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Genotype to phenotype: challenges and opportunities

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The application of genetic engineering methods to mice has greatly expanded the ability to study the molecular basis of physiology and behavior in health and disease. Targeted disruptions or insertions of single genes are unique in that they promise to elucidate the precise role of many endogenous factors important during ontogeny and adult life in a manner which is relatively precise and free of the side effects encountered with many pharmacologic probes. Though the deletion or insertion of a gene may be a relatively ‘clean’ process, interpretation of the resulting phenotype may not be straightforward, since most behaviors and physiologic processes are controlled by more than a single gene. In fact, the knocking out or insertion of a single gene may result in no phenotypic change. This may be the case, in particular, if there exist gene redundancy mechanisms whose presence may prevent abnormal phenotypes from becoming unmasked. Conversely, single genes are often essential in a number of different behaviors and physiologic processes. Hence, ablation of an individual gene may prove so drastic as to be lethal, or so widespread as to create an amalgam of phenotypes whose interpretation becomes confounded by the interactions of the various new physiologic changes or behaviors.

A legacy of the conventional knock-out methodology is the fact that many genetic mutant mice are a hybrid of two strains. Aberrant traits linked to unusual alleles in the genetic background of the progenitor strains may confound interpretation of the behavioral phenotypes of the offspring. Further, different breeding strategies will result in mutants with different genetic loading from the maternal and paternal genomes. An exceptional case in point can be found in mice lacking the gene for transforming growth factor b1 (Tgfb1), a protein important in growth, differentiation and the functioning of most cells. In these knock-outs, expression of the genetic defect in a C57BL/6, C3H, or 129 mouse strain, or else in a mixed 129 × CF1, 129 × C57BL/6, 129 × C3H, or a 129 × C57 × NIH/O1a strain, all produce a wide range of phenotypes with regard to preimplantation development, yolk sac development, bowel and gastric inflammation and colon tumor suppression [7]. Strain-

related problems have been reviewed by a number of authors, and solutions such as systematically backcrossing the mutation into a single strain, improving statistical sampling by increasing the number of experimental animals, and the use of parental controls in the evaluation of phenotypes are currently in use [6,14,15].

Since genetic mutations in conventional transgenic and knock-out mice exist from the earliest stages of embryogenesis throughout development, phenotypes examined in adulthood will reflect their ontogenetic histories. In monoamine oxidase A (MAO-A) deficient mice, excessive whole brain levels of serotonin (5-HT) during the critical period of development disrupts the formation of the mouse barrelfield by impeding the normal patterning of thalamocortical axons and cortical somatosensory cells [2,24]. Such neuroanatomic defects may contribute to the alterations noted postnatally in these animals in regional cerebral cortical blood flow in somatosensory and barrelfield cortex [10], as well as in the alteration of emotional learning [11], aggression and social behavior [2].

Another concern with using knock-out or transgenic mice is that compensatory systems may be activated in these animals which may mask the expression of the resulting phenotypes. Presence of physiologic adaptation is increasingly being described in a number of genetic mutant mice, including reports of compensatory changes in the localization, density [17] and sensitivity [3] of postsynaptic receptors, and changes in cell metabolism [23]. Adaptive changes frequently occur within the same system as that of the genomic ablation (isomodal). Changes are most marked when there are no immediate redundancy mechanisms such as receptor crossreactivity or availability of alternate peptides which might serve a similar function. Thus, deletion of the MAO-A gene results in a significant isomodal increase in 5-HT, the enzyme's substrate, whose catabolism to 5-hydroxy-indoleacetic-acid (5-HIAA) is largely dependent on MAO-A. Adaptive changes may also be due to the loss of a gene product that is downstream from the functions of the disrupted gene. Thus, in association with the increase in 5-HT levels in brain, MAO-A deficient mice show a downregulation of post-synaptic 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors [21]. Decreases in the 5-HT_{2A} receptors have been reported also in mice lacking the serotonin transporter [17]. Presumably this reflects a downregulation of the receptor in response to increased synaptic concentrations of 5-HT. However, regional differences were noted suggesting that this adaptive regulation does not occur throughout the central nervous system [17]. This further illustrates the complexity of compensatory mechanisms which may lead to marked regional differences in functional response.

Compensatory changes may also be due to a differential expression of another gene whose function may normally be indirectly regulated by the downstream gene product of the ablated gene (crossmodal). Cross-modal changes have been reported, for example, in mice carrying a mutant superoxide dismutase 1 (SOD1) gene, in which there is an unexpected age-dependent increase in choline acetyl transferase mRNA in the striatum [1]. It is hypothesized in these mice that dysfunction in the cholinergic system may be the result of a loss of dopaminergic afferents which is an end-stage feature of the SOD1 mutation [4,12].

Though many physiologic compensatory changes may fall short of 'normalizing' the animal's altered physiology, others may 'overshoot' their mark. An example of such

overcompensation can be seen in MAO A deficient mice, whose chronic elevations in brain levels of 5-HT are associated with a cerebral cortical blood flow (CBF) whose topography largely represents a mirror image of that found in wild-type mice. Areas of high relative CBF in MAO-A knock-outs demonstrate a low flow in the wild-type mice, and vice-versa. When MAO-A knock-outs are administered an acute bolus of d-fenfluramine, an indirect releaser and reuptake inhibitor of serotonin, the relative regional CBF distribution returns to that seen in wild-type mice. This example highlights the fact that in MAO-A deficient mice compensatory physiologic changes due to a chronic increase in 5-HT may act to reset the homeostatic state in a manner opposite to that seen during an acute serotonergic challenge. Such a resetting of the homeostatic baseline state may function to attenuate the physiologic consequences of acute, exaggerated shifts in regional concentrations of 5-HT.

Attention has recently been drawn to the problem of the variability in the phenotypic characterization reported by different laboratories for several transgenic and knock-out mouse strains [5]. Such variability may reflect differences in the behavioral/physiologic paradigms employed in testing, environmental differences (e.g., time of day of the testing), social differences (e.g. individual vs group housing), or experiential factors (e.g., early vs late postnatal weaning). In addition, genotypic differences elicited between the mutant and wild-type mice may reflect differences in the interaction of genotype with incidental factors such as handling stress, to which different animals may demonstrate a greater or lesser sensitivity. Care needs to be exercised in the interpretation of behavioral data. Motor and visual deficits in mice are well known to alter performance in the Morris water maze, and thus, it may be incorrectly concluded that such animals demonstrate deficits in learning and memory. Changes in pain threshold may alter performance in operant tasks involving footshocks. Further, any changes in olfaction in rodents may significantly influence behaviors heavily dependent on this sensory function such as aggression, social exploration, mating, maternal behaviors, feeding, and exploration of a novel environment. If a phenotype appears late because of a developmental delay, it may be missed in studies that examine animals crosssectionally at an early age. False negatives may also appear if animals are tested for phenotypes under conditions in which they normally do not display the phenotype ('floor' effect). For example, females may show no increase in aggression in a standard paradigm in which a female is exposed in her homecage to an intruding male mouse. Rather, aggression may be more reliably tested in a paradigm in which female mice normally show aggression, that is, in a postpartum female protecting her young. Conversely, subtle differences between wild-type and mutant mice in a baseline physiologic parameter such as, for instance, sympathetic tone may go undetected if variables such as heart rate and blood pressure are examined under experimental conditions whose inherent stress results in maximal physiologic elevations of these parameters in both genotypes ('ceiling' effect).

The recent introduction of conditional knock-outs represents a major scientific advance. Inducible knockouts, a specific type of such mutant mice, allow the investigator to switch the gene of interest on or o. at particular times [13,16,18,22]. This allows any given animal to be used as its own control, avoiding some of the problems associated with the variation encountered between animals. In addition, the availability of inducible knock-outs should provide an important tool to address the current problem of ontogenetic effects on the adult phenotype.

Though inducible knock-outs may avoid some of the ontogenetic problems inherent with conventional knock-outs, caution is required in interpreting the resulting phenotypes. The potential for redundancy mechanisms masking changes in behavior or physiologic function remains, even if a gene is acutely turned 'on' in late life. Furthermore, the adult phenotype is determined as much by its ontogenetic history as it is by the turning 'on' or 'off' of genes at the time of testing. The complex orchestration of gene activation and inactivation during development is essential in determining neuronal migration and brain structure in a manner which may either facilitate or constrain the postnatal repertoire of functional changes. Eliciting functional changes in adult life by turning a gene 'on' or 'off' may, in specific instances, require gene activation/inactivation at specified antenatal stages in such a manner as to reproduce 'normal' development.

Region-specific gene knock-outs and transgenics have also been described [8,9,19]. In these animals activity of a given gene is linked to a tissue-specific promoter, allowing targeted examination of gene function rather than the widespread changes seen with conventional knock-outs. For example, linking MAO A cDNA to the promoter of Calcium Calmodulin Kinase II allowed targeted forebrain expression of MAO A in mice otherwise deficient for MAO A [20]. Reintroduction of the MAO A gene into these mice decreased their level of aggression, suggesting that MAO A plays an important role within the forebrain in modulating aggression. Such studies represent a first step in understanding the orchestration of facilitating, as well as inhibitory inputs from multiple brain regions that determine a behavior as complex as aggression. Region-specific knock-outs and transgenics should also allow us to address the possibility that biological factors such as MAO-A may exert opposing effects in different brain regions.

Far from being examples of genetic determinism, mutant animals underscore the importance of experience and environmental factors in determining an animal's phenotype. Standardization, particularly of behavioral tests between different laboratories, should help to reduce the variability in the assessment of behavioral phenotypes. Evaluation of models of functional testing for potential 'ceiling' and 'floor' effects should reduce the potential for false negative results. If feasible, replacement therapy should be used to verify that the behavioral or physiologic defects are actually due to the lack of gene product. Because of the possibility of different genes subserving similar functions, as well as the developmental adaptation in any biologic system, greater consideration should be given to describing phenotypic function under conditions of a challenge test. Such pharmacologic, physiologic or behavioral 'stress tests' may serve to better unmask any underlying functional abnormalities. With increased use of inducible knock-outs and the greater availability of tissue-specific promoters, there will be a growing need for understanding the complex timing and orchestration of the multiple factors active during ontogeny.

A distinct utility will remain for conventional methodology in studies of gene effects during ontogeny and in animal models of chronic human genetic diseases expressed in a generalized manner throughout the body. Conventional knock-outs and transgenics may prove particularly useful in animal models of disorders, whose onset in adult life depends on the occurrence of early developmental defects, as has been proposed for schizophrenia. Furthermore, conventional knock-outs may help us better understand the plasticity of the

nervous system and its various potential compensatory mechanisms. In some cases such studies may elucidate related proteins or pathways whose existence or functions were previously masked. Such compensatory mechanisms may be active at transcriptional, translational, as well as post-translational levels. Hence, investigations of compensatory mechanisms using DNA microarrays need to be supplemented with techniques such as tissue-specific protein panels and studies of signal transduction. Effort should be made to broaden the scope of phenotypic characterization to include not only behavioral, physiologic, and biochemical systems predictably affected by a targeted mutation, but also other related (crossmodal) systems. Surprisingly, transgenic and knock-out animals, though designed for specificity, may, in fact, in coming years reveal much about the interdependence of related systems. Such studies may help elucidate, for example, the functional relationship between serotonergic, dopaminergic and cholinergic neural pathways in the brain. The search for the key to disorders of likely genetic etiology may be most productive, if one looks not only under the 'light of the nearest lamp post', but also in regions less obviously illuminated.

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Abbreviations

CBF	cerebral blood flow
5-HIAA	5-hydroxy-indoleacetic-acid
MAO-A	monoamine oxidase A
5-HT	serotonin
SOD1	superoxide dismutase
Tgfb1	transforming growth factor b1

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