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Genetic Pharmacotherapy

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1. Introduction

In current drug development, *proof-of-concept*—determining whether a ligand engaging its target is likely to be therapeutic—requires specific ligands. This presents a catch-22, as the motivation to develop ligands requires proof-of-concept studies that cannot be conducted without ligands. A strategy we term *genetic pharmacotherapy*—a refinement of genetic blockade focused on druggable targets—obviates the catch-22 by enabling proof-of-concept studies *prior* to the development of specific ligands via genetic means in mouse models. In this strategy, which could help avert investment in molecular entities that will ultimately prove therapeutically inefficacious, a gene is conditionally down-regulated via a molecular *switch* in adult mice. Both the precise temporal control of the intervention and the consequent change in target protein function parallel the administration of drugs, with the additional advantage of perfect specificity. Moreover genetic pharmacotherapy overcomes the impediment of the blood-brain barrier, which makes developing ligands for psychiatric disorders particularly challenging. Here, we describe the transgenic technologies that form the basis for the strategy, discuss the advantages and limitations in juxtaposition with other gene expression modification approaches, contrast examples of prior implementation, address the feasibility for systematic use, and illustrate past and future opportunities. Although the molecular tools are widely available, genetic pharmacotherapy has only been implemented outside the central nervous system (CNS), despite its particular utility for CNS disorders. The systematic application of this strategy should foster the development of new, innovative molecular therapies.

2. The conceptual basis and requisite components of genetic pharmacotherapy

We define genetic pharmacotherapy as the use of a genetic intervention to achieve a pharmacological effect. Genetic pharmacotherapy has two requirements. First, genetic blockade must be universal — reaching all cells in the body, including the brain — to simulate organism-wide drug distribution. Second, induction of gene-modulation must be temporally controllable, as opposed to originating during embryogenesis, so that target modulation occurs as it would with drug administration. While traditional knockout

strategies have been used extensively to study the roles of proteins in a broad range of disorders, constitutive mutations are often lethal in early life precluding study of target gene function in adulthood (Lewandoski, 2001). Moreover, many non-lethal knockouts of genes of interest elicit paradoxical phenotypes — phenotypes that are opposite to the effects of pharmacologic blockade of the same target in adulthood — as a result of developmental compensations (Gingrich & Hen, 2000). The genetic strategy that satisfies these two requirements is a refinement of Cre-lox recombination, in which two individually silent mutations are introduced. One mutation drives a ligand-inducible effector enzyme that enables target-modulation, and the other mutation makes the target gene of interest susceptible to inactivation by the effector enzyme. This involves breeding a mouse carrying the inducible effector — CreERT — with an animal carrying the effector-sensitized *floxed* gene-of-interest. In the resulting progeny, inducing the CreERT produces irreversible target modulation paralleling the institution of pharmacotherapy.

2.1 Origins of the inducible-Cre strategy

In Cre-lox recombination, Cre recombinase — from P1 bacteriophage — recognizes two closely spaced 34-base pair loxP sequences, excises the intervening sequence, and recombines the flanking strands. When placed strategically by homologous recombination, the excision inactivates the so-called floxed gene (Nagy, 2000; Sternberg & Hamilton, 1981) (**Figure 1a**). To inactivate the target gene, a mouse that carries the Cre transgene is bred with a mouse carrying loxP sites flanking a portion of the gene of interest (the floxed gene); in the resulting F_1 progeny the floxed gene is inactivated in all cells where Cre is expressed (**Figure 1b**).

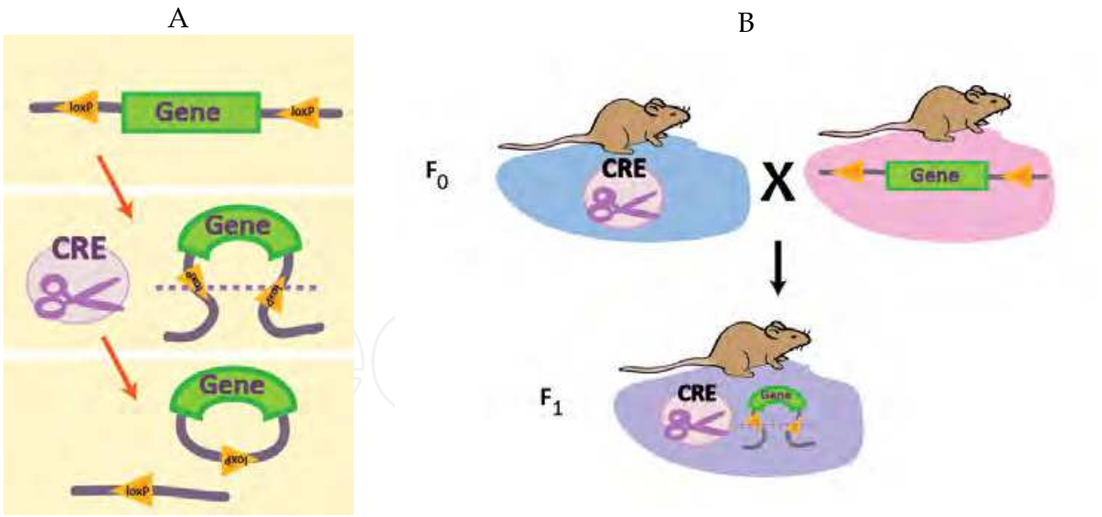

Fig. 1. Removal of targeted DNA sequences by Cre-lox recombination. A. Cre recombinase excises the portion of DNA between the loxP recognition sites (the floxed gene), recombining the flanking strands. B. To accomplish this in mice, a mouse carrying the Cre transgene is bred with a mouse carrying the floxed gene (F_0 generation). In the progeny (F_1), the floxed gene will be recombined in all Cre-expressing cells. If Cre is driven by a cell-specific promoter, recombination will be restricted to just those cells; if Cre is driven by a universally-expressed promoter, recombination will be universal.

In 1992, two groups (Lakso et al., 1992; Orban et al., 1992) reported the first use of Cre in mice to achieve tissue-specific expression. In 1994, Rajewsky and colleagues (Gu et al., 1994) employed Cre as a conditional gene-targeting tool to circumvent the pleiotropic embryonic lethality of the same null mutation. Tissue-specific Cre-mediated conditional gene inactivation has enabled investigators to address questions that would be otherwise intractable in global knockouts (Lewandoski, 2001). However, as mentioned, to best simulate treatment with a drug, which permeates the entire body, inactivation of the target gene should take place in every tissue type — and thus Cre expression should be driven by a universally-expressed promoter such as the hybrid chicken beta-actin promoter and cytomegalovirus enhancer (CAG) promoter. This promoter was dramatically shown to drive expression of enhanced green fluorescent protein (EGFP) in virtually every cell type to produce green mice (Okabe et al., 1997) (**Figure 2**).

Fig. 2. The transgenic CAG promoter drives expression universally. Five mouse pups are seen under normal light (left); under blue light excitation (right), two of the pups fluoresce green as a result of expression of the ubiquitous CAG promoter that drives EGFP expression in all cell types (in the mice, only red blood cells and hair are not green) (From: Okabe et al., 1997, with permission).

2.1 Modified, ligand-activated CreERT enables temporal control over target gene modulation

To achieve temporal control of Cre recombination, Chambon and colleagues (Feil et al., 1996; Metzger & Chambon, 2001; Metzger et al., 1995) created a ligand-dependent version of Cre, the chimeric protein CreERT, which mediates recombination only in the presence of the drug tamoxifen or its derivatives. In CreERT, Cre is fused to the mutated ligand-binding domain of the estrogen receptor, which recognizes tamoxifen and 4OH-tamoxifen, but not endogenous estrogen (**Figure 3a**). As a steroid receptor of the nuclear receptor family, the estrogen receptor in its inactive form is restricted to the cytoplasm via association with chaperone proteins (Giguère, 2003). As with estrogen activation of the native steroid receptor, tamoxifen releases CreERT from chaperone proteins, enabling the recombinase to diffuse into the nucleus to mediate site-specific recombination (**Figure 3b**). Consequently, target gene-inactivation is temporally controlled and tamoxifen-dependent, as CreERT

remains in the cytoplasm until tamoxifen-administration, and returns to the cytoplasm once tamoxifen is no longer in the system.

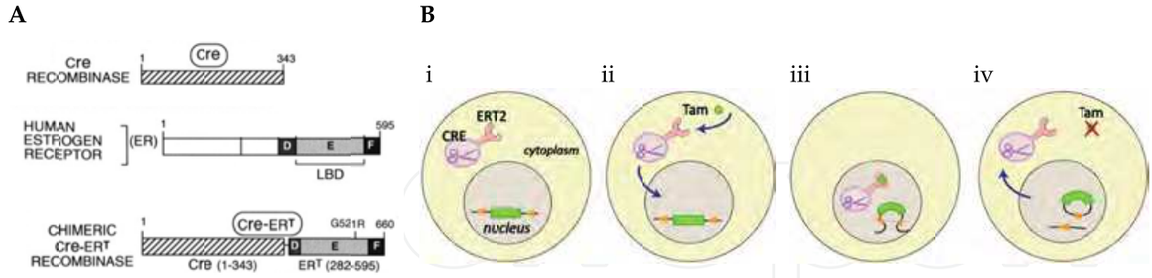

Fig. 3. Temporally controlled, ligand-dependent Cre-lox recombination. A. A mutated version (Gly521→Arg) of the estrogen receptor ligand binding domain (LBD) that recognizes tamoxifen but not estrogen was fused to the Cre protein, resulting in the chimeric protein CreERT (From Metzger & Chambon, 2001, with permission). B. In the absence of tamoxifen or derivatives, the location of CreERT is restricted to the cytoplasm by association with chaperone proteins (i). When engaged by its high-affinity, high-specificity ligand tamoxifen, CreERT is released from the chaperone proteins and diffuses into the nucleus (ii), only then allowing Cre access to recombine genomic DNA irreversibly (iii & iv). After tamoxifen clearance, CreERT will again become sequestered in the cytoplasm (iv).

An enhanced version, CreERT2, is now generally used as it has about a 4-fold greater induction efficiency over CreERT (Indra et al., 1999; Lewandoski, 2001 citations 112-115). For some transgene combinations, there may be some recombination in the absence of tamoxifen (**Figure 4**) (Hayashi, 2002), so evaluation of the magnitude of pre-tamoxifen recombination will therefore be a necessary control. Several ubiquitous inducible Cre lines are available (**Table 1**). An alternate strategy employs a modified progesterone receptor that is activated by RU486 (Kellendonk et al., 1999).

Cre line	Strain name	Stock Number
CagCreERT1	B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J	004682
RosaCreERT2	B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J	008463
UBC-CreERT2	B6.Cg-Tg(UBC-cre/ERT2)1Ejb/J	008085

Table 1. Ubiquitously expressed inducible Cre lines. Mice are readily available (jaxmice.jax.org).

Until recently, mice with floxed alleles were generated in individual laboratories and the range of commercially available lines of mice with floxed alleles was limited. Now, floxed mice are being made systematically through a multinational consortium, the Knockout Mouse Project (www.komp.org) (Skarnes et al., 2011). The goal of KOMP is to produce conditional alleles of all expressed mouse genes. So far, 9,000 floxed alleles are or will soon be available, and floxed alleles for the remainder of mouse genes should be accessible in the near term. This comprehensive resource provides the basis for systematic target evaluation using genetic pharmacotherapy.

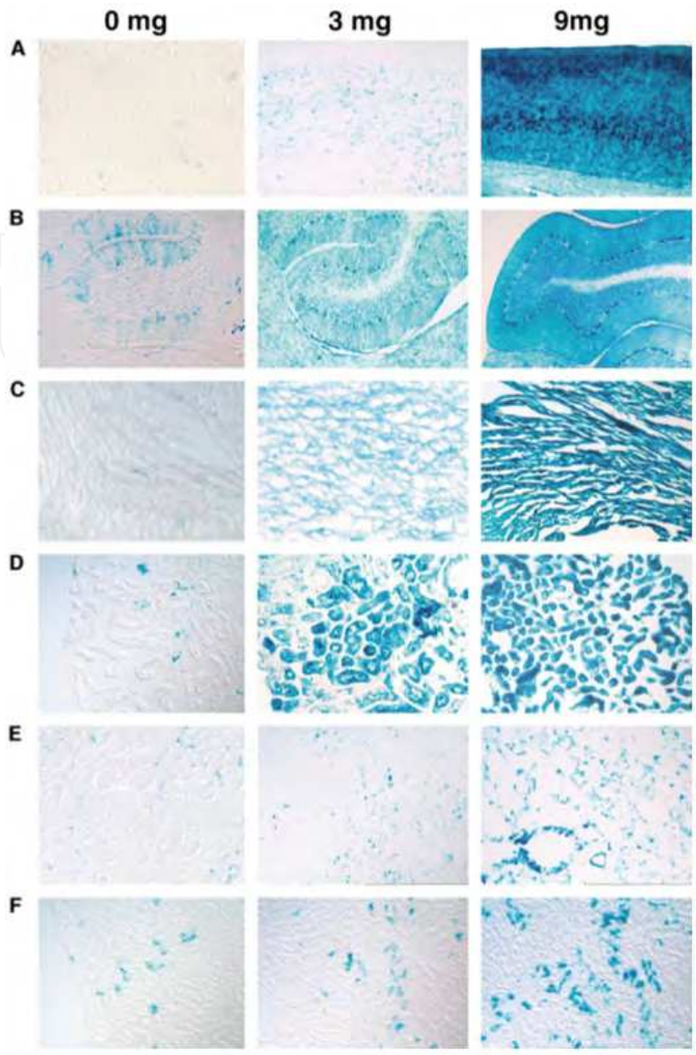

Fig. 4. CreERT2-mediated recombination prior to tamoxifen induction. In a small percentage of cells, recombination occurs in (A) cerebral cortex, (B) cerebellum, (C) heart, (D) kidney, (E) lung, and (F) liver in the absence of tamoxifen (left-most column), as shown by β-galactosidase expression, with the CagCreERT2 mouse line. After tamoxifen (doses shown on top) there is a massive induction of expression (From: Hayashi, 2002, with permission).

3. Comparability to target-specific drugs, with advantages and limitations

Using inducible Cre-lox recombination to inhibit target gene expression offers comparability to, as well as many advantages over, ligand-based inhibition for proof-of-concept studies. Comparable to pharmacologic treatment, genetic pharmacotherapy enables control over the degree of target modulation, analogous to adjustments in drug dose, to assess dose-response; and it also mimics the global, organism-wide action of drug intervention, which enables assessment of possible side effects due to pleiotropic target expression. The strategy's advantages include preclusion of off-target effects via perfect target-specificity, and access to targets in the CNS via permeation of the blood-brain-barrier, which stymies

the evaluation of many drug candidates for CNS disorders. There are limitations; these include relative difficulty in targeting splice variants (protein isoforms from the same gene), as the strategy works at the DNA level, and an inability to capture the subtleties of drug action at targets that exhibit functional heterogeneity, i.e. differential structural conformations, drug affinities, and function of a single gene product. We examine each of these points in further illustration below.

3.1 Relative change induced in target-protein function is commensurate to agonism and antagonism

Often, in order to evoke a response in the host cell system, a drug need occupy only a fraction of the total receptors available—a function of both the drug's affinity and the intrinsic ability of the drug-receptor interaction to induce cellular change. This causes the dose-response curve to shift to the left of the receptor occupancy curve, so that a drug dose that elicits maximal tissue response may cause only partial occupation of the available receptors (**Figure 5**).

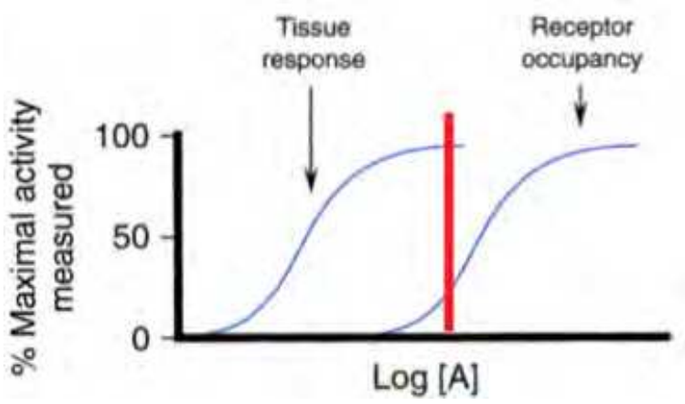

Fig. 5. Maximal tissue response at sub-maximal receptor occupancy. Based on efficacy, drug doses generally need to activate only a fraction of the total available receptors to induce a maximal tissue response. In this illustration, the drug dose that induces maximal tissue response (red line) activates only a fraction of the total available receptors. (Modified from: Ross & Kenakin, 2001, with permission from the McGraw-Hill Companies, Inc.)

For example, opiate agonists etorphine and sufentanil have significant analgesic activity at very low receptor occupancy — approximately 2% at the ED_{50} (Rosenbaum et al., 1984); and in another example, only 22% receptor occupancy is needed for half-maximal stimulation by PEG-TPOm, a mimetic peptide agonist in development for protection against chemotherapy-induced thrombocytopenia (loss of blood platelets) (Samtani et al., 2009). The antipsychotic dopamine D2 receptor antagonist olanzapine achieves optimal clinical efficacy at about 60% receptor occupancy (Mamo et al., 2007).

Similarly, genetic pharmacotherapy need not produce full target inhibition in order to elicit a response. Quantitative gradation in target protein expression (in analogy to a drug dose range) can be controlled by floxing one or both alleles of the gene of interest, and by tamoxifen dose (**Figure 6**) and frequency of administration; in adult mice, tamoxifen is generally administered once daily for 5 days to achieve full recombination. Varying tamoxifen dosing can then mimic the range of drug action.

While the Cre-lox strategy can also be used to simulate agonism, as discussed in later sections, we focus mainly on its application to simulate target antagonism, as the majority of drugs are inhibitors (Copeland et al., 2007; Li et al., 2007) and the accessibility of both ubiquitous CreERT2 mice and the floxed target of choice should now enable systematic *in vivo* target evaluation.

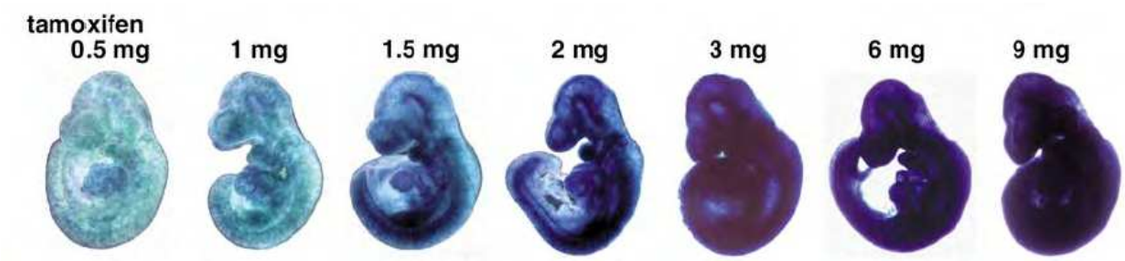

Fig. 6. Tamoxifen dose-dependent recombination in embryos with CreERT driven by a universally expressed promoter. Although Cre-mediated gene modulation is irreversible, "dosing" as a percentage of cells undergoing recombination can still be controlled. Whole-mount transgenic embryos were assayed 9.5 days post-coitum for activity of target gene, β-galactosidase, whose expression had been induced by tamoxifen at the indicated doses (per 40 kg mouse weight) 24 hours prior via intraperitoneal injection of the pregnant dam. (From: Hayashi, 2002, with permission).

To evaluate potential therapeutic efficacy of target modulation, biomarkers or surrogate endpoints specific to the disease model can be used as a response measure—for example, the lowering of blood glucose for genetic pharmacotherapy interventions in diabetes models, LDL cholesterol levels in dyslipidemia models, or PET imaging of ligand binding in CNS models.

3.2 Ubiquitously expressed effector simulates organism-wide pharmacologic actions

In this gene expression modulation system, the effector protein CreERT2 controls the expression of the target gene (for the candidate drug target). In order to achieve the closest simulation of a pharmacologic agent, which has organism-wide effects, it is paramount that a ubiquitously expressed locus such as Rosa26 (Soriano, 1999; Ventura et al., 2007) be used to drive the effector protein expression (**Table 1**). This way it is possible to assess the phenotypic consequences of pleiotropy occurring in targets — that is, the side effects arising from inhibiting a gene product that participates in multiple signaling or metabolic pathways or in different tissues (for further discussion of the relevance of pleiotropies to pharmacotherapy, see Hodgkin, 1998; Searls, 2003). While small interfering RNA (siRNA) has been used extensively for controlled gene inhibition in proof-of-concept studies, siRNA is not drug-like, as it must be delivered to the tissue of interest (Davidson & McCray, 2011).

3.3 Superiority over pharmacologics: preclusion of off-target effects via perfect target-specificity

Since Paul Ehrlich, drug design has aspired to the ideal of "magic bullet" drugs that seek out only "enemy targets" involved in pathology while leaving the body unharmed (Parascandola, 1981). Lack of target specificity not only severely limits the use of small molecules as therapeutics — for example, the clinical use of anti-Parkinsonian drugs pergolide and cabergoline has been greatly limited because their off-target effects cause

valvular heart disease (Keiser et al., 2009) — but also restricts their use as experimental tools in proof-of-concept evaluation (Peterson, 2008). Although promiscuous targeting and multi-receptor activity do produce therapeutic benefits when single-receptor action does not — for example, in the case of statins and psychotropic medications (Keiser et al., 2009; Peterson, 2008) — it is nonetheless preferable that well-defined functional outcomes be understood through well-delineated biochemical actions. Even a drug with high specificity that achieves a desirable outcome in an animal model or clinically still cannot be concluded to work only via the pathway assumed. Genetic pharmacotherapy, on the other hand, enables demonstration of precise, controlled causality in *in vivo* studies.

This is not to say that benefits of polypharmacy cannot be addressed with genetic pharmacotherapy; indeed, the synergy of inhibiting multiple targets simultaneously could be assessed in mice with multiple floxed alleles or by combining genetic pharmacotherapy with traditional pharmacotherapy. It may be impractical to translate findings from such an experiment into a ligand that accomplishes the same synergistic inhibition, but it would enable investigators to parse precisely which targets mediate the desired results, and could guide drug optimization efforts.

Along these lines, genetic pharmacotherapy raises the possibility of validating previously established targets. For example, every antipsychotic drug approved so far has dopamine D2 receptor-blocking activity based on the presumed mode of action of first-generation antipsychotics, which was an entirely serendipitous discovery. However, it likely that their therapeutic efficacy involves interaction with other targets; for instance, antipsychotics inhibit *KCNH2*, a recently described potassium channel, found to be overexpressed in the brains of patients with schizophrenia (Huffaker et al., 2009). The authors state:

> "Whereas D2 receptor affinity is thought to account for the therapeutic effects of antipsychotics, KCNH2 binding is responsible at least for side effects such as altered QT interval or even sudden cardiac failure. Given that KCNH2 controls neuronal excitability and firing patterns, could the therapeutic effects of antipsychotic drugs also be related to their affinities for the brain-specific isoforms of KCNH2? (Huffaker et al., 2009)"

With the present lack of specific KCNH2 ligands, genetic pharmacotherapy could enable further dissection of this possibility and resolve the conceptual justifications guiding the development of compounds with similarly intended actions, pharmacologic or therapeutic.

3.4 Specific advantages with regard to CNS targets

Genetic pharmacotherapy offers particular advantages for proof-of-concept studies for CNS disorders. Such studies face not only the obstacle of designing high-affinity, high-specificity ligands, as in evaluation of targets in other tissues, but also the blood-brain barrier — the superfine filter composed of endothelial cells lining brain capillaries and astrocytes — that either blocks or actively transports out more than 98% of candidate drugs (Miller, 2002). Genetic pharmacotherapy circumvents this obstacle because tamoxifen permeates the blood-brain barrier freely.

3.5 Limitations of achieving target inhibition via DNA modification

While drugs can distinguish between different protein isoforms arising from RNA splice variants (Thompson et al., 2011) that may have different anatomical and functional

specificities (Huffaker et al., 2009), genetic pharmacotherapy cannot make such a distinction easily, as target inhibition is mediated via changes at the DNA level. Similarly, a drug may interact differentially with receptors exhibiting functional heterogeneity — e.g. a receptor with distinct allosteric conformations and signaling complexes, varying by anatomical distribution (Mailman, 2007; Mailman & Murthy, 2010) — a subtlety less amenable to simulation with genetic pharmacotherapy but achievable in some cases with pharmacology. The D2 dopamine receptor provides a classic example: agonist stimulation of presynaptic D2 dopamine autoreceptors diminishes dopamine synthesis and release, which may achieve a dopamine antagonist-like effect post-synaptically via decreased dopamine neurotransmission -- whereas preferential agonism of post-synaptic D2 dopamine receptors would achieve the opposite effect. Selective targeting of distinct cell-type D2 receptors cannot be achieved with a universal CreERT line, but the D2 receptor partial agonist aripiprazole appears to exhibit such functional selectivity. Nevertheless, a judiciously chosen tissue-specific inducible CreERT2 strategy may allow such issues to be addressed with better precision than with pharmacology. For instance, presynaptic dopamine D2 receptors have been selectively targeted using a DAT-Cre driver (Bello et al., 2011), and DAT-CreERT2 mice have been reported (Engblom et al., 2008), so that such changes could be induced in adulthood to model an autoreceptor selective dopamine D2 receptor antagonist.

4. Evolution of genetic pharmacotherapy

Genetic pharmacotherapy builds on molecular developments of the last two decades. Below we describe seminal applications of aspects of the genetic pharmacotherapy strategy, and, in certain instances, the molecular entities with actions mirroring the corresponding genetic intervention. Where we wish to build on these foundations is in illustrating the feasibility and advantages of applying this concept — specifically, by employing inducible Cre-lox technology — to evaluate new potential therapeutic drug targets systematically, particularly in the CNS, a possibility that has not yet been articulated until now.

4.1 Genetic blockade *in lieu* or absence of pharmacologic blockade

The potential advantages of using genetic blockade in place of a pharmacologic blockade for CNS studies were described in 1996 in studies reporting the first transgenic expression of Cre in the mouse nervous system (Tsien et al., 1996):

> "Studies of … mechanisms underlying … vertebrate animal [behavior] have traditionally been carried out using pharmacological blockades…. gene knockouts provide an alternative means. While the two methods are complementary, genetic deletion is generally superior to pharmacological blockade with respect to molecular and anatomical specificities and animal-to-animal reproducibility. For instance, while many antagonists cannot distinguish receptor isoforms, genetic blockade can make that distinction. Likewise… a genetic blockade can be highly confined and reproducible."

The juxtaposition of genetic and pharmacologic blockade, in this instance, pertains to their relative merits as experimental tools for addressing questions regarding memory formation as opposed to new therapeutic targets. In a related, concurrent study, the tetracycline transactivator system (described below) was used for regional and temporal control over

calcium-calmodulin-dependent kinase II (CamKII) expression to demonstrate the requirement of CamKII for both implicit and explicit memory formation (Mayford et al., 1996).

4.1.1 Tetracycline-regulatable gene expression

The tetracycline transactivator system (Gossen & Bujard, 1992) offers another ligand-controlled gene expression system, in which the targeted gene is turned off or on by the administration of tetracycline or (more commonly) doxycycline (**Figure 7a**). In this system, the *E. coli*-derived tetracycline-controlled transactivator (tTA) drives target-gene transcription by binding to a modified *tet* operator (*tet*O) sequence, and this activity can be diminished and switched off depending on varying concentrations of doxycycline. This strategy is adapted from the *E. coli* tetracycline-resistance operon, in which transcription of tetracycline resistance-mediating genes is negatively regulated by the tetracycline repressor (*tet*R). The presence of tetracycline causes the dissociation of *tet*R from the promoter region of the operon and enables transcription of resistance genes. In contrast, as just mentioned, the modified transactivator tTA, based on *tet*R, *stimulates* (as opposed to repressing) transcription when bound to minimal promoters fused to *tet*O sequences, and the presence of low concentrations of tetracycline (<100 nM) or doxycycline prevents the binding of tTA to the tetO sequences and thereby halts transcription. In the modified tet-on version of this system (Kistner et al., 1996), doxycycline turns on target gene expression via a reverse tTA (rtTA). In this case, rtTA will bind tetO to activate transcription only in the presence of doxycycline (**Figure 7b**).

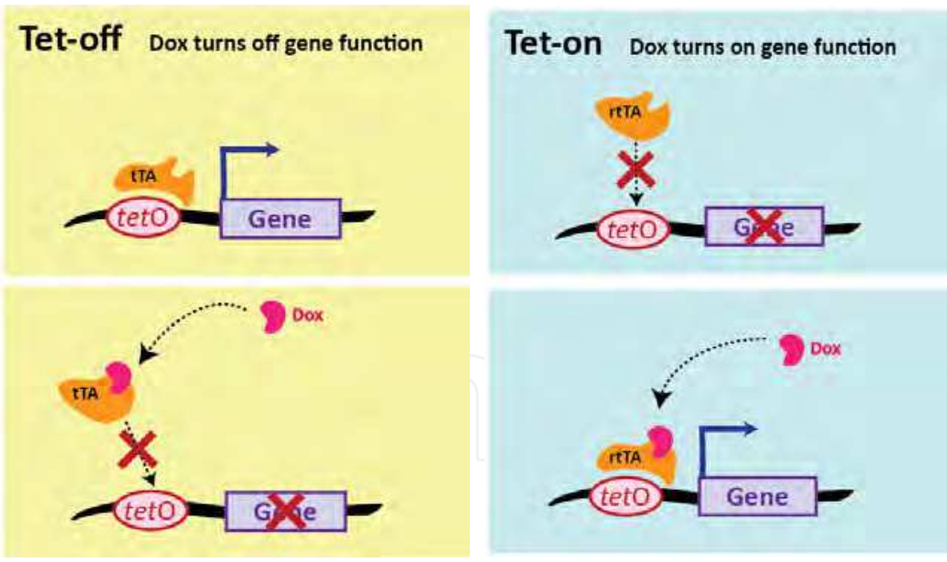

Fig. 7. Tetracycline transactivator (tTA)-controlled gene expression. (a) In tet-off gene regulation, the effector protein tTA binds to the *tet*O promoter, which activates transcription of the target gene. Upon the administration of doxycycline, tTA changes conformation and ceases to activate the target gene's transcription. (b) In the tet-on system, a modified *reverse* tTA (rtTA) binds *tet*O only in the presence of doxycycline.

While the tet-regulatable strategy is appealing for the reversibility of target modulation (with the addition or removal of doxycycline administration) in inhibition studies, it is

impossible to produce a silent mutation in the target gene, such as the introduction of loxP sites. In order to control the gene of interest, the *tet*O promoter must be substituted for the native promoter on one allele of the target gene, and the native promoter on the other allele, in turn, used to drive tTA. A study employed this strategy (**Figure 8**) to examine the role of the dopamine transporter (DAT) in scaling learning (Cagniard et al., 2006).

In this tandem design, tissue specificity of target gene expression is maintained, but pre-doxycycline expression levels vary considerably; this is problematic for genetic pharmacotherapy because target gene function should be at basal levels prior to induction. In the strategy illustrated in **Figure 8**, the mutant mice expressed modestly reduced levels of DAT, and it took several weeks of doxycycline administration to achieve significant suppression of DAT expression (Cagniard et al., 2006). In another study examining the role of potassium SK3 channels, SK3 expression was several fold higher than control, and was suppressed with doxycycline (Bond et al., 2000).

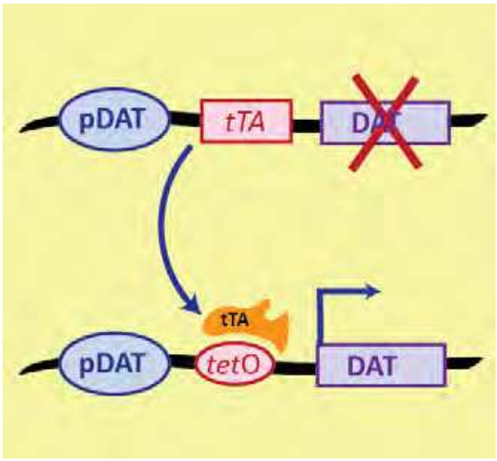

Fig. 8. Target inhibition with tet-off system. For doxycycline-induced knockdown of the targeted dopamine transporter (DAT) function, tTA is expressed under the control of the DAT promoter pDAT on one allele, abrogating DAT expression at that locus; endogenous DAT expression at the other allele is also disrupted by placement of the *tet*O promoter ahead of the gene—but in cells with endogenous pDAT promoter activity driving tTA expression, DAT will be expressed via the *tet*O promoter. Thus tissue specificity of target expression is maintained. Doxycycline then blocks tTA action and so inhibits all DAT expression.

Though less ideal for systematic target evaluation when compared to CreERT2, the tetracycline-transactivator system has nevertheless been particularly powerful in modeling a disease state and subsequently assaying the proof-of-concept of its reversibility via doxycycline administration. In a landmark paper, Yamamoto and colleagues (2000) created a mouse model of Huntington's disease (HD) by *tet*O-driven, striatum-restricted expression of a pathogenic version of the Huntingtin protein. By 4 weeks of age, the mice began to exhibit choreic movements and dystonia, and by 8 weeks showed striatal Huntingtin aggregates — both hallmarks of HD. HD is progressive, without a specific treatment or cure, and prior to this study was assumed to be inexorable in its course. However, abolishing the expression of mutant Huntingtin by doxycycline administration in symptomatic mice not only halted but also *reversed* the accumulation of protein aggregates and progressive motor

decline. Although developing a pharmacologic intervention for HD has yet to be achieved, this study demonstrated that blocking expression of pathogenic Huntingtin in symptomatic subjects reversed manifestations of the disease, and indeed could be viewed as having achieved *a cure*. This proof-of-concept clearly motivates a search for drugs that would reverse or prevent Huntingtin protein aggregate formation.

Kellendonk and colleagues (2006) used the tetracycline-transactivator system to address the possibility of reversing schizophrenia-like abnormalities induced by dopamine D2 receptor overexpression (D2OE) in the striatum. Imaging studies have shown that dopamine transmission is increased in patients with schizophrenia, involving both increased dopamine release and increased dopamine D2 receptor binding (Guillin et al., 2007). The D2OE mice, in which tTA drove striatally restricted expression of an extra human D2R allele, exhibited elevated receptor binding capacity — 15% higher than control littermates. The pivotal finding was that dopamine dysfunction — previously thought only to account for the positive symptoms, such as hallucinations and delusions — could be causally linked to cognitive deficits (Simpson et al., 2010). D2OE mice showed altered dopamine transmission in the prefrontal cortex as well as selective cognitive impairment in working memory tasks, a prefrontal cortex-dependent process, without more general cognitive deficits — similar to cognitive impairments in patients with schizophrenia. Administering doxycycline to reverse the D2OE did not reverse the working memory impairment, suggesting that the cognitive deficits in these mice arose not from continued D2OE but as a consequence of D2OE during development. Whether D2OE occurs prior to the onset of schizophrenia in patients is not known; however, dopamine release is increased (Howes et al., 2008), so there is clear evidence for increased dopamine transmission prior to the onset of schizophrenic symptoms. The earlier consequences of increased dopamine transmission in the D2OE mice are consistent with the well-recognized inability of dopamine D2 receptor antagonists to ameliorate cognitive impairments. It must also be noted that while genetic blockade of transgene expression accomplished down-regulation of D2 receptor overexpression, it did not mimic actual D2 receptor antagonist pharmacotherapy fully, as doxycycline inhibited only the transgenic D2 receptors while D2 antagonists would block both the transgenic as well as native D2 receptors.

In these studies, the tetracycline-transactivator system was used to model a disease, and subsequently doxycycline administration was used to turn off pathogenic protein expression to establish causality between protein and disease. In genetic pharmacotherapy studies, introduction of an extra tet-regulatable allele could be used to test the effects of increasing target expression, mimicking the actions of a target agonist. One benefit of this strategy, as mentioned, is reversibility of the target modulation; however, the transgenic mice would need to be engineered on a per-target basis, as there is no repository analogous to KOMP for tet-regulatable alleles. Using the tet-off system in inhibition studies, as in the DAT study, disadvantageously requires engineering two transgenic mice (for both the *tet*O and tTA alleles).

4.2 Genetic blockade techniques beyond inducible Cre and tTA

Genetic blockade as a proof-of-concept tool has also been used in a number of oncogenic signaling pathway studies. Although the blockade in the following two examples was accomplished via methods difficult to translate to other druggable targets, the studies successfully established a therapeutic proof-of-concept via genetic means in the absence of a high-specificity ligand against the endogenous target. In one study, a silent mutation engineered into an oncogenic kinase allowed for its selective inhibition, whereas kinase

inhibitor non-specificity had previously deterred such an investigation (Fan et al., 2002). In a second study, tumorigenic expression of an endocrine receptor was suppressed by a truncated version of the same receptor (delivered via an adenovirus) that acted as a dominant-negative inhibitor (Min et al., 2003). In both cases, the subsequently developed pharmacologic inhibitors produced the same effect as the genetic blockade, corroborating the parallel between genetic and pharmacologic target blockade.

4.2.1 Engineering a kinase for selective inhibition to determine its role in oncogenic signaling

To overcome the lack of specific kinase inhibitors, Fan and colleagues engineered a silent mutation into a target kinase gene that allowed selective inhibition while retaining kinase activity prior to administration of the mutant kinase-specific antagonist, NaPP1 (Fan et al., 2002). Mice subcutaneously injected with cancer cells containing endogenous or NaPP1-sensitized versions of the epithelial growth factor receptor (EGFP) oncogene v-erbB grew tumors of similar size and with similar latencies — but the inhibitor NaPP1 suppressed growth only in tumors with the sensitized allele (**Figure 9**).

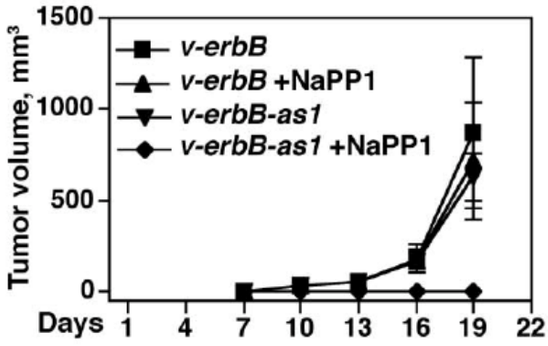

Fig. 9. Chemical genetic blockade in a proof-of-concept study. Assay of tumor growth inhibition in nude mice injected with *v-erbB-* or *v-erbB-as1*-transformed cells. NaPP1 blocks tumor growth only in the mice with the mutagenized *v-erbB-as1* allele that renders the kinase susceptible to selective inhibition by NaPP1 (From: Fan et al., 2002, with permission).

Fan and colleagues (2002) concluded that selective inhibitors of EGFR may effectively arrest cancer cell proliferation at a favorable therapeutic index, as basal signaling in normal cells is unlikely to be affected. Although inhibitors selective for the ErbB EGFR were not available at the time of this chemical-genetic blockade study, genetically engineering inhibitor sensitivity demonstrated the target's proof-of-concept. This incentivized further efforts to develop ligands specific for EGFR subtypes such as ErbB. Indeed, initial marketing of Gefitinib, an EGFR tyrosine kinase inhibitor, was approved in 2003 for patients with non-small cell lung cancer, and Erlotinib, another EGFR tyrosine kinase inhibitor, was approved for the same indication in 2004 and for pancreatic cancer in 2005 (National Cancer Institute Online Drug Information, Pao, 2005).

4.2.2 Virally delivered truncated IGF-I receptor as dominant-negative inhibitor of tumorigenesis

Like EGFR, the insulin-like growth factor I receptor (IGF-Ir) is a mitotic growth factor that stimulates cell growth and is implicated in tumorigenesis (Prager et al., 1994). In certain

tumors, IGF-Ir appears to be essential for both malignant transformation and maintenance of the malignant state (Baserga, 1995; Sell et al., 1993). Additionally, initial studies indicated potential tumor selectivity in targeting IGF-Ir for therapeutic applications: reduction of IGF-Ir function induced apoptosis in tumor cells but produced only growth arrest in untransformed cells; and IGF-Ir knockout mice are viable, indicating it is not indispensable for relatively normal development (Min et al., 2003). Prior to the availability of selective ligands, blocking receptor function was accomplished by introducing into tumor cells a recombinant, truncated version of the IGF-Ir with a deleted intracellular tyrosine kinase domain, enabling non-functional heterodimerization. Genetic blockade via dominant-negative receptor expression prevented the formation of sarcomas in nude mice (Prager et al., 1994) and was successful in treating lung, colorectal, and pancreatic xenograft models (Min et al., 2003). Since then, a number of small-molecule IGF-Ir inhibitors have been discovered, with anti-neoplastic action in a wide range of cancers (Carboni et al., 2009; Flanigan et al., 2010; García-Echeverría et al., 2004; He et al., 2010; Iwasa et al., 2009; Kurio et al., 2011), including several that have now moved into clinical trials (Carboni et al., 2009; Iwasa et al., 2009).

4.3 Genetic pharmacotherapy simulates ligand-mediated restoration of p53 anti-tumor activity

Ventura and colleagues did the study — outside the CNS — that most closely embodies our vision of genetic pharmacotherapy (Ventura et al., 2007). They restored function of the transcription factor p53, which has tumor suppressor activity, via a global inducible Cre strategy. p53 was shown in 1991 to be the most frequently inactivated protein in human cancer (Hollstein et al., 1991) so its pathway has been of longstanding interest, and has been the subject of molecular therapeutic development efforts (Vassilev, 2004). Pharmacological restoration of p53 protein function to achieve tumor regression was achieved indirectly by disrupting its interaction with its suppressor protein, MDM2. Indeed, a series of small molecules named Nutlins were discovered that are active in the mid-nanomolar range (Vassilev, 2004).

Ventura and colleagues sought to determine whether rescuing p53 function directly would induce apoptosis and tumor regression. They made p53-LSL mice in which p53 is knocked out due to the insertion of a floxed-Stop (lox-Stop-lox, "LSL") cassette — which stops transcription ahead of the p53 gene — but can be restored by Cre-mediated removal of the cassette. These mice were bred with global ROSA26-CreERT2 mice to produce p53 knockout mice in which tamoxifen could restore p53 function. After irradiating double mutant progeny shortly after birth to accelerate tumor formation, they then showed that tamoxifen treatment of the CreERT2-bearing p53-LSL mice resulted in regression of autochthonous lymphomas and sarcomas, without affecting normal tissues (**Figure 10**).

4.4 Contrasting inducible Cre-lox genetic pharmacotherapy with other modes of genetic blockade

For each of the three targets discussed above for cancer therapy — EGFR, IGF-Ir, and p53 — the drug effects mirror those of the genetic pharmacotherapy intervention, corroborating the technique's comparability as a means of testing a pharmacologic proof-of-concept. Although the Nutlin molecules increase p53 activity indirectly via MDM2 inhibition, and a genetic intervention more directly analogous to the corresponding drug might have targeted a floxed-MDM2 allele, the two studies nonetheless demonstrate the anti-tumor effects (proof-of-concept) of p53-up-regulation (**Figure 11**).

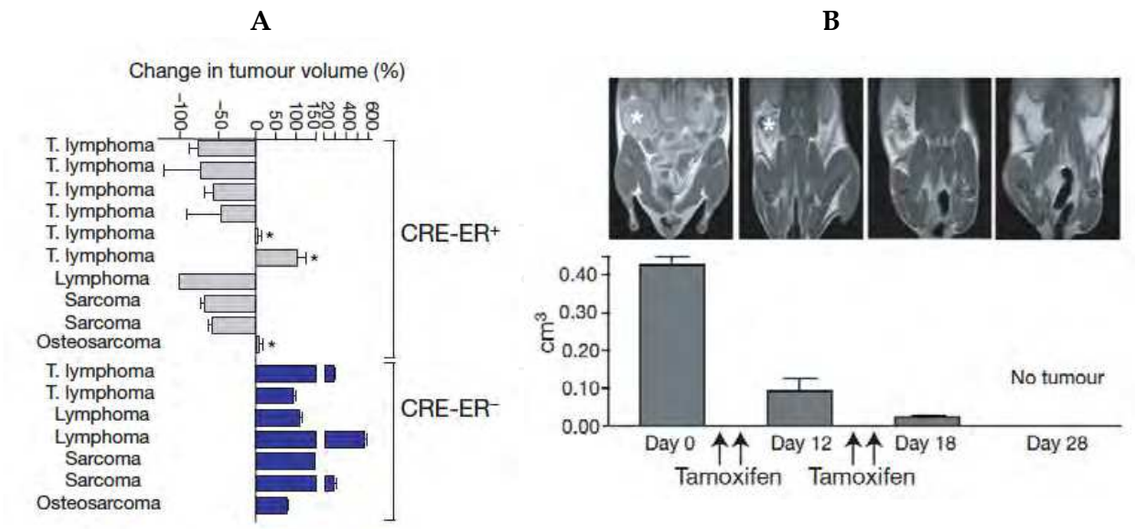

Fig. 10. Genetic pharmacotherapy mediates up-regulation of p53 expression and causes regression of autochthonous sarcomas and lymphomas. A. Tamoxifen administration caused tumor regression in p53-LSL mice expressing tamoxifen-inducible CreERT2, but not in CreERT2-negative p53-LSL mice. B. A series of MRI images (top) shows the regression of an abdominal lymphoma (indicated by the white asterisk) after the administration of tamoxifen. Below, the tumor volume at the time point of each corresponding MRI image. (From: Ventura et al., 2007, with permission)

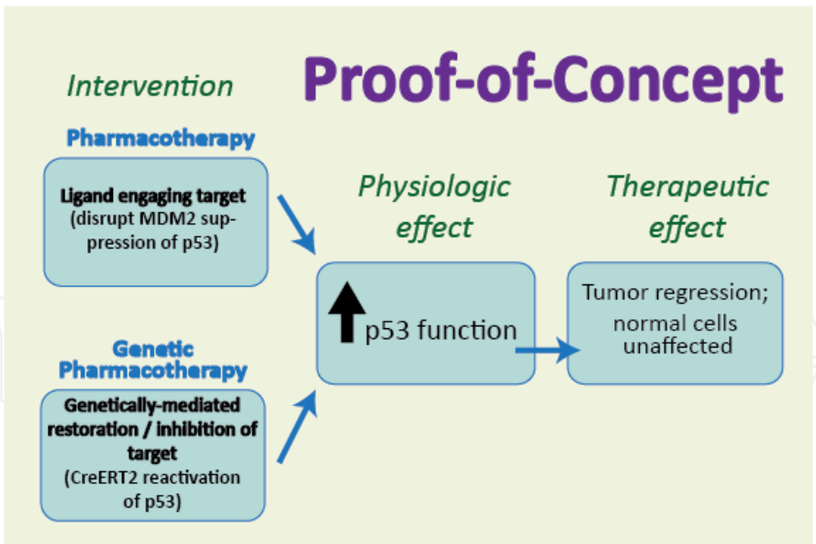

Fig. 11. Parallels in proof-of-concept studies for drug development between ligand-based pharmacotherapy and genetic pharmacotherapy, as exemplified by p53 up-regulation for cancer pharmacotherapy (Vassilev, 2004; Ventura et al., 2007). Both ligand inhibition of MDM2 and tamoxifen-inducible Cre-mediated rescue of homozygous null p53 alleles result in an up-regulation of p53 function that halts tumor cell growth and causes apoptosis, leading to tumor reduction in mice.

While genetically engineering a target for selective inhibition successfully demonstrated the proof-of-concept of EGFR knockdown as cancer therapy — as did expression of a dominant-negative protein to inactivate the endogenous target IGF-Ir — these techniques are highly system-specific and impractical for application to systematic target validation. Inducible Cre-lox recombination technology, on the other hand, can function as a *wild card* enabling ubiquitous modulation of the target of choice.

5. Past and current opportunities for genetic pharmacotherapy applications

A striking example of how genetic pharmacotherapy could have sped up target-validation and substantiated drug discovery efforts is illustrated by dopamine D3 receptor ligand development for the treatment of schizophrenia. Improved treatments that target novel mechanisms implicated in schizophrenia pathophysiology are urgently needed, as current therapies provide little if any alleviation for negative symptoms (anhedonia, social withdrawal) or cognitive symptoms (working memory, attention processing, cognitive flexibility) and furthermore bear a significant side effect profile that contributes to dramatically high patient non-adherence (>74%) (Lieberman et al., 2005). In the 1980s, studies on propsychotic drugs such as amphetamine showed that the drugs elicit maximal dopamine release in the ventral striatum (Di Chiara & Imperato, 1988), whereas antipsychotic extrapyramidal side effects (movement disorders) were thought to arise from interrupting dopamine release in nigrostriatal connections (Grace & Bunney, 1986). It thus became a longstanding pharmacologic goal to block ventral dopaminergic transmission selectively while leaving the dorsal nigrostriatal connections implicated in motor control unaffected in order to achieve a better side effect profile. So, when cloned in 1990, and seen to be distributed just in the ventral striatum (**Figure 12**), the dopamine D3 receptor immediately became a promising drug target for the treatment of schizophrenia (Sokoloff et al., 1990). The prediction was that D3 selective antagonists would prove therapeutic with reduced motor side effects.

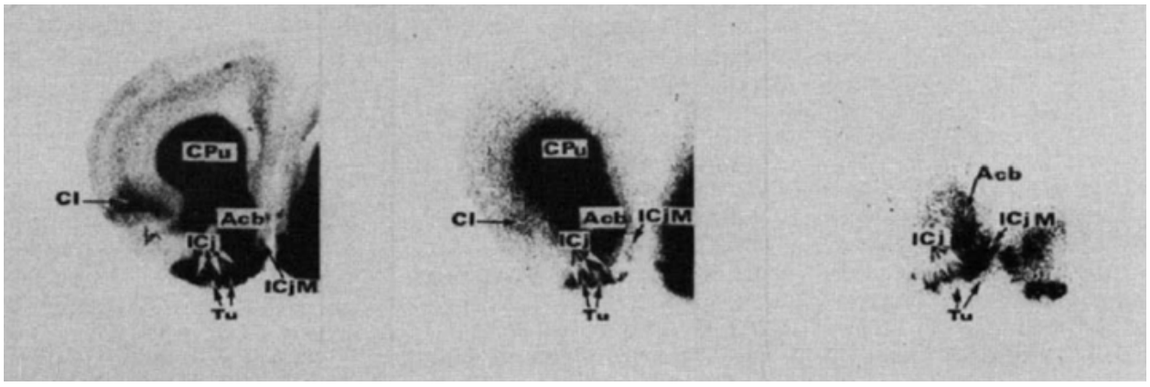

Fig. 12. Distribution of dopamine D2 and D3 receptors in coronal sections of the rat brain. Autoradiographic visualization of the dopamine D2/D3 receptor ligand [^{125}I] iodosulpride binding in a coronal section through the caudate-putamen (CPu) reveals the distribution of dopamine D2 receptors (left). While the distribution of dopamine D2 mRNA revealed by ^{32}P labeled probes (center) matches the iodosulpiride binding, D3 mRNA is localized preferentially in the ventral striatum, principally the nucleus accumbens (Acb), the islands of Calleja (ICj) and the olfactory tubercle (Tu). (From: Sokoloff et al., 1990, with permission).

Although a recently revealed amino acid difference between the highly homologous D2 and D3 receptors will provide new guidance for the design of new D3 receptor-selective ligands together with resolution of the crystal structure (Chien et al., 2010), sufficiently selective ligands are still not available (Lã Ber et al., 2011). Because of the catch-22 that therapeutic efficacy of drug targeting cannot be tested until selective ligands are available, and because the past two decades have yet to yield a sufficiently selective ligand, the proof-of-concept study to demonstrate the therapeutic potential of selective D3 inhibition has yet to be done. Inducing D3 knockdown via inducible-Cre technology in mice modeling schizophrenia endophenotypes could show whether selective dopamine D3 receptor antagonists would be worth pursuing.

5.1 Genetic pharmacotherapy to test proof-of-concept of glutaminase inhibition

While all antipsychotic drugs in clinical use currently target the dopamine system, dysfunction of glutamatergic synaptic transmission has been repeatedly implicated in the pathophysiology of schizophrenia (Javitt, 2010). Schizophrenia appears to involve NMDA-type glutamate receptor hypofunction, as phencyclidine (PCP), ketamine and other NMDA receptor antagonists induce schizophrenia-like symptoms in normals, exacerbate the condition of patients with schizophrenia, and mimic aspects of the disorder in animals (Javitt, 2007; Moghaddam, 2003). However, PCP and its congeners paradoxically induce glutamate release, and overactivate AMPA/kainate-type glutamate receptors (Moghaddam & Adams, 1998), suggesting that tempering release might prove therapeutic. Indeed, mGluR2/3 agonists attenuate both PCP-induced glutamate release and PCP-induced motor stimulation in rodents, and the mGluR2/3 agonist LY214002 has shown significant promise in early clinical trials (Patil et al., 2007). However, therapeutic benefit has yet to be demonstrated in subsequent clinical trials, suggesting that other ways of modulating glutamatergic transmission should be pursued. One promising target is glutaminase, the mitochondrial enzyme that is the rate-limiting step in the recycling of neurotransmitter glutamate from glutamine, that is thought to catalyze the production of the majority of neurotransmitter glutamate (Kvamme et al., 2001). However, there are no known CNS-active glutaminase inhibiters.

Recent clinical studies identify hyperactivity in hippocampal CA1 as being most associated with schizophrenia and predictive in prodromal patients of the transition to diagnosed schizophrenia (Schobel et al., 2009). This builds on a growing body of evidence pointing to hyperactivity in the hippocampus —presumably due to excessive glutamate transmission — as being a primary node in the pathophysiology of the disorder (Lodge & Grace, 2011); hippocampal hyperactivity drives dopamine neuron firing leading to excessive dopamine release and a positive feedback loop that may drive the transition to schizophrenia (Lisman et al., 2010). Interestingly, mice heterozygous for *Gls1*, the gene encoding glutaminase, exhibit a relative hypoactivity in hippocampal CA1 (Gaisler-Salomon et al., 2009)(**Figure 13**) that is the exact *inverse* of the findings of hyperactivity in hippocampal CA1 in prodromal and diagnosed patients with schizophrenia (Schobel et al., 2009).

Reduced glutaminase expression may therefore confer protection from the pathological processes that engender hippocampal hyperactivity in schizophrenia. Glutaminase inhibition should temper the upstream driving cause of disease symptomatology, rather than block downstream effects such as excessive dopamine release.

Consistent with the therapeutic potential of glutaminase inhibition, *Gls1* heterozygous mice showed diminished amphetamine-induced locomotor stimulation and striatal dopamine release (Gaisler-Salomon et al., 2009), two animal-model correlates of positive symptoms in

schizophrenia. They showed diminished sensitization to amphetamine (Gaisler-Salomon et al., unpublished) — sensitization is thought to be a key process involved in the progression of schizophrenia (Duncan et al., 1999), and in contrast to patients with schizophrenia, *Gls1* hets showed diminished ketamine-induced frontal cortex activation. The mice showed enhanced latent inhibition, a behavioral measure typically diminished in schizophrenia and enhanced by administration of antipsychotic drugs (Weiner & Arad, 2009). Other measures affected in schizophrenia, including pre-pulse inhibition and working memory, were unaffected. Thus, reduction in *Gls1* function engendered endophenotypic changes suggestive of potential resilience to schizophrenia. Moreover, the global glutaminase deficiency seen in *Gls1* hets had its strongest impact in the hippocampus, suggesting that systemic *Gls1* inhibitors should have a similar focal action. Lacking CNS-active glutaminase inhibiters, genetic pharmacotherapy offers a way to test the therapeutic potential of glutaminase inhibition (Gellman et al., 2011).

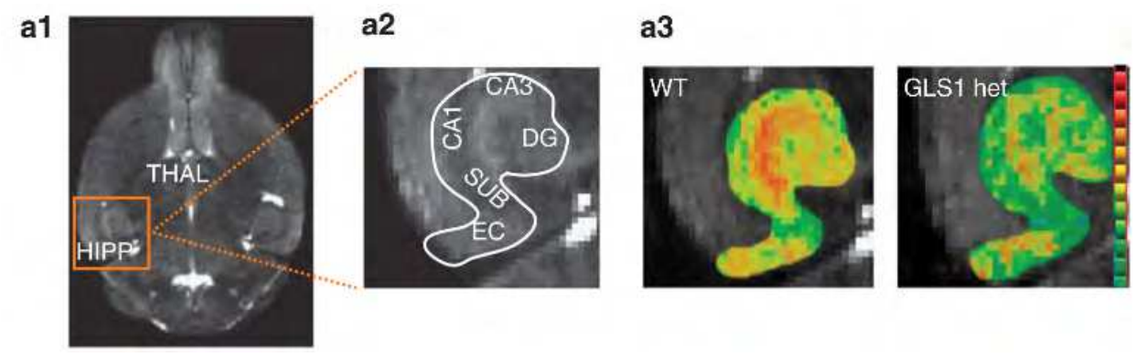

Fig. 13. Cerebral blood volume (CBV) imaging in the hippocampus of *GLs1* het mice. The hippocampal formation (a1, a2) is color-coded in an individual CBV map (a3) such that warmer colors reflect higher CBV values. Regional CBV is fairly uniform across hippocampal subregions in WT mice, but *Gls1* het mice show a selective reduction in the subiculum and CA1. (From: Gaisler-Salomon *et al.* (2009), with permission of the authors).

6. Feasibility and recommendations for systematic use

For disorders with well-defined molecular targets, genetic pharmacotherapy offers a direct way to test the therapeutic potential of inhibiting the target in advance of ligand development. The investigator administers tamoxifen to a disease-modeling mouse carrying both the global-inducible CreERT2 allele and the floxed version of the targeted allele. The resulting DNA recombination event and ensuing change in target expression levels simulates pharmacologic target-modulation. The Knockout Mouse Project (www.komp.org) (Skarnes et al., 2011) now makes available floxed alleles of genes of interest so that the requisite tools are readily or will shortly be available.

While the Cre-ERT2 approach is most directly applicable to antagonism studies, it can also be used for tests of agonism with transgenic mice bearing flox-Stop alleles so that tamoxifen administration would turn on an additional allele or alleles; however, such mice are not widely available. The tTA-tetO system is similarly useful for studies of agonism, which could be most straightforwardly accomplished in transgenic mice bearing the promoter for the target gene driving tTA in tandem with tetO driving the target gene. In contrast,

antagonism with tTA-tetO, as noted above, is inherently limited by the inability to design mutant mice in which the target gene is unperturbed. The utility of these approaches is summarized in **Table 2**.

In psychiatric disorders such as schizophrenia, molecular targets are less well defined. Diagnosis remains symptom-based due to lack of specific biomarkers or objective diagnostic tests. Mouse models capture many dimensions of the disorder but cannot be said to be models of the disorder (Arguello & Gogos, 2006; Nestler & Hyman, 2010). As such, for psychiatric disorders, the search for therapeutics is conducted in two stages. In the first stage, recognized behavioral dimensions are evaluated for desired effects of target modulation. For schizophrenia, these dimensions include reduction in correlates of positive symptoms (Carpenter & Koenig, 2008) and negative symptoms (O'Tuathaigh et al., 2010). In the second stage, a mouse model with construct validity — that is, a mouse carrying a mutation known to be implicated in the disease state, or that has been exposed to exogenous agents known to elicit the disorder — is subjected to the same genetic pharmacotherapy and phenomenological assays. Many genetic mouse models have been developed for schizophrenia based on genetic mutations that have been associated with the disorder, both constitutive (Carpenter & Koenig, 2008) and inducible (Pletnikov, 2009). Models based on exogenous agents include *in utero* challenges (Lodge & Grace, 2009), perinatal lesions (Tseng et al., 2009) and drug-induced states (Bickel & Javitt, 2009; Gonzalez-Maeso & Sealfon, 2009).

	CreERT2	tTA-tetO
Antagonism	Ubiquitous CreERT2 :: flox-Target	*(unperturbed control impossible)*
Agonism	Ubiquitous CreERT2:: flox-Stop-Target	pTarget-tTA :: tetO-Target

Table 2. Mouse genetic strategies for genetic pharmacotherapy. Genetic pharmacotherapy can mimic pharmacotherapy for both inhibition (target antagonism) or stimulation (target agonism). CreERT2 is most practical for inhibition studies as the requisite mouse lines (ubiquitous inducible Cre mice, and komp.com-accessible floxed-target mice) are widely available. tTA-tetO cannot be used for inhibition as it is impossible to make a doxycycline-regulatable line without perturbing control expression. Both CreERT2 and tTA-tetO strategies are suitable for stimulation studies, limited by the need to make the requisite mouse lines.

7. Conclusions

The genetic pharmacotherapy strategy enables testing the therapeutic proof-of-concept of target modulation in the absence of specific ligands. It allows circumvention of the proof-of-concept catch-22 in drug development, where the risk of investing in targets with limited validation often impedes the pursuit of specific ligands that would eventually be validated. It also minimizes the complementary problem of investment in selective ligands that would ultimately fail in clinical proof-of-concept studies (**Figure 14**). The recently announced conditional allele library resource (Skarnes et al., 2011) will facilitate efforts to test multiple targets with the inducible Cre strategy. Druggable targets that are likely to be disease modifying have now been identified (Hajduk et al., 2005; Knox et al., 2011; Zhu et al., 2010). The concept and tools of genetic pharmacotherapy have been well established for some time, but their potential for systematic application to proof-of-concept evaluation for drug

development efforts has not been fully appreciated. Genetic pharmacotherapy should prove to be a powerful orthogonal tool (Hardy & Peet, 2004) for drug development.

The lack of investment in truly innovative psychiatric drugs over the past decades underpins the vastly unmet need for better treatments, as the burden of psychiatric illness remains high and current treatments remain ineffective or nonexistent (Brundtland, 2001; Miller, 2010). Critics of psychiatric drug development argue that because the etiology of major mental illnesses remains so poorly understood, adequate treatments cannot be developed (Conn & Roth, 2008). However, applying genetic pharmacotherapy in increasingly sophisticated mouse models of psychiatric disorders promises to make the full mouse genome accessible to drug discovery and so expand greatly the accessibility of molecular targets for pharmacotherapies. We believe that this strategy will expedite the development of innovative new molecular therapies, particularly for CNS disorders.

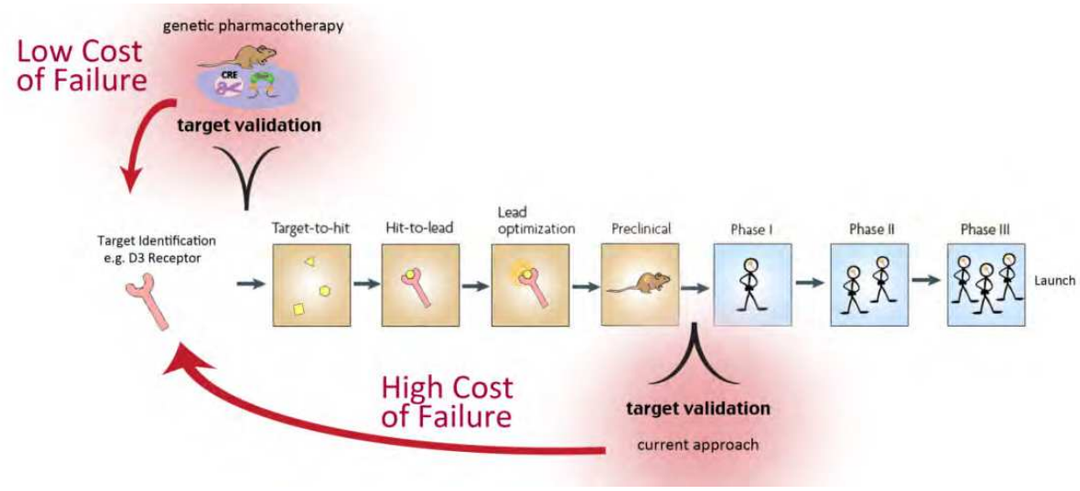

Fig. 14. Role for genetic pharmacotherapy in early stages of the drug development pipeline. Genetic pharmacotherapy (low cost of failure) bypasses ligand development (high cost of failure) in enabling target validation; ligand development (tan shading) can then be implemented for the most promising targets for which proof-of-concept studies demonstrate higher likelihood of success in clinical development (blue shading).

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Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

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