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ARTICLE

Inverse Drug Screens: A Rapid and Inexpensive Method for Implicating Molecular Targets

Dany S. Adams and Michael Levin*

Center for Regenerative and Developmental Biology, Forsyth Institute and Developmental Biology Department, Harvard School of Dental Medicine, Boston, Massachusetts

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Summary: Identification of gene products that function in some specific process of interest is a common goal in developmental biology. Although use of drug compounds to probe biological systems has a very long history in teratology and toxicology, systematic hierarchical drug screening has not been capitalized upon by the developmental biology community. This "chemical genetics" approach can greatly benefit the study of embryonic and regenerative systems, and we have formalized a strategy for using known pharmacological compounds to implicate specific molecular candidates in any chosen biological phenomenon. Taking advantage of a hierarchical structure that can be imposed on drug reagents in a number of fields such as ion transport, neurotransmitter function, metabolism, and cytoskeleton, any assay can be carried out as a binary search algorithm. This inverse drug screen methodology is much more efficient than exhaustive testing of large numbers of drugs, and reveals the identity of a manageable number of specific molecular candidates that can then be validated and targeted using more expensive and specific molecular reagents. Here, we describe the process of this loss-of-function screen and illustrate its use in uncovering novel bioelectrical and serotonergic mechanisms in embryonic patterning. This technique is an inexpensive and rapid complement to existing molecular screening strategies. Moreover, it is applicable to maternal proteins, and model species in which traditional genetic screens are not feasible, significantly extending the opportunities to identify key endogenous players in biological processes. genesis 44:530–540, 2006. **Published 2006 Wiley-Liss, Inc.**†

Key words: screen; pharmacology; blockers; inhibitors

INTRODUCTION

One common first step of basic and biomedical research is to discover gene products that function in some specific process (tumor progression, limb development, liver differentiation, etc.). In this discussion, the process of interest (POI) is defined generally as any biological event or system that one wishes to understand at a molecular level by identifying the proteins involved. Once these targets are located, experimental or clinical applications can be developed that target that specific protein.

The identification of novel targets for therapeutics is currently done by performing forward genetic screens (Amsterdam *et al*., 1999; Hamilton *et al*., 2005; Hansen *et al*., 2005; Neuhauss *et al*., 1999; Reddien *et al*., 2005). However, applying this process to the discovery of biomedically relevant targets is currently hampered by the following problems: (a) many model systems good for physiological or biomedical research, such as *Xenopus laevis* and chick, are inaccessible to forward genetics; (b) genetic knock-out may result in an early-lethal phenotype (for any target that is utilized in early development as well as later physiology), thus masking interesting phenotypes and making it difficult to understand the later roles of many gene products; and (c) this technology is extremely expensive and time-consuming. For example, to find out which ion transporter gene(s) may be involved in specification of kidney fate for embryonic cells, many thousands of possible ion channels/pumps would have to be knocked out, (an essential first step toward biomedical applications targeting the gene product). Any channel that was also utilized prior to kidney specification would not be located, since the loss of function animal would be unlikely to develop normally to the desired stage. The expense involved also serves as an energy barrier for testing novel hypotheses: it is

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*Correspondence to: Michael Levin, Center for Regenerative and Developmental Biology, Forsyth Institute and Developmental Biology Department, Harvard School of Dental Medicine, 140 The Fenway, Boston, MA 02115.

E-mail: mlevin@forsyth.org

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unlikely that one would embark on an expensive search for ion transporters involved in the POI for example, unless there was already strong evidence to support such a possibility. In contrast, new areas of biology would be uncovered if a method existed to easily test the involvement of a broad class of proteins in an important POI.

We have formalized a powerful strategy for using known pharmacological compounds to rapidly and inexpensively implicate specific candidates for roles in any process (Adams and Levin, 2005). Of course, the use of individual drugs to probe development has a long history; in particular, workers in toxicology, neuropharmacology, and teratology are comfortable using drug compounds to perturb their systems. However, developmental biologists have not heretofore benefited from the many advantages that hierarchical drug screens possess. Work is often limited to either highly time-consuming and inefficient exhaustive testing of large numbers of drugs (so-called "Sigma screens"), or use of single compounds to confirm roles of a target that is already known to be involved. In contrast, a systematic approach can quickly suggest likely molecular candidates and allow one to determine whether a particular pathway should be investigated further.

Our approach is to systematize the process and capitalize on the hierarchical specificity of well-characterized reagents to uncover molecular players in interesting developmental events. This results in a binary search, which is a highly efficient way to narrow candidates from broad categories and provides some significant advantages over traditional genetic screens. Using this technique, we recently found and characterized novel roles for four ion transporters crucial for embryonic left-right patterning, eye development, and spinal cord regeneration, as well as new functions of neurotransmitters during embryonic development, i.e. prior to the formation of neurons (Adams *et al*., 2006; Bunney *et al*., 2003; Chen and Levin, 2004; Cheng *et al*., 2002; Fukumoto *et al*., 2005a,b; Levin *et al*., 2002; Nogi *et al*., 2005; Qiu *et al*., 2005). The strategy is appropriate for vertebrate and invertebrate systems (Bunney *et al*., 2003; Hibino *et al*., 2006; Shimeld and Levin, 2006; Tomlinson *et al*., 2005), and has revealed a number of important aspects of physiological and morphogenetic regulation. To bring this powerful methodology to wider use in the study of developmental systems, we describe here the strategy, give several specific examples of its use, provide sample databases, and discuss its limitations and future potential.

RESULTS AND DISCUSSION

The General Strategy

This strategy is an application of "chemical genetics" (Crews and Splittgerber, 1999; Koh and Crews, 2002; Lunn and Stockwell, 2005; Mayer, 2003; Mitchison, 1994; Smukste and Stockwell, 2005; Stockwell, 2000a,b; Yeh and Crews, 2003) and relies on grouping candidate target proteins into hierarchical trees along functional and often phylogenetic lines. Broadly, it consists of several rounds of application of various drug blockers to a POI assay, beginning with compounds of broad targeting, and moving on to inhibitors of increasing specificity. The choice of drug at each step is guided by the previous results, and the tree structure allows the user to rapidly rule out large classes of targets as unlikely to be involved, and conversely, to implicate a small number of targets for further validation and analysis.

For example (see Fig. 1), a tree of ion translocators branches into channels, pumps, and pores. The channel node splits into K^+, Ca^{2+}, H^+, Cl^-, and Na^+ conductances, and the K^+ channel node has subnodes for each kind of potassium channel (voltage-gated, inward-rectifying, etc.). Each of those nodes has subnodes for the specific, known channels belonging to that category. This tree organization can be applied to many other pathways, including molecular motor and cytoskeleton-driven processes, metabolic networks, neurotransmission, and many others (Arita, 2003; Barnes and Sharp, 1999; Borodina and Nielsen, 2005; Fiehn and Weckwerth, 2003; Hartig, 1994; Mitchison, 1994; van Helden *et al*., 2002). A binary search of this tree can be performed by assaying the effect on the POI of compounds that block specific nodes in the tree; the next node in the search is chosen based on results obtained in the previous round: promising (effective) branches are followed, and ineffective branches are stricken from further consideration. It is thus possible to very rapidly focus efforts on proteins that are likely to be involved in the process under investigation.

For example, if the POI is pancreatic cell differentiation, for which a convenient assay exists, and a broad K^+ channel inhibitor (such as barium chloride) has no effect, no K^+ channels need to be considered as having a role in this process. In contrast, if the broad K^+ channel blocker interferes with this process, then the screen suggests testing a number of blockers of different types of K^+ channels to see which one(s) may be involved. The hierarchical testing process continues until a specific channel family (or even channel) is implicated; thus it leads quickly and directly to the most promising targets for study using molecular technology. By taking advantage of existing and ever-increasing pharmacological information, this technique achieves a significant decrease in the number of targets to be validated using expensive and time consuming molecular biological tools. Thus, it is an extremely efficient primary search engine for candidate proteins likely to be important in any phenomenon. As a bonus, very early in the process one learns whether the whole family is involved. Thus, the lack of specificity of reagents at the top of the tree is a benefit, and can be capitalized upon to quickly ask whether the involvement of K^+ flows is involved and worth pursuing further, lowering the investment required to explore a novel hypothesis.

Developmental biologists, neurobiologists, neuropharmacologists, cardiologists, and physiologists have long

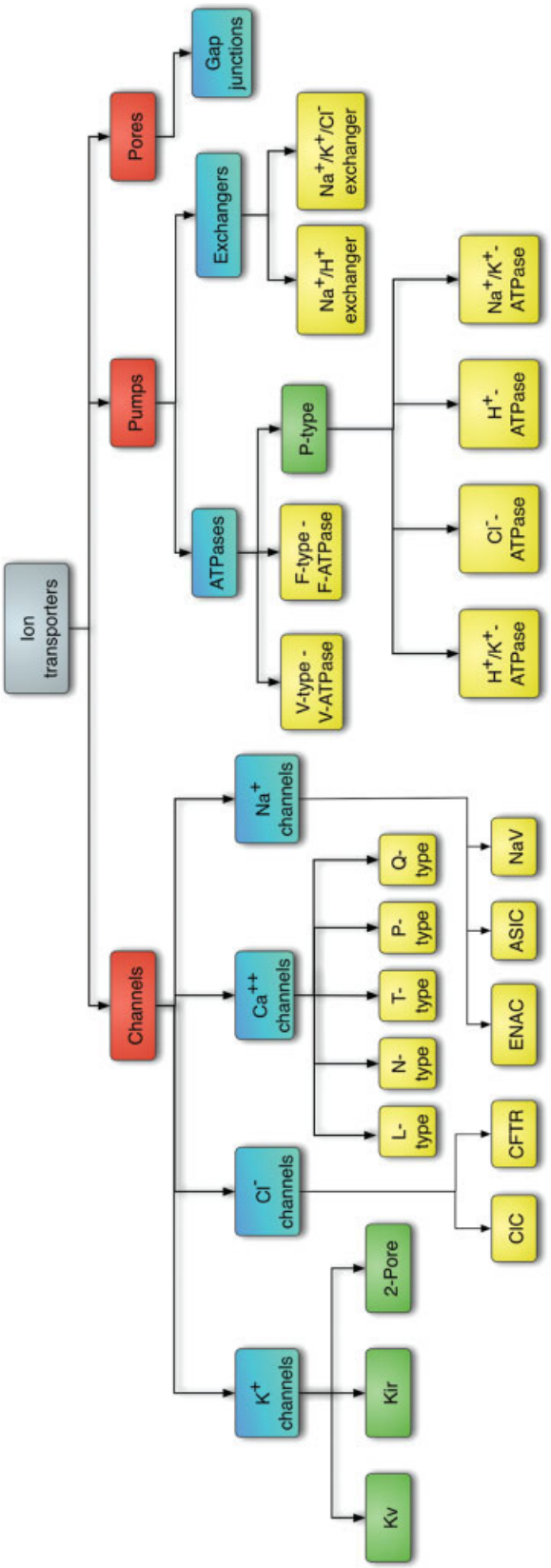

FIG. 1. This diagram illustrates arrangement of possible targets (in this case, a subset of ion transporters) in a hierarchical tree. The groups are arranged from the broadest classes at the top to more specific subfamilies at the bottom. The individual colors of each element serve only to demarcate different levels (hierarchical layers) of the tree.

been using specific poisons and toxins to probe biological processes (for an excellent compendium of information, see www.pharmabase.org). It is this immense body of pharmacological work that can be utilized to create a loss of function screen to rapidly, and usually inexpensively, determine whether a cellular mechanism is specifically involved in your favorite biological context (Mitchison, 1994; Tomlinson *et al.*, 2005). The crucial steps are (1) to develop an assay that makes the phenomenon of interest amenable to application of pharmacological reagents, and (2) to define an optimal ordering of drug blockers into a hierarchy from least to most specific, and use the results of each step to dictate the choice of the next step. Of course, pharmacological techniques cannot conclusively prove the involvement of any target; similarly, since one, sometimes, cannot be certain that a given compound has penetrated to the target cell or organelle, one cannot rule out a target family with 100% assurance. However, this process is a loss-of-function screen that is used to identify candidates for subsequent validation; unlike expensive and time-consuming molecular techniques such as morpholinos or RNAi, this approach can be an extremely effective way to narrow down from many thousands of possible candidates to a manageable number of high-priority targets that can then be characterized using molecular reagents.

Sample Previous Applications

As with all screening methodologies, the devil is in the details. Can the strategy actually work, given realistic constraints? Are there enough specific drugs available? Can they actually be arranged in hierarchies? Does overall toxicity or drug penetration pose severe problems? For some assays and pathways, these issues will preclude application. However, for many types of screens, these problems can be minimized to rapidly derive extremely useful information.

To illustrate this approach, we briefly discuss several applications in which it has been successfully used. The first large-scale screen was applied to determine whether ion flows were important for embryonic left–right asymmetry and if so, to learn what ion transporters were specifically involved. The POI was embryonic laterality, and the assay used was the development of *Xenopus* embryos exposed to blockers at early stages and scored for specific alterations in the position of three visceral organs 1 week later. Cleavage-stage embryos were soaked in drugs for 6 h after fertilization; a candidate translocator was implicated if treatment resulted in the randomization of the sidedness of the heart, gut, and gall bladder in the absence of general toxicity or nonspecific morphogenetic defects (such as dorso-anterior developmental alterations). Broad inhibitors were first used to determine that Ca^{+}, Cl^{-}, and Na^{+} ions are probably not crucial in the early steps of determination of laterality, while K^{+} and H^{+} fluxes are involved. More specific compounds targeting different members of various K^{+} and H^{+} channel and pump families ultimately implicated the

V-ATPase and H^+/K^+-ATPase pumps, and the KvLQT-1 and K_{atp} channels; all of these targets were then successfully validated using molecular dominant negative constructs. The identification of this small number of proteins led to the successful characterization of their native expression, uncovering new subcellular localization events in early embryogenesis (Adams *et al*., 2006; Chen and Levin, 2004; Levin, 2004; Levin *et al*., 2002); a number of laboratories subsequently extended this work (Duboc *et al*., 2005; Hibino *et al*., 2006; Kawakami *et al*., 2005; Raya *et al*., 2004; Shimeld and Levin, 2006).

This same strategy was then applied to ion transporters in a number of other embryonic patterning events, and to uncovering novel prenervous roles for the neurotransmitter serotonin (Fukumoto *et al*., 2005a,b; Levin *et al*., 2006). The initial left–right asymmetry screen took ~6 months; at the time, the whole strategy had to be worked out, caveats and troubleshooting steps identified, and the drug and corresponding target trees had to be created from the literature. Once all of this was in place, subsequent screens for neurotransmitter roles in embryonic patterning and ion transporters in spinal cord/muscle regeneration took 4 and 2 months, respectively. All of the necessary algorithms and data for those two fields have now been assembled, allowing very rapid progress for laboratories using the existing databases in their chosen POIs. Moreover, the binary search nature of this algorithm ensures a logarithmic decrease in the number of drugs that ultimately need to be tested. While the exact number is impossible to predict in advance (every combination of pathway and POI will have its own traversal through the tree), in practice one ends up using very few of the drugs in the complete tree's list. In each of the screens described earlier, no more than ~20–30 compounds needed to be tested before implicating a short list of targets that were ultimately validated.

It is also useful to estimate the cost of this approach, to compare with other screening technologies. We estimate that it cost our group less than $1,000 to assemble a large library of compounds that we have not only used in all of the screens in our laboratory but also continuously share with collaborators performing different screens. With the exception of some exotic venoms useful for probing Ca^{2+} signaling, the compounds are relatively inexpensive and once purchased, are available in large enough quantities to enable many screens for years to come.

Specific Methodology

One first designs an assay relevant to the POI in which drug effects can be readily investigated. Important components include estimating the accessibility of the relevant cells to reagents, deciding how many reagents can be tested in parallel (the degree of throughput of the assay), and designing specific criteria for recognizing interesting loss-of-function effects vs. potential toxicity. The next step is to obtain a drug tree (if one exists for the pathway to be tested) or construct one if necessary. One then begins at the top of the tree, with drugs or other reagents of low specificity, that target for example, large families of transporters, or all processes involving a particular ion (Step 1); apply the drugs to the preparation (making sure that *N* is large enough to enable statistical comparison with controls). If toxicity or nonspecific effects result, the dose must be reduced, or a different reagent (with the same targeting) chosen from the database. Reagents affecting targets on the same horizontal level of the tree can be tested in parallel since their outcomes will be independent.

For any reagent tested, if no effect is seen on the phenotype being assayed, the whole subfamily (all of the nodes below that drug's node in the tree) can be crossed off the list and need not be considered further (although it is usually good to confirm the lack of involvement using a different drug of similar targeting, to reduce the number of false negatives). In contrast, if an interesting phenotype results from the treatment, one goes on to the next round of the screen, using more specific drugs (Step 2) that can distinguish among members of the broad family implicated in Step 1. This process continues as long as increasingly specific drugs exist (Steps 3, 4, etc); how long that is depends on which families happen to be involved in your phenomenon but usually allows at least 2 or 3 rounds of the screen. It is often possible to rule out large categories of candidates, and thus narrow down to a reasonably small list of candidate proteins that can then be pursued using molecular techniques (see below). The point of the screen is to create a list of candidate proteins that are quite likely to play a necessary role in the POI. The hierarchical structure allows one to improve either the precision or the accuracy of your list; at each step of the screen, a negative result improves your precision (helps you narrow down the list) while a positive result improves your accuracy (helps confirm the contribution of a particular candidate). Information needed to carry out such a screen for serotonin signaling is given in Supplemental Table 1.

Two Simple Examples: Are Chloride Transport or Ca^{2+} Involved in My POI?

Figure 2a shows one scheme that could reveal a requirement for activity of a particular type of calcium channel (CaC). Step 1: Calcicludine blocks all CaCs; thus, if it has no effect on the POI, CaCs are not involved and one can quit. Step 2: ω-conotoxin MVIIC blocks P/Q and N-type CaCs; thus, if it has no effect, those CaCs can be removed from the list. If the POI is affected by ω-conotoxin MVIIC, P/Q- and/or N-type CaC may play a role; note that one has not excluded T-, R-, or L- type CaCs at this point, since more than one subfamily may be involved. Step 3 is either ω-Agatoxin IIIA (if ω-conotoxin MVIIC had no effect) or ω-Agatoxin IIIA and ω-conotoxin GVIA (if ω-conotoxin MVIIC did have an effect). Again, negative results allow one to cross channels off this list,

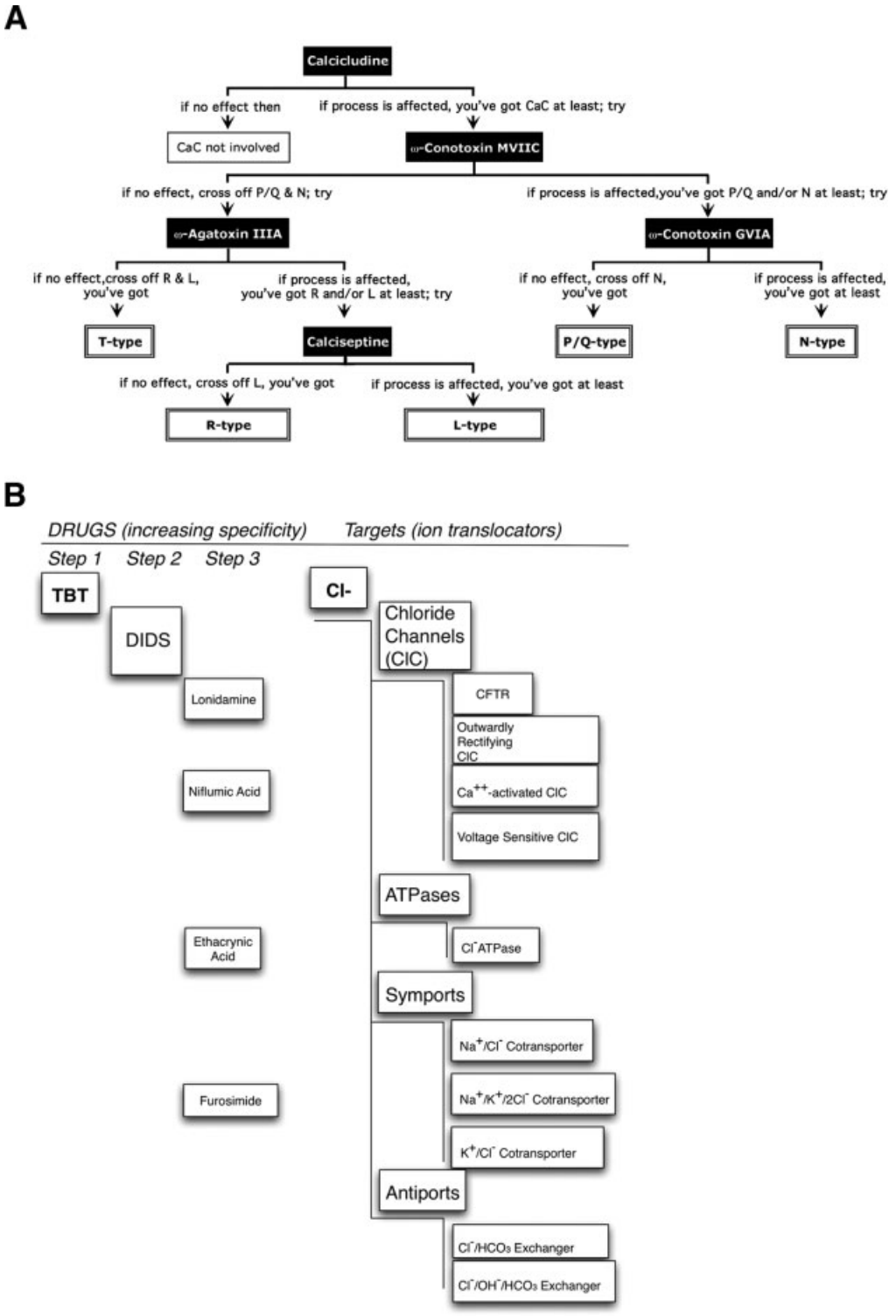

FIG. 2. Sample decision trees for testing the involvement of calcium (A) and chloride (B) signaling in a POI. Panel (a) illustrates the detailed logic for traversing a data tree to identify a calcium conductance. Panel (b) simply illustrates the corresponding drug and transporter trees for chloride.

while positive results enhance confidence in targets downstream of that node. Step 4 part I: Calciseptine, to distinguish between R- and L-type channels if ω-Agatoxin IIIA causes a phenotype. Step 4 part II: if needed, something to test for T-type channels, since a positive result with ω-Agatoxin IIIA does not rule out T-type CaCs.

To understand the structure of the chart in Figure 2a, it can also be helpful to start at the end. That is, if the POI required R-type calcium channels, the result of the calcicludin test (Step 1) will be positive; the result of the ω-conotoxin MVIIC test (Step 2) will be negative; the result of the ω-Agatoxin IIIA test (Step 3) will be positive; the result of the calciseptine test (Step 4) will be negative. If one is simply looking for candidates, a good one is now provided: R-type CaC's are quite likely to have a role in the POI. However, if one wishes to be sure that no other CaC is important, one would also have to test T-type CaC's, because a positive result was obtained at Step 3. Mibefradil can be used to distinguish between R- and T- type CaCs. If mibefradil has no effect, then only R-type CaCs have a role; if it has an effect, T-type CaCs are probably important also.

The chloride chart in Figure 2b shows three levels of specificity (three steps). At each step, negative results from drugs listed on the left allow one to delete from the list of candidates all the targets to the right; a positive result suggests that, in addition to completing that step, one should also proceed to the next step within that category of transporter. Step 1 for a chloride screen would be treatment with tributyl tin (TBT), a Cl^-/OH^- exchanger. If, at different pHs, TBT has no effect (the negative result), Cl^- is very unlikely to be involved in the POI, and thus Cl^- transporters can be crossed off of the list of candidates. If TBT does cause a relevant phenotype, one moves on to Step 2, perhaps trying DIDS next. If the result was negative, one would cross all chloride channels (ClC) off the list, and focus the remaining work on active Cl^- transporters. If the result was positive, Step 3 part I could be niflumic acid, to test for the relevance of the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated ClC, respectively. If those results are negative, only two ClC types remain on the list, outwardly rectifying ClC and voltage sensitive ClCs. If either of those results was positive, all four ClCs remain candidates. Step 3 part II would be ethacrynic acid and furosimide to test for contributions of Cl^--ATPase and the $Na^+/K^+/2Cl^-$ cotransporter. If these cause no phenotype, one adds only the Na^+/Cl^- co-transporter, the K^+/Cl^- co-transporter, and two known Cl^- antiports to your list of candidates. If either result is positive, one has not ruled out the other transporters; however, one has obtained important evidence consistent with involvement of the Cl^--ATPase and/or the $Na^+/K^+/2Cl^-$ cotransporter in the POI. So, a negative result, particularly at an early step, greatly reduces the number of candidates to be tested, while a positive result increases the confidence with which one can identify candidates.

Although the overall number of available reagents is huge, this screen harnesses the power of tiered organization to allow a binary search that quickly yields a small number of compounds needing to be tested more carefully. Thus, like a dichotomous key, a step screen either reduces the number of alternatives that have to be considered, or increases the efficiency and accuracy with which one can choose candidates to characterize using molecular techniques such as morpholinos, RNAi, or knock-outs. Alongside blockers and inhibitors of channels and pumps, useful reagents also include chelators, ionophores, gap junctional communication inhibitors, and direct alterations of ion concentrations in the extracellular medium. More complex strategies can often also make use of activator drugs (in contrast to blockers).

To perform a step screen, the most important things are: knowing the drugs that are available for each category, and having an effective ordering structure. While no definitive list can be compiled (since new pharmacological reagents are constantly being developed, and new specificity information sometimes appears for known drugs), we have assembled a very detailed list that should greatly reduce the effort needed to carry out such a screen to find ion translocators. This table is generally applicable to most model systems, but, of course, depending on the particular area of transporter biology to which the data will point, one must then delve deeper into the specific literature about the reagents used and the candidates to understand the nuances specific to each result and group of transporters.

Once effective and specific drugs are found that perturb the POI, they will serve as important reagents in subsequent steps. For example, they can be used (1) to ensure that the ion flows one detects directly in electrophysiological approaches are in fact due to the target (by ensuring that the flows cease upon application of the relevant blocker) and (2) to determine the timing of the POI. For example, if one discovers that application of a particular blocker during early development results in craniofacial defects, one can apply the drug at different stages to determine exactly which developmental process might be sensitive. One important point is that because one can rarely demonstrate complete wash-out of the drug, it is most useful to compare exposures that begin early vs. begin late, rather than exposures that end at various time periods. Despite the inability to formally prove wash-out, such experiments can give very valuable temporal information in cases where the drug does indeed leave the cells quickly.

A More Complex Example: Serotonergic Signaling

To illustrate a more complex application of this approach, we now consider serotonergic signaling. This pathway has been extensively characterized by the neuropharmacology community (Hoyer *et al.*, 1994) and is rich in the ways that positive and negative serotonin signals may be provided to cells. It is now becoming appreciated that neurotransmitters such as serotonin (5-HT) have interesting patterning roles outside of the nervous system (Buznikov *et al.*, 2005; Buznikov and Shmukler, 1981; Levin *et al.*, 2006), and Figure 3 illustrates the serotonergic component of a large-scale neurotransmitter screen as might be performed in a regeneration or embryonic patterning assay. Each node in this tree repre-

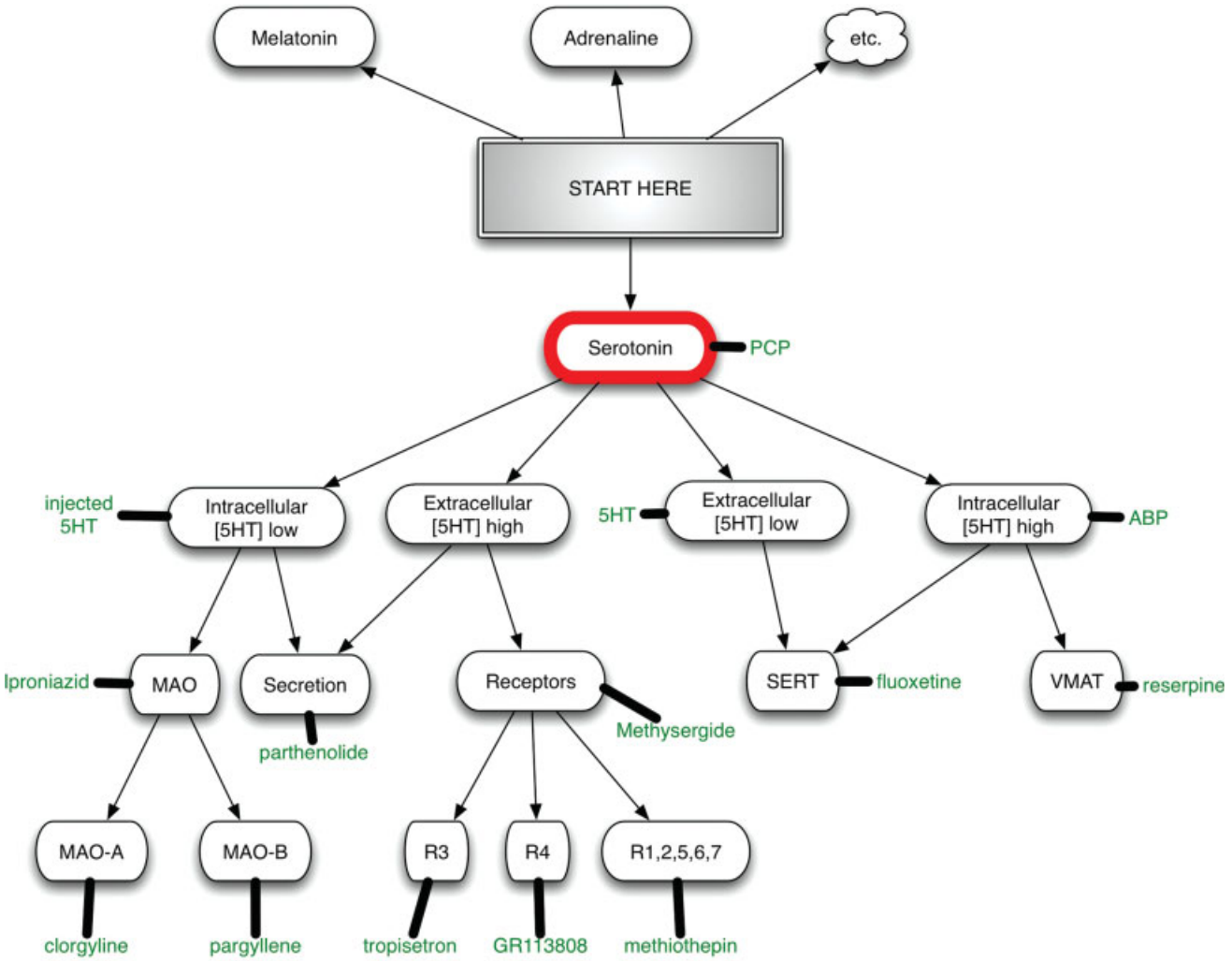

FIG. 3. A more complex strategy for probing neurotransmitter effects in a POI. Oval nodes represent specific functional modules (proteins or physiological conditions) whose involvement is to be tested. For reasons of space, lower tiers have been left out of this diagram. For example, the serotonin receptor 1 family is subdivided into types 1a, 1b, 1d, 1e, 1f, 1p, and 1s. Green labels indicate an example of reagents that can be used to test the node to which they are attached. Arrows between two nodes indicate suggested screen paths to be taken depending on the outcome of each result. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sents a particular mechanistic target. The treatments in green attached to each node represent an example of a reagent that can be used to probe the involvement of the given target (Supplemental Table 1). To begin, the user hypothesizes that the POI can be controlled by signals provided by four situations: low intracellular [5HT], high intracellular [5HT], low extracellular [5HT], and high extracellular [5HT]. Each of those conditions can in principle activate specific downstream programs and they can be tested using the methods indicated. For example, if 5HT applied externally does not perturb the POI, it is likely that external [5HT] is not a major regulator in this case and serotonin receptors can likely be ignored. If external 5HT application does perturb the POI however, one needs to test the involvement of any of seven receptor subtypes, as well as a serotonin transporter (by the activity of which external 5HT can raise internal [5HT]). Intracellular [5HT] can be lowered by the introduction of serotonin-binding protein (SPB). If SPB application has no effect, then minimum internal [5HT] levels are probably not crucial. Likewise, low intracellular [5HT] is normally maintained by degradation via monoamine oxidase or secretion. The importance of this parameter can be tested by injection of serotonin into cells (to increase internal [5HT]), or inhibition of the two pathways that result in lower internal [5HT] levels. All of the nodes in this tree can be targeted in gain- or loss-of-function tests using various compounds. Thus, this hierarchical traversal using pharmacological reagents can probe not only individual proteins but also cell signaling modules, and can utilize assays more complex than simple introduction of drugs into medium.

Note also that the involvement of a given node can potentially implicate more than one upstream mechanism. If the results of fluoxetine testing implicate the serotonin transporter SERT in the POI, it may suggest the involvement of low extracellular [5HT] or high intracellular [5HT], both of which would be perturbed by inhibition of serotonin import. These possibilities can easily be distinguished by the methodology described above.

Specific Considerations, Potential Problems, and Trouble-shooting

Organisms and choices of assays. In our laboratory, such step screens have been used successfully to study axial patterning in *Xenopus,* zebrafish, and chick (Adams *et al*., 2006; Bunney *et al*., 2003; Cheng *et al*., 2002; Fukumoto *et al*., 2005a,b; Levin and Mercola, 1998; Levin *et al*., 2002). Others have extended them to *Ciona* and sea urchins (Hibino *et al*., 2006; Shimeld and Levin, 2006). Each of these model organisms offers a different advantage. Below, we briefly describe the advantages and disadvantages of three model vertebrates to illustrate issues that may make step screens easier or more difficult in a given assay system.

Xenopus laevis. *Xenopus* is excellent for a drug screen, in contrast to their unsuitability for traditional genetics. Advantages: embryos can be synchronously collected in very large numbers, facilitating biochemical, pharmacological, and statistical analyses. They develop in a simple salt solution in room temperature, and are easily injected, enabling exposure to almost any kind of compound. There is a detailed fate map for *Xenopus* blastomeres, enabling some control over the distribution of injected reagents. The early embryonic cells are large and easily accessible, providing ample opportunity for introduction of morpholinos and dominant negative constructs (for target validation). Well-developed protocols for electrophysiology and in situ hybridization ensure that endogenous activity and expression of many different kinds of targets can be readily analyzed. The vitelline membrane surrounding the early embryo is highly porous and thus it is usually safe to assume the accessibility of drugs. Finally, and uniquely among model organisms, is the fact that *Xenopus* oocytes are frequently used as "laboratories" for the study of ion translocators, neurotransmitter receptors, 2nd messenger cascades, calcium-dependent events, and cytoskeletal rearrangements. This is a crucial advantage because the literature contains a huge number of available plasmids encoding w.t. and mutant proteins that have already been physiologically characterized in *Xenopus* (although usually in oocytes, not embryos). This is a particular advantage in screens involving ion and other small molecule transport. In a number of cases, targets implicated in *Xenopus* have been validated in other model systems; degree of conservation is surely highly dependent on the pathway involved but in many cases the advantages of *Xenopus* may favor this system even if the ultimate validation is to be done in another species. Disadvantages: The cells of *Xenopus* embryos are relatively large and round, and this can make in vivo wholemount imaging difficult. In addition, the blastomeres are opaque, complicating light-microscopy, and the yolk is autofluorescent, which complicates the use of fluorescent markers to localize proteins or ions.

Gallus gallus (chick). Advantages: chick embryos are flat and transparent, making them amendable to a variety of imaging techniques (most of which were designed for use on cultured cells), including the use of fluorescent indicators of ion flux and neurotransmitter release. The cleavage pattern of chicks is similar to that of most mammals. Disadvantages: chick embryos are not available for study until there are already several tens of thousands of cells, thus they cannot be used to study phenomena that occur during early cleavage stages. Delivery of reagents in ovo is possible, but the *N* will necessarily be smaller than for *Xenopus*.

Danio rerio (zebrafish). Advantages: zebrafish embryos are available at all stages, from gamete onward, and cohorts can be synchronized if needed for subsequent biochemical analysis. The cells are relatively large and transparent, thus making them excellent for imaging, including with fluorescent indicators. Embryos can be collected in large numbers (for biochemistry and statistics), cultured relatively simply, and are readily injected, facilitating delivery of reagents to intracellular locales. Finally, it is possible to create transgenic lines of zebrafish. Disadvantages: cell migration and mixing during gastrulation can make it difficult to relate early events to later effects. Moreover, the incomplete early cleavage can make it difficult to target individual blastomeres separately.

Caveats and trouble-shooting. One of the difficult aspects of using pharmacological agents is determining the correct dose, that is, a dose that will clearly affect your phenomenon without affecting anything else, and without being toxic. Remarkably, however, this is often possible. Even drugs as potentially lethal as palytoxin (listed in catalogs as the most toxic nonproteinaceous natural reagent now known) can be used at a low enough dosage to affect patterning without causing any other defects (Adams *et al*., 2006). Thus, titrating the dose of each drug is a critical part of the preliminary work for a screen; a good starting point is the concentrations at which the drugs are used in the literature. Nonetheless, certain drugs will undoubtedly be toxic, and certain drugs will do nothing in a given assay. Luckily, however, there is often another drug with the same target but a slightly different mechanism of action, which can be used as an alternative. For example, if early steps (1 and 2) indicate that Ca^{2+} channels may have a role in the POI, Step 3 might include treatment with BayK8644, an interesting drug because the R and S isomers have opposite effects on L-type Ca^{2+} channels (Bellemann and Franckowiak, 1985). It might be found, though, that while there seems to be an effect, it is not clear enough to interpret. The next step might be to try FPL64176, another L-type channel inhibitor that is known to be 40-

fold more potent than BayK8644 (Rampe *et al.*, 1993). Another approach to solving this problem is to use a less-potent analog of the drug of interest, or a slightly different member of the same family of drugs. Saxitoxin, a blocker of Na^+ channels, has many natural and synthetic analogs that might give results if saxitoxin itself proves toxic or inconclusive. Importantly, even if one drug gives a phenotype, it is still valuable to show that other drugs with the same target cause the same phenotype; these are positive controls showing that it is the desired effect of the drug that is causing the phenotype. Thus, one should plan on testing alternatives (if they exist) regardless of whether one chooses well the first time. The goal cannot be to unequivocally rule targets in or out, but rather to accumulate evidence that a small number of particular gene products are worthy of further investigation using more specific (and more time-consuming and expensive) molecular techniques.

Another difficulty is that there is often no positive control to prove that the drug being used has reached the target of interest. This may lead to false negatives that result from the failure of a drug to penetrate a chorion, vitelline membrane, cell layer, or subcellular compartment. Labeled drugs (for example FITC-conjugated glibenclimide for the study of K_{ATP} channels) are increasingly available however; so before dismissing a favorite reagent, one can research whether a labeled version is available or possible to create. Nonetheless, false negatives can happen, especially because some families (as well as potential as yet undiscovered targets) have no good reagents that target them. Thus, this screen will not saturate, but is a very useful tool to focus later efforts on a manageable number of interesting candidates. Another source of false negatives is that some systems can adjust after a loss of function treatment by up-regulating a different superfamily member to do the same job (Borgens, 1982). Judicious use of drug cocktails may reveal some of these fascinating mechanisms.

Also complicating the interpretation of some results is the lack of specificity of some of the drugs. For example, if Step 1 indicates that H^+ flux has a role in your phenomenon, Step 2 might include oligomycin, an inhibitor of the V-type H^+-ATPase. If that inhibitor causes an effect, however, one still needs to control for the fact that oligomycin also inhibits Cl^- flux through CFTR; one solution would be to determine whether lonidamine, a CFTR-specific inhibitor, gives the same phenotype. Thus, it is the intersection of one's data-set that implicates specific targets. This is another reason it is important to know as much as possible about the reagents, and it is important to remember that each result must be interpreted in the context of the entire screen.

The duration of exposure must be carefully considered. The basic parameters are dictated by the POI in question and whether one is attempting to probe different temporal stages for involvement of specific targets. Most small molecules reach their targets quickly, but it is important to ensure that one's assay has given each drug a fair chance to affect the target cells. In contrast, longer exposures raise the possibility of indirect effects. Thus, one generally tries to use the shortest exposure period that still encompasses the relevant POI. Many processes are regulative and can mask real effects once the blocker is withdrawn if it is removed too soon. For example, in the case of left–right patterning, GJC blockers that are withdrawn after 1 h have no effect (Levin and Mercola, 1998) because the embryo is apparently able to correct for this short inhibition of the relevant signal.

Finally, it is important to consider that much drug-mechanism data comes from the study of cultured mammalian cells. Thus, in extrapolating to other experimental contexts and species, specificity may be different (for example as a result of sequence and structure divergence between mammalian and other versions of relevant protein targets). In some cases (such as in *Drosophila*), data is available on the interaction of drug reagents with nonmammalian targets (Alshuaib and Mathew, 2004; Etter *et al.*, 1999; Gasque *et al.*, 2005; Jiang *et al.*, 2002).

CONCLUSION

The fundamental logic of this approach is very simple, but it has not sufficiently benefited the developmental biology field because no systematic guide to using this approach, nor specific examples of its use, have been available. Inverse drug screens have a relatively low start-up cost and provide a short list of candidates for further analysis. They must inevitably be followed by validation (using specific genetic knock-down) and then characterization of endogenous components (for example, expression studies of whatever protein set was implicated by the drug results). The set of strategies we have described is complementary to, and offers the following advantages over, current methodology. It can be applied to almost any model system, including those that are not amenable to genetic or genomic screens (including mammalian cell culture). It allows extremely rapid identification of promising targets, being orders of magnitude faster than genetic techniques. It is also relatively inexpensive since far fewer targets need to be examined (unlike a genetic screen, which is an exhaustive search, this is a binary search and is thus logarithmically faster). Drug reagents are generally far less expensive than producing knock-outs genetically or with morpholino technology (in terms of both cost, and bench-time). This process uncovers targets that are otherwise masked by early embryonic lethal phenotypes, since the reagents can be added at a stage after normal development has taken place. It reveals maternal effects, since the drugs down-regulate activity of maternal proteins that would remain untouched by morpholino or siRNA-based approaches. By adding reagents at defined time-points, one can obtain timing (stage-specific) information on the POI; inducible expression technologies provide a similar functionality (Adam *et al.*, 2000; Blau and Rossi, 1999; Wheeler *et al.*, 2000), but drug experiments are often faster and cheaper than the molecular genetics necessary to create a

clean inducible effect, and work even in model systems where no satisfactory inducible technique is available.

The contributions of this technique to a number of fields will continue to increase as pharmacologists continue to expand the set of reagents available and new drug trees are created by the community to probe important protein classes. We call on experts in the pharmacology of various fields to produce published or electronically available hierarchical drug trees. It is an essential feature of this strategy that any such tree that is created immediately becomes useful to countless possible model systems and assays. Next step approaches include statistical methods to deconvolute pathway information from proteomic or genomic datasets derived from experiments that use inhibitors and activators to modulate signaling (such as referenced at http://www.signaling-gateway.org). Such information ultimately enables modeling and reconstruction of signaling pathways from proteomics and genomics datasets in response to biological modifiers (De Young and Keizer, 1992; Lemon *et al*., 2003a,b; Papin *et al*., 2005; Pradervand *et al*., 2006).

The data sets we have assembled, focusing on ion transporters, will be available at www.cellregeneration.org. A crucial next step is the automation of this process. We are currently in the process of designing and implementing an expert system (Roach *et al*., 1985)—a software suite that will utilize arbitrary drug/target tree databases and take a user through a step-by-step screen in any POI. This system will encompass a simple binary tree search as well as more complex fuzzy-logic components to deal with overlapping targets and low specificity of some reagents to derive probability-based orderings of targets for validation. The use of this system and of such tiered pharmacological screens in general will significantly hasten the discovery of novel targets for important biological and biomedical regulatory processes.

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