹⁶, we defined a “regulon” as a regulator (e.g., transcription factor, membrane receptor, small RNA, etc.) and the predicted gene targets under that regulator’s influence (i.e., a “gene’s” sphere of influence over the expression of other genes). A regulome is therefore the global (i.e., genome-wide) network of individual regulons. Regulomes can be knowledge-based (e.g., the extraction of relationships between regulators and targets from published literature) or reverse engineered using empirical gene expression data (e.g., using the Algorithm for the Reconstruction of Accurate Cellular Networks, ARACNe)²⁰. In both scenarios, the regulator-target interactions can be signed to reflect gene activation or repression allowing the global transcriptional consequences of regulator blockade or activation to be predicted. For CRAFT, we created a knowledge-based regulome by computationally extracting information relating to known interactions between membrane receptors and transcription factors (TFs) along canonical linear pathways and between TFs and their target genes according to published experimental evidence. Taking into account the direction of the receptor>TF>target gene interactions and the desired shift in the disease-related gene expression signature, CRAFT computationally ranks membrane receptors (genome-wide) in terms of their predicted magnitude and direction of effect over a disease’s gene expression signature and therefore identifies putative disease modifying drug targets. As >50% of approved drugs target receptors²¹, by connecting gene expression profiles to membrane receptors CRAFT maximizes the likelihood of finding targets which are druggable by small molecules (as opposed to say TFs which are difficult to drug by conventional small molecule approaches). In proof-of-concept, we applied CRAFT to epilepsy resulting in the discovery of the microglial receptor Csf1R as a novel drug target for epilepsy and in a series of

pre-clinical experiments repositioned the Csf1R blocker PLX3397 to the treatment of epilepsy¹⁶. Critically, by starting from a gene expression signature ascertained from disease tissue that was conserved across species, we were able to impart disease-context specificity on our drug target predictions – a fact confirmed by the observation that whilst PLX3397 is effective in attenuating seizures in preclinical models of chronic epilepsy, it has no effect when seizures are acutely induced in non-epileptic (naïve) mice (i.e., the conventional seizures models traditionally used in ASD discovery). As acute seizure models have been the gatekeepers for ASD discovery for >50 years²², this clearly distinguishes our regulome framework to drug discovery from all current antiepileptic drugs and in our view provides a framework for the future direction of disease modifying drug discovery in epilepsy and more widely.

As referred to above, these systems-genetic analyses have to date been based on gene expression data from bulk brain tissue samples. However, the development of methods to assay the transcriptomes of individual cells (scRNA-seq) now offers the opportunity to accelerate progress in the both the identification of cell-type specific perturbed functional pathways and in the re-construction of regulomes as a tool for predicting novel disease modifying drug targets. Thus scRNA-seq can de-convolve not just CNS cellular heterogeneity but also heterogeneity within a cell population to reveal novel disease associated cell subsets and their transcriptional features. As examples, scRNA-seq has identified transcriptionally distinct classes of microglia²³, dopaminergic neurons²⁴ and interneurons²⁵ enriched in genetic risk to AD, Parkinson's disease (PD) and schizophrenia respectively.

For schizophrenia, the approach taken was to assess the relative enrichment for genetic susceptibility among different cell-types and subtypes²⁵. This required first estimating a specificity index for each cell-type by assessing the proportion of the total expression of each gene in a cell-type compared to all other cell-types. For each cell-type, genes are then ranked according to their cell-type specific expression, with the hypothesis being that if a disease is

associated with a particular cell-type then a higher amount of heritability for the disease should be located in sets of genes specific to a particular cell-type. For GWAS data, enrichment of heritability among cell-type specific gene sets is assessed using either LDSC²⁶ or MAGMA²⁷, whereas for rare variants a gene-based collapsing analysis can be used (e.g., see references ¹⁵ and ²⁸). To control for type 1 error, it is standard to apply a pre-defined study-wide significance level based on the number of gene sets (i.e., cell-types or pathways) tested. Gene sets contributing to the enrichments can then be functionally annotated (GO, KEGG, etc.) to provide clues to the cell-type specific biological pathways impacted by genetic risk.

Many of the early scRNA-seq datasets of the mammalian brain were based on the use of fresh brain tissue and therefore precluded detailed analysis of the human brain. More recently however, the development of single nuclei RNA-seq (snRNA-seq) has allowed the benefits of scRNA-seq to be extended to archival frozen brain tissue samples^{29,30}, thus making available the many human brain tissue banks to single-cell RNA-seq analysis. The question of how comparable the nuclear transcriptome is to that of the whole cell has been addressed in several studies and provides a degree of confidence in the value of snRNA-seq compared to scRNA-seq; for example, multiple groups have shown that snRNA-seq on population of nuclei faithfully represents tissue-level RNA^{30,31}, the expression profile of single nuclei is well correlated with the average profile of single whole cells (Pearson $r=0.87$)³⁰, and 98% of transcripts are represented in whole nuclei versus whole cells³². On the other hand, genes related to mitochondrial respiration have been found to under-represented in nuclear samples²⁹ and transcripts destined for export to synaptic neuropil are reported to be relatively depleted in snRNA-seq²⁵. Nevertheless, the challenges of fully capturing the complete expression profile of structurally complex neurons is a long-standing concern, which has also been raised in regard to laser-capture microdissection, and currently no “perfect” method exists. Ongoing snRNA-seq studies will determine if the data quality and sparseness of snRNA-seq is a major barrier to the identification of cell-type specific subsets or pathways underpinning disease.

As well as using snRNA-seq to identify cell-types and pathways underpinning disease states, these same single cell RNA-seq datasets can be leveraged for drug target discovery based on the regulome (i.e., GRN) approach. By developing CRAFT we established proof-of-concept for regulome-based target discovery¹⁶. However, drug target predictions using CRAFT are limited by the accuracy and completeness of the “knowledge” on which the CRAFT regulome is built. For example, the direction of effect of a TF>gene interaction is often not specified in a database, and such gene regulatory interactions may be both tissue and disease-context specific depending on context specific chromatin modifications – information which may not have been fully captured by the published study reporting the regulatory interaction. Although knowledge of gene regulatory relationships is expected to improve with time, knowledge-based regulomes are unlikely to ever reach the context and cell-type specificity that empirical data from a diseased human brain can potentially achieve. Therefore, in addition to using knowledge-based regulomes, there is increasing interest in the use of cell-type and disease-context specific gene expression data for re-constructing gene regulatory networks (regulomes) for drug target discovery, with current approaches including the use of multivariate information theory³³ and data diffusion³⁴. However, building informative regulomes from single cell gene expression data faces several hurdles including potential hidden batch effects, non-normality of the data (particularly for lowly expressed genes), the need for data normalization (to bring cell-specific expression counts to a common scale) and the high levels of unexplained technical variability.

Future directions.

The importance of CRAFT is that it established the principle that targeting cell-type specific gene expression signatures is a means to novel drug target discovery. The next few years are likely to see an explosion in both statistical methodology and single-cell gene expression datasets for informing cell-type and disease-context specific pathways and regulomes for target

discovery in human disease. In addition to single-cell RNA-seq, a variety of other technologies are being rapidly developed that allow measurement of epigenetics at a single cell level including DNA accessibility (scNOME-seq, scATAC-seq, scDNase-seq), chromosome organisation (scHIC), DNA modifications (scBS-seq, scAba-seq, CLEVER-seq), histone modifications (scChIP-seq) and sc-Small RNA-seq³⁵ which will improve our ability to characterize cell-type specific disease pathways and the accuracy of the gene regulatory interaction inferences. Moreover, increasingly, we see that the repertoire of potential drug targets is extending beyond the traditional single protein targets. For example, RNA can have a persistent tertiary structure that allows the development of drug-like small molecules against RNA targets³⁶. Regulome technologies based on single-cell multi-omics including the relationships between regulatory small RNAs and gene expression at a cell-type and disease-context specific level thus open up a new area of drug target discovery for disease modification that extends beyond the classical single membrane receptors. In our view, the challenge of the next decade resides in harnessing the conceptual, technological and methodological advances in single-cell multi-omic technologies to epilepsy. For this we need to engage scientists beyond academia and re-invigorate Pharma's enthusiasm for epilepsy drug discovery. Such discovery efforts, particularly in the precompetitive space of drug target identification should be leveraged by generation of publicly available multi-omic data and public-private funding schemes. Close working relationships and true partnership between Pharma and academic research groups will be essential to generate the critical mass needed to discover truly innovative disease modification therapies for patients with epilepsy.

Figure Legend

Figure 1a: Cell-type specific gene expression profiles at the microscale underpin cellular and multi-cellular phenotypes at the macro-scale.

Figure 1b: Our research aims to understand the specific gene expression changes in specific cell-types that underpin disease states. It follows that if these gene expression changes underpin

a disease state, then reversion of the cellular gene expression from its disease state toward health would, by definition, be disease modifying. This is termed the signature reversion paradigm.

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Figure 1a.

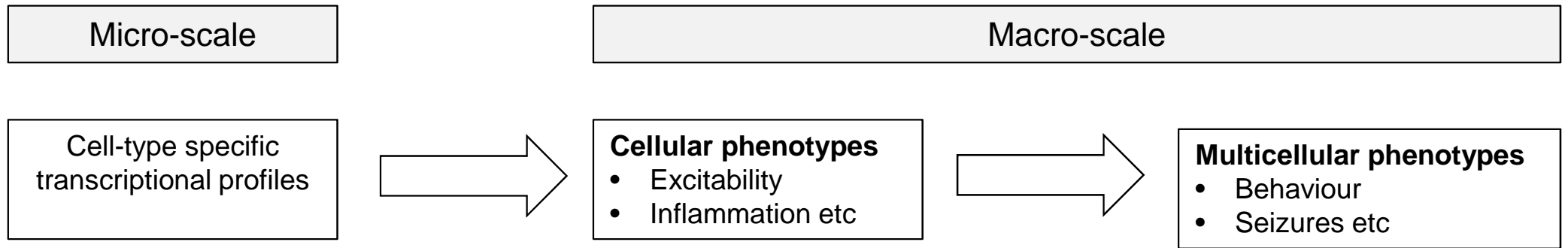
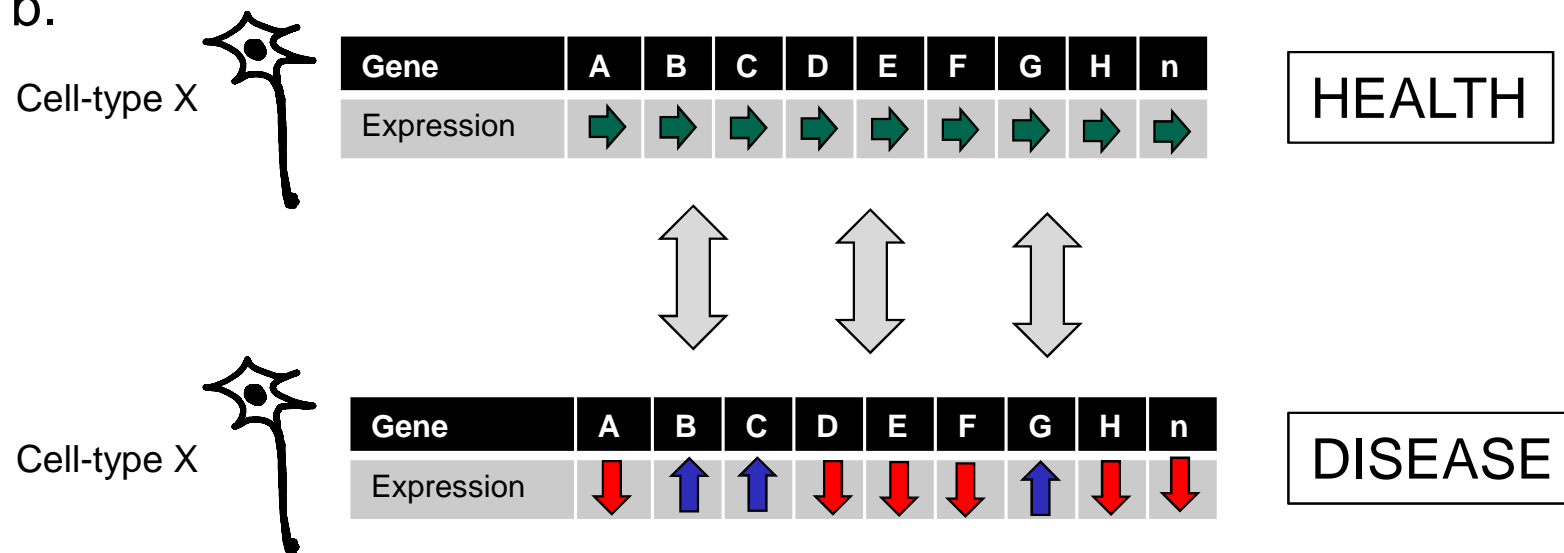


Figure 1b.



- Modern anti-epilepsy drug development has failed to deliver more effective anti-seizure drugs
- None of the current anti-seizure drugs are disease modifying or prevent the development of epilepsy
- The signature reversal paradigm and systems genetics provide a framework for the rapid discovery of disease modifying therapies
- Systems-level approaches using gene regulatory networks are likely to become increasingly important with the advent of high throughput single-cell RNA-sequencing (scRNA-seq) technologies