METHODS IN GENOMIC NEUROSCIENCE

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1 Gene–Environment Interaction and Complex Behavior

Marla B. Sokolowski and Douglas Wahlsten

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1.1 INTRODUCTION

During the early decades of the last century, the statistical laws but not the molecular mechanisms of heredity were well understood, whereas the last two decades witnessed an explosive growth in knowledge of genes and their functions at the molecular level. Psychologists and developmental biologists have always been interested in interactions between heredity and environment, but only recently have we acquired

the tools needed to understand how interactions actually work. This chapter focuses on both statistical and molecular biological methods applicable to the detection and analysis of gene–environment (or gene by environment, G×E) interactions. We highlight theories, techniques, and experimental strategies relevant to studies of individual differences in behavior, including natural behavioral variants and genetic mutants. Although the use of molecular approaches to study G×E interactions is still in its infancy, the advent of the genome projects combined with some of the latest technologies for analyses of genome-wide responses to the environment make investigations of this type timely and promising.

1.2 THE MEANING AND IMPORTANCE OF INTERACTION

Well before the dawn of modern genetics, the theory that preformed characters undergo quantitative enlargement or unfolding was rejected by embryologists in favor of epigenetic development, the notion that the parts of an organism emerge through qualitative transformation.[1] Early Mendelism, especially the doctrine of unit characters championed by Bateson,[2] revived preformationism by asserting that genes specify the properties of adult organisms in a one gene–one character fashion. Gottlieb et al.[3] characterize this view as predetermined epigenesis, in contrast with probabilistic epigenesis that allows many possible outcomes from the same set of genes. As pointed out by Strohman,[4] exclusively genetic determination of adult characters is still a widely held opinion, as expressed in mosaic theories of development.[5]

The concept we now term genotype–environment interaction was formulated early in the last century as an alternative to unit characters. Johannsen[6] proposed that the genotype, the set of all the individual's genes, is inherited from the parents, whereas the observable phenotype develops and may have many values. He observed that "some strains of wheat yield relatively much better than others on rich soil, while the reverse is realized on poorer soils." Woltereck[7] proposed that the organism inherits not the character but the "Norm of Reaction with all its numberless specific relations" to all conceivable conditions, a lawful norm that can lead to many different "biotypes." He equated the Norm of Reaction with Johannsen's genotype concept. In a more recent expression, Lewontin[8] refers to the norm of reaction as the graph of the phenotype values of "a particular genotype as a function of the environment," and emphasizes, as did Woltereck and Johannsen, that these graph lines may take rather different forms and even intersect. Nijhout[9] uses the term "reaction norm" to denote continuous variation in phenotypes in response to graded changes in environment and "polyphenic development" for situations where the phenotype is expressed in qualitatively different forms as a consequence of environmental conditions.

Hogben[10] set forth a view that is widely held today. "Characteristics of organisms are the result of interaction between a certain genetic equipment inherent in the fertilized egg and a certain configuration of extrinsic agencies." He cited many instances where the quantitative effects of changing the environment depended strongly on the genotype. Concerning the issue of how much of a difference is due to heredity and how much to the environment, Hogben argued: "The question is

easily seen to be devoid of a definite meaning." He also said, "When we understand the *modus operandi* of the gene, we can state the kind of knowledge we need in order to control the conditions in which its presence will be recognized." This doctrine has now become the principal rationale for the Human Genome Project as a source of new therapies for medical disorders.

G×E interaction asserts that the response of an organism to an environmental treatment depends on its genotype, and the manifestation of genetic differences between individuals depends on the environment. This concept is presented graphically in Figure 1.1A. On the other hand, the reaction range concept claims that environmental effects are essentially the same for all genotypes, such that rank orders of genotypes are maintained over a wide range of environments, consistent with simple addition of genetic and environmental effects, and there is a gene-imposed upper limit on phenotypic development (Figure 1.1B; see References 11 and 12).

Interactionism as a doctrine assumes several forms. In psychology, Hull[13] maintained that behavior is governed by mathematical laws and that "the forms of the equation" are the same for all species and individuals, emphasizing, "Innate individual and species differences find expression in the 'empirical constants' which are essential constituents of the equation expressing the primary and secondary laws of behavior." For the behaviorism of Watson, Hull, and Skinner, the forms of the laws were identified with the biological structure of a nervous system that determined how environmental stimuli are sensed and associated. In the study of animal learning, this view led psychologists away from genetic research and justified the almost universal use of albino rats in the lab because the functional laws themselves were thought to be the same for all, including humans (see previous critique[14]).

In biology, Waddington[15] proposed a complex epigenetic landscape that governed how an individual would develop under different environmental conditions. The topography of the landscape, however, was said to be genetically specified. Like the notions of Hull, this theory of passive gene-related responsiveness to environment was reductionist, being based on strict genetic determination of an earlier phase of structural development and/or a lower level of organization (see Gottlieb[16]); genetic effects were held to be unidirectional from molecule upwards to morphology and behavior.

Interdependence or interpenetration of heredity and environment, on the other hand, holds that environment is an essential factor at all levels of organization; interactions are bidirectional, and the organism is an active agent in constructing and transforming its own environment.[8,17-19] According to Oyama,[20] a developmental system comes into being "…not as the reading off of a preexisting code, but as a complex of interacting influences, some inside the organism's skin, some external to it … It is in this ontogenetic crucible that form appears and is transformed, not because it is immanent in some interactants and nourished by others … but because any form is created by the precise activity of the system."

Thus, the fact that different genotypes evidence different shapes of reaction norms should be taken only as the starting point for investigation of development.[21] While the mathematical shapes of functions provide important clues, a deeper theory of development cannot be encapsulated in a few formulas.[20]

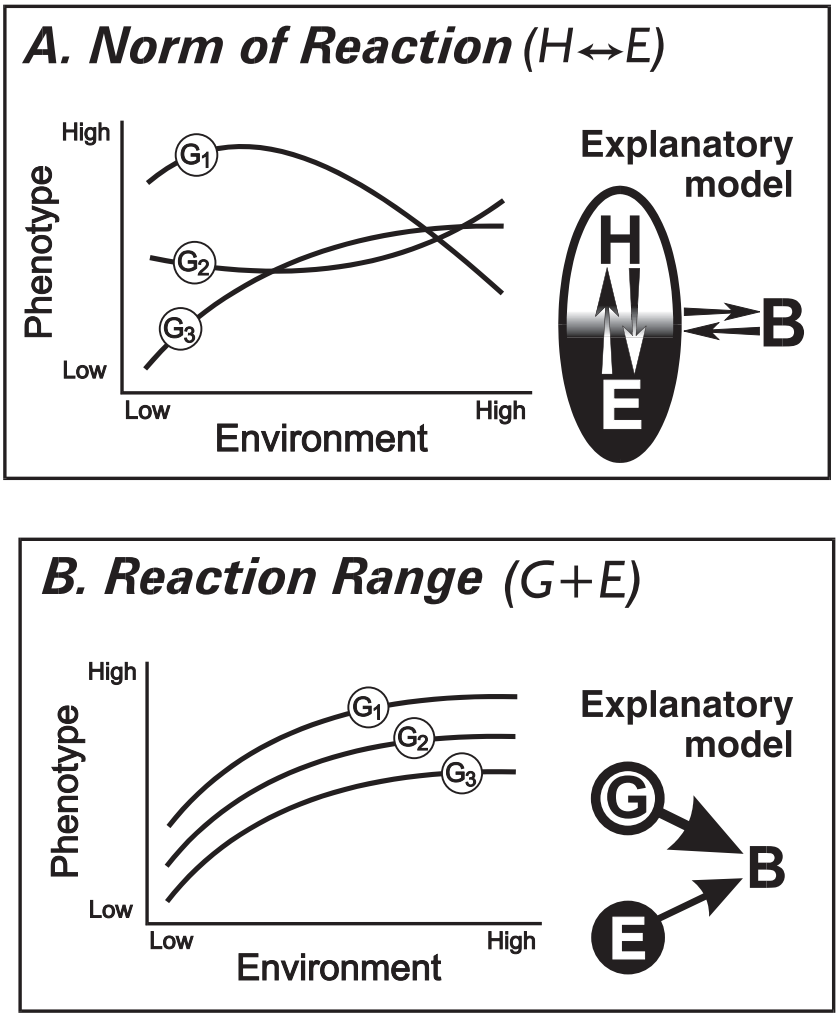

FIGURE 1.1 Two conceptual models of the functional relations between 3 genotypes and environment. A. The norm of reaction expects that the shape of the function will depend on genotype, and it allows for reversals of rank orders in different environments. This is possible because genotype and environment are interdependent causes whose interaction produces development. B. The reaction range expects essentially the same shape of the function for all genotypes. It asserts the developmental separation and hence additivity of genetic and environmental causes.

Ongoing disputes about appropriate models and credible assumptions for the analysis of human behavior continue to generate interest in G×E interaction. The prevalent view in quantitative genetic analysis in psychology denies the existence or importance of G×E interaction and instead asserts that genetic differences and environmental variations have additive effects, as expressed in the equation Phenotype = Genotype + Environment (P + G + E; see Plomin et al.[22]). When two

variables are statistically independent and additive, their phenotypic variance (V_P) can be partitioned into two components attributable to genetic (V_G) and environmental (V_E) variance. The broad-sense heritability ratio is then $h^2 = V_G/V_P$. The meaning and magnitude of h^2 for IQ in particular has been hotly debated for many years and continues to be contested.[23–25]

Additivity implies that the manifestation of genetic differences is unaffected by the rearing environment and the consequences of environmental change should be the same for all genotypes (Figure 1.1B). Only when factors act separately in the process of development will their effects generally be separable statistically. Some behavior geneticists, while not outright denying the existence of G×E interaction, argue that interaction effects pertinent to human psychology are generally so small that an additive model is a good approximation of reality.[22] In this respect, there is a stark contrast between prevailing conceptions in genetic studies of human and nonhuman animals.[26,27]

Three kinds of criticisms have been directed at heritability analysis of human behavior: (1) available research designs with humans are incapable of cleanly separating genetic and environmental effects;[28-31] (2) statistical methods of analyzing variance into components are relatively insensitive to the presence of real interactions[26] and yield a false impression of additivity; and (3) enough is known about the regulation of gene expression during development to warrant rejection of additivity as a general principle.[8,11,27,32]

Research on human behavior does not require an assumption of additivity. In psychiatric genetics, for example, models involving G×E interaction have been prominent for many years and published in recent reviews.[33] The diathesis-stress theory of Gottesman and Shields,[34] the model-fitting methods of Kendler and Eaves,[35] and the interaction hypothesis of Wahlberg et al.[36] provide clear examples. Cloninger[37] concludes that schizophrenia involves the "nonlinear interaction of multiple genetic and environmental factors."

In essence, two questions are raised by this discussion. First, is research with animals in the laboratory pertinent for models of human behavior, or can a theory of human exceptionalism be defended? At the molecular level, there is so much in common between human and mouse that broad generality of basic principles is expected. For further discussion of this question see Skuse.[38] Second, could it be that medically significant disorders involve interaction while variations within the normal range that are of central interest to psychologists do not? Little has been published on this latter question; however, in the few cases where a genetic and molecular basis for normal individual differences in behavior is understood, G×E interaction cannot be excluded.[39]

1.3 GLOBAL APPROACHES TO STUDYING INTERACTION

1.3.1 Experimental Designs

To ascertain the impact of an environmental variable, independent groups of animals having equivalent genotypes must be reared in different environments. This kind of one-way research design (Figure 1.2A) is easily arranged with an inbred mouse

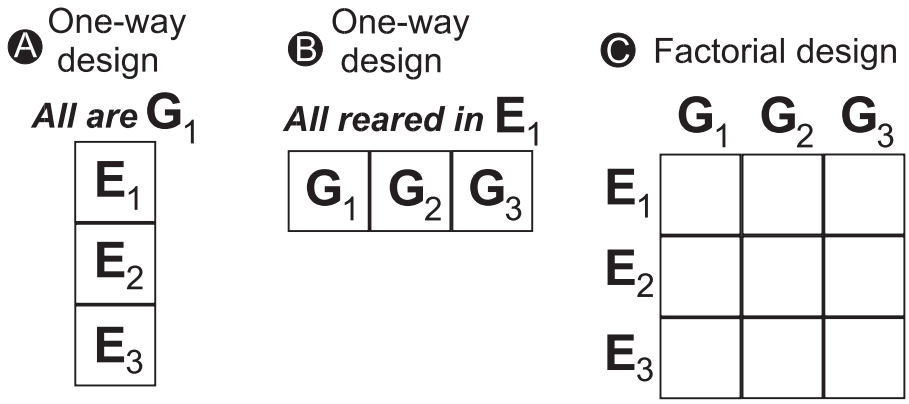

FIGURE 1.2 Experimental designs involving three genotypes and three environments. A. Raising genetically identical individuals in different environments. B. Raising different genotypes in the same environment. C. Raising each genotype in three different environments. Only the factorial design can reveal the presence of genotype × environment interaction, even though the two factors interact in the developmental sense even in the one-way designs.

strain, an isofemale line of *Drosophila*, or a clone of *Daphnia*. Likewise, genetic variation can be studied with a one-way design (Figure 1.2B) wherein different genotypes are reared in the same environment, as was done carefully by Mendel. Heredity and environment may be strongly interacting factors in the developmental sense in either kind of experiment, yet in neither case will the statistical interaction be apparent. Interaction is visible only when animals with different heredities are reared in different environments using a factorial design (Figure 1.2C). The smallest experiment conceivable is the 2×2 design, but the generality of findings will be greater when several strains are subjected to a wide variety of environments so that genotype-specific norms of reaction may be observed.

Following in Mendel's footsteps, modern geneticists usually strive to rear their subjects in a uniform environment in the lab or a carefully cultivated field so that data for different genotypes will not be confounded with environmental differences. Hence, results of many superb genetic analyses tell us nothing at all about the presence or magnitude of G×E interaction. A clue that interaction lurks in the background is sometimes seen when different labs fail to replicate effects of the same mutation. For example, three recent studies published simultaneously in *Nature Genetics* reported discrepant results of effects of a null mutation in the corticotrophin-releasing hormone (*Crh*) and its receptors (*Crhr1* and *Crhr2*) on mouse anxiety.[40-42] Because the genetic backgrounds of their strains as well as the details of the tests for measuring anxiety also differed between labs, it was not possible to attribute discrepancies to the rearing environments in the three labs.

Crabbe et al.[43] addressed this problem by testing the same eight genetic strains with identical test apparatus and protocols simultaneously in three labs. For certain phenotypes, ethanol preference in particular, the three labs observed essentially the same results, whereas measures of activity, anxiety, and activating effects of a cocaine injection yielded different patterns of data for certain strains in the three labs. The

study was explicitly designed to yield large genetic effects by choosing strains known to differ greatly on several phenotypes, and great efforts were made to equate many aspects of the lab environment. Nevertheless, substantial G×E interaction was seen. This study contradicts the contention that interaction is to be expected only when extreme differences in environment are employed.[44]

A few large studies in behavioral genetics have replicated a complete genetic crossing experiment in two different environments. Henderson[45] conducted a diallel cross of four inbred strains to create 12 F_1 hybrids, and all 16 groups were reared in either standard lab cages or larger, enriched environments. He found that evidence of genetic influences was markedly suppressed by rearing in the small, impoverished lab cages. Carlier et al.[46] repeated an entire reciprocal crossing study with eight genetic groups derived from ovaries that were grafted into a hybrid female, and they found that an effect of the Y chromosome on fighting behavior of male mice having inbred mothers was not evident in the F_1 maternal environment.

Interaction also occupies an important place in the laboratory as a research tool for analyzing mechanisms of development. In *Drosophila*, for example, temperature-sensitive mutations make it possible to delineate critical periods for genetic effects and to study interactions among gene products; wild-type and mutant transgenes can also be engineered to be expressed at certain times during development or targeted to specific tissue.[47] Inducible mutations in mice, whereby production of a specific protein is shut down when an animal drinks water containing an antibiotic, make it possible to assess the role of the gene in formation of memories in the adult without the confounding developmental effects that are typical for most targeted mutations.[48] By inserting special regulatory sequences near a gene, its expression may also be limited to a particular kind of tissue in the brain.[49]

1.3.2 Single Genes and Pleiotropy

How can we meaningfully analyze the effect of alterations in a single gene on the performance of a behavior in several environments when we know that most genes have pleiotropic functions? When a gene known to influence behavior is knocked out or inactivated, severe disruptions in a number of phenotypes are often observed. This usually reflects a role for this gene in both development and behavior. Indeed many genes that alter behavior are vital genes that cause lethality when inactivated (e.g., in fly food search — *foraging,*[50] *scribbler;*[51] courtship- *fruitless;*[52] learning — *latheo*[53]). It is of interest that more subtle alterations in the gene, for example hypomorphic mutations that cause a small reduction in the amount of gene product, often exhibit the behavioral alteration but not the other pleiotropic phenotypes. Greenspan[54] in his review entitled "A Kinder, Gentler Genetic Analysis of Behavior: Dissection Gives Way to Modulation," argues for the importance of studying milder mutations because they are more similar to the subtler genetic influences on behavior found in nature. These milder mutations and the ability to target the expression of a gene to certain times in development and to certain tissues in the organisms may allow us to disentangle a gene's role in development from its role in behavioral functioning. As an example, variants with partial loss of function of the major serine/threonine protein kinases — cAMP-dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinase type II

(CaMKII), or protein kinase C (PKC) — all cause effects in behavioral plasticity specific to learning and memory, while severe mutations in these genes are lethal.[54] These effects on learning and memory are seen when the level of kinase is reduced by only 10–20%. A 12% difference in the expression of the *foraging* gene which encodes a cGMP-dependent protein kinase (PKG) explains rover compared to sitter natural foraging behavior variants.[50] All of these kinases are involved in a wide range of biological processes; however, a subtle shift in kinase activity exerts a potent effect on the phenotype. It is likely that natural variants that have been selected under natural conditions involve these types of subtle mutations, because more severe mutations with their prevalent pleiotropic effects would be selected against. Future research on the molecular basis of natural behavioral variants will enable us to test this prediction.

One conclusion from the discussion above is that studies of the molecular mechanisms underlying G×E interactions on behavior should be done using natural variants, mutations, or transgenes that have subtle effects on the behavioral phenotype. If mutants with large effects are used, then one is more likely to identify genes and processes important to the many pleiotropic functions of the gene rather than to the behavior specifically. The task of teasing apart which specific mechanisms are associated with the behavioral function would then be overwhelming. In some cases, however, the developmental alterations in mutants may be the cause of the behavioral variation; for example, the presence of an altered level of a specific kinase or a second messenger such as cAMP during nervous system development may cause alterations in the morphology of the neurons and on their functioning.[55] In this case the connection between the developmental and behavioral phenomena can be determined using inducible transgenes prior to the molecular analyses of G×E interactions.

1.3.3 Research Outside the Lab

Research with wild populations indicates that G×E interaction is not merely some oddity confined in a laboratory. On the contrary, genotype-dependent responsiveness to environment is crucial as a means of adapting organisms to a wide range of circumstances. Certain reptiles lack sex chromosomes, and sexual differentiation depends on clutches of eggs being laid in soils having different temperatures,[56] whereas many other species are strongly buffered against temperature effects. The specific kind of food, oak catkins or leaves, on which larvae of the geometrid moth *Nemoria arizonaria* dine leads to a remarkable development of morphology that matches the caterpillar to the texture and color of its host.[57] Many other examples are cited by Nijhout.[9] G×E interaction is seen in all parts of the animal and plant kingdoms. Knowledge of which aspects of development are sensitive to which features of the environment can teach us a great deal about the mode of life and evolution of a species.

1.3.4 Studies of Humans

Research on G×E interaction is particularly difficult with humans because replicate genotypes simply do not exist. Monozygotic twins provide only two copies of a

genotype, and the environments of the pair also tend to be correlated. In the study of schizophrenia, there is evidence for G×E interaction in the Finnish adoption studies that show elevated psychopathology in adopted-away offspring of schizophrenic mothers only when they are reared in psychologically inferior homes.[36] The data are consistent with the hypothesis of gene-related vulnerability to stressful or confusing environments, but the case is weak because there is no identification of genotype per se. Instead, the probands and matched controls are selected on the basis of a maternal phenotype that is not a reliable proxy for a genetic abnormality.

When a specific, major gene effect on human development is established, evidence for G×E interaction may be obtained. The classic case is the phenylalanine hydroxylase (PAH) mutation that leads to phenylketonuria.[28] Children homozygous for the recessive allele are much more sensitive to the level of phenylalanine in the diet and can thrive only with rearing on a low phenylalanine diet and careful monitoring of blood levels of the amino acid. In work on genetic diseases, the studies are not as well controlled as lab experiments with mice or flies, but large effects of a mutation nevertheless permit conclusions about interactions in many instances.

In psychology, strong claims have been made that G×E interaction effects involving intelligence in particular have been sought but cannot be detected.[58,59] A closer look at the nature of IQ tests reveals a very large interaction, however, one that is obscured by the manner in which test items are chosen and the raw test score is transformed into an IQ score. The rationale for intelligence test interpretation was stated clearly by Goodenough:[60] "…the intelligence tests in present use are indirect rather than direct measures. They deal with the results of learning, from which capacity to learn is inferred. When opportunity and incentives have been reasonably similar, the inference is sound, but its validity may be questioned when a comparison is to be made between two or more groups for whom these factors have been markedly different." In other words, given similar exposure to relevant educational material, if one child learns faster and therefore more than another, psychologists infer this must be because of differences in an inherent property of the nervous system termed intelligence (see Wahlsten[61]). Intelligence is believed to cause *differences in the ease or rate of learning*, as expressed in the slope of the function relating amount of acquired knowledge to cumulative experience (see Figure 1.3). This is an example of interaction *par excellence*. It has become customary to avoid discussion of raw intelligence test scores and instead convert the raw scores to standardized scores based on large, representative samples of children of different ages. The desired scaling results in a mean IQ of 100 and a standard deviation of 15 at every age, no matter what kind of items are on the test. This practice effectively obscures the real rates of growth of intelligence and conceals the interaction. It gives rise to perplexing facts, such as different brands of IQ tests that yield the same mean and variance of IQ but are far from perfectly correlated with each other. It also tends to minimize the indications of a dramatic increase in intelligence test score over a period of one or two decades in a society, because most IQ tests are altered and restandardized every few years, which forces the mean back to 100.[62] Insisting that critics of heritability analysis should be able to show G×E interaction in IQ scores requires a large, gene-related difference in the *second derivative* of the function relating knowledge to experience.

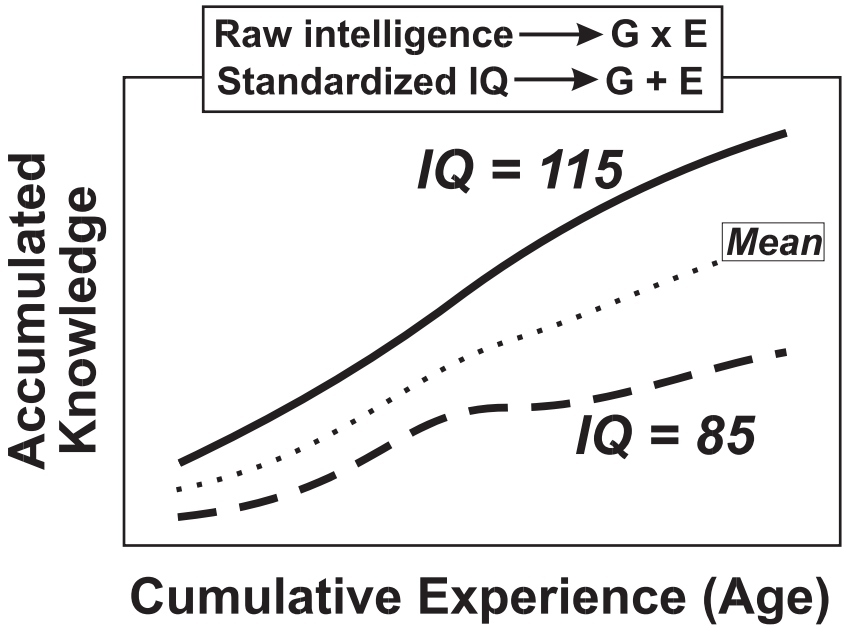

FIGURE 1.3 Accumulated knowledge, as expressed on an intelligence test, as a function of cumulative experience, for which age is a proxy variable. Hypothetical profiles are shown for two genetically unique individuals who have substantially different slopes of the experience–knowledge relation at certain ages, but nevertheless have stable IQ test scores across age. Converting the test score to a standard score tends to obscure the presence of interaction and make the factors appear to be additive.

1.4 STATISTICAL ANALYSIS OF INTERACTION

1.4.1 Factorial Designs

When J genotypes are reared in K different environments, the experiment with JK groups can be appraised with the analysis of variance (ANOVA) for fixed factors. Fisher and Mackenzie[63] devised this method to evaluate yield of 12 potato varieties under six conditions of manure. They divided the variance between the 72 groups into three portions, the two main effects and a third term, the "deviations from summation formula," a quantity we now assign the appellation "interaction." Statistically, interaction is defined as the variation among the JK group means that cannot be accounted for by the addition of the separate main effects of genotype and environment.

Whereas execution and interpretation of ANOVA are now quite routine, one crucial aspect of this methodology is not widely appreciated. For many interesting kinds of interaction that may exist in the real world, the ANOVA tends to be much less sensitive to presence of interaction than to the main effects.[26,64] That is, the statistical power of the test of interaction is often pathetically low and Type II errors (failure to reject a false null hypothesis that G and E are additive) are probably very

TABLE 1.1
Hypothetical Means for an Experiment with and without Strain by Lab Interaction

	Additive Main Effects				Model with Substantial Interaction			
Strain	**Lab A**	**Lab B**	**Difference**	**Mean**	**Lab A**	**Lab B**	**Difference**	**Mean**
A	30	40	10	35	30	50	20	40
B	35	45	10	40	35	35	0	35
C	40	50	10	45	40	50	10	45
D	45	55	10	50	45	65	20	55
E	50	60	10	55	50	50	0	50
F	55	65	10	60	55	65	10	60
G	60	70	10	65	60	80	20	70
H	65	75	10	70	65	65	0	65
I	70	80	10	75	70	80	10	75
Mean	50	60	10	55	50	60	10	55
				Lab effect:	$\sigma_M = 5.0$	$f = 0.2$	$n = 22$	
				Strain effect:	$\sigma_M = 12.9$	$f = 0.5$	$n = 7$	
				Lab × Strain interaction:	$\sigma_M = 5.8$	$f = 0.23$	$n = 31$	

Note: Standard deviation within each group is set at 25 units. Sample size is calculated in order to yield power of 90% when Type I error probability is set at $\alpha = 0.01$. Values obtained from Tables 8.4.1 and 8.4.2 in Cohen[66] must be adjusted with the formula on page 396 in order to adapt tables computed for a one-way design for use with a factorial design.

common. Great attention is usually devoted to the proper choice of a criterion for Type I error (rejection of a true null hypothesis), especially in linkage studies, and this concern is appropriate because we expect that most genetic markers in a genome scan are not linked to a gene causing individual differences in a particular behavior.[65] On the other hand, in a study of inbred strains in different environments, we have good reason to suspect that the factors really do interact, and the null hypothesis lacks credibility; hence the central issue is the sensitivity of the test of interaction.

An effective remedy for low power of a test of interaction is readily prescribed. Larger samples are required to confer adequate power on an assessment of what, to the ANOVA procedure, appears to be a relatively small effect. Power and sample size calculations should be done before the data are collected, and we must propose credible but hypothetical values of group means, guided by previous studies. The method of Cohen[66] is convenient when working with effects having more than one degree of freedom.

An example is provided in Table 1.1 for a study where nine inbred strains are reared and tested with the same apparatus in two labs. Suppose that in Lab A the strain means on a test range evenly from 30 to 70, and in Lab B each strain scores 10 units higher, which is an instance of additive effects. Next we must propose a model of group means that expresses the kind of interaction we would like to be able to detect. It would be silly to suggest that in Lab B there will be no strain differences at all; this would be a huge interaction effect but not one we could

plausibly expect to find, given decades of research with inbred strains. The model of interaction in Table 1.1 entails three strains that have identical means in both labs, three strains that differ by 10 points, and three strains that differ by 20 points. Note that the distributions of strain and lab means are the same under both models. The method of Cohen[66] requires that we find the standard deviation between group means (σ_M), and the effect size *f* is the ratio σ_M/σ, where σ is the standard deviation within groups (set at 25 in this example). For the strain main effect, σ_M is based on nine means, whereas it is based on only two for the lab main effect. For the interaction having eight degrees of freedom, we must take the average squared difference between all 18 group means expected under the hypothesis of interaction and the means expected from simple additivity. When criteria for Type I (α) and II (β) errors are set at 0.01 and 0.10 (90% power), respectively, only 7 mice per group and a total of 126 in the study would be needed to detect the large strain main effect and 22 would be needed to detect the medium-sized lab main effect, but one must test *31 per group* and 558 in the whole study in order to be able to detect the moderate interaction effect. Precisely how many more observations are needed to detect the interaction vs. main effects depends strongly on the specific kind of interaction that is likely to occur.[26]

In the specific case of a small factorial study of two strains in two labs, a general guideline can be proposed if we can agree on a criterion for the size of an interaction that would be considered noteworthy in our field of study. Wahlsten[61,67] proposes that we should certainly want to detect the interaction if the treatment effect on one genotype is *twice as large* as the effect on the other genotype. In this case, one must test *at least six times as many mice* in order to detect the interaction compared with the number needed to detect a substantial main effect. Considering the sample sizes commonly employed in neurobehavioral genetics, many researchers appear to be satisfied with studying main effects and rarely employ sample sizes that are adequate for the evaluation of substantial interactions. The problem is particularly severe for a simple 2×2 design where each effect in the ANOVA has only one degree of freedom.

1.4.2 Contrast Analysis

Some of the more elegant experimental designs in behavioral and neural genetics cannot be evaluated with the usual ANOVA. Consider the reciprocal crossing and backcrossing experiment that can be used to study maternal environment, cytoplasmic, and Y chromosome effects.[46,68,69] As illustrated in Table 1.2, the 16 groups may be arranged conceptually as a 4×4 factorial design, but the main effects and the global interaction term are almost impossible to interpret scientifically. Clarity emerges, however, when specific pairs of groups or linear combinations of group means are compared with each other in a logical series of biologically informative questions, each embodied in a one degree of freedom contrast (see Wahlsten[5,64]).

The challenge of achieving sufficient power for tests of interaction is present for contrast analyses as well as factorial ANOVA methods. The required sample size to detect a particular kind of interaction effect can be determined conveniently with a formula that is a good approximation for the noncentral *t* distribution.[64] When an experiment is to be analyzed with several orthogonal contrasts, it is inevitable that

TABLE 1.2
Factorial Design that Is Better Analyzed with Logical Contrasts

Origin of Mother	Origin of Father			
	Strain A	**Strain B**	**A × B Hybrid**	**B × A Hybrid**
Strain A	1. Inbred	3. F_1 hybrid	5. Backcross to A	6. Reciprocal of cross 5
Strain B	4. F_1 hybrid	2. Inbred	9. Backcross to B	10. Reciprocal of cross 9
A × B Hybrid	7. Backcross to A	11. Backcross to B	13. F_2 hybrid	14. F_2 hybrid
B × A Hybrid	8. Reciprocal of cross 7	12. Reciprocal of cross 11	15. F_2 hybrid	16. F_2 hybrid

Note: Abbreviated contrast analysis; see Sokolowski[68] or Wahlsten[69] for a more complete presentation.

i. Do inbred strains differ? (1 vs. 2)
ii. Is there an effect of genes in groups with an inbred mother? Note that the question whether there is hybrid vigor is logically equivalent to this question. ([1 vs. 3] and [2 vs. 4])
iii. Is there a Y effect in backcrosses with inbred mothers? ([5 vs. 6] and [9 vs. 10])
iv. Is there a Y chromosome effect in F_2 hybrids? ([13 vs. 14] and [15 vs. 16])
v. Is the magnitude of the Y effect different with inbred and hybrid mothers? {([5 vs. 6] and [9 vs. 10]) vs. ([13 vs. 14] and [15 vs. 16])}
vi. Is there an effect of cytoplasmic organelles in backcrosses and F_2 hybrids? ([7 vs. 8] and [11 vs. 12] and [13 vs. 15] and [14 vs. 16])
vii. Is there an effect of autosomal genes? ([3 vs. 5] and [4 vs. 10] and [7 vs. 14] and [11 vs. 13] and [8 vs. 16] and [12 vs. 15])
viii. Is the autosomal gene effect larger when the mother is inbred? {([3 and 4] vs. [5 and 10]) vs. ([7 and 11] vs. [14 and 13])}

a larger sample size will be required to detect some effects than for others. In such a case, the experimenter should choose a sample size for the entire experiment that is adequate to allow detection of the smallest effect that he or she is seriously interested in evaluating. An example of the application of this method to the reciprocal cross breeding design in Table 1.2 is provided by Wahlsten,[64] and other examples are given by Wahlsten.[27,70]

1.4.3 Multiple-Regression Analysis

Factorial ANOVA and contrast analysis are best employed when the study involves carefully controlled treatment conditions given to independent groups of subjects. These kinds of analyses can also be performed using multiple-regression methods. Multiple regression offers the added advantage of being able to incorporate continuous variables in the list of predictors in order to account for the influence of covariates. A model can even include terms to assess group differences in the slopes of response to a covariate or nonlinear trend of response. Along with the elegance of the method come many hazards that can undermine the credibility of an analysis.[71,72] Only one aspect of this very large topic will be discussed here.

In multiple regression, an equation is computed that gives the best prediction or expected value of a dependent variable (Y) from several predictors (X) using the method of least squares: $E(Y) = b_0 + \Sigma b_j X_j$, where b_0 is the Y-intercept when all predictors are zero and b_j is the regression coefficient for the jth predictor. A predictor X may be a "dummy" variable to code the difference between a particular strain and a reference group or an orthogonal contrast in a contrast analysis. One of the most valuable pieces of information disgorged from a computer analysis is the "tolerance" that shows the extent to which the predictors are independent from one another. When the predictors are indeed independent, tolerance is 1.0, the standard errors of the regression coefficients tend to be low, and the multiple R^2 for the entire equation can be decomposed into fractions, each of which is attributable to a single predictor; that is, the model is perfectly additive. When a contrast effect and a covariate are themselves correlated, on the other hand, tolerance will be less than 1.0, sometimes much less, and effects will be confounded, so that the variance can no longer be partitioned into non-overlapping portions. As discussed in detail by Aiken and West,[71] interaction effects in a multiple-regression model will usually have very low tolerance unless each variable is "centered" by expressing it as the deviation from the mean. Instead of coding the interaction term as $X_1 * X_2$, one should use (X_1 – Mean of X_1) * (X_2 – Mean of X_2).

1.5 MOLECULAR TECHNIQUES FOR QUANTITATIVE ANALYSIS

Many examples of G×E interaction have been well documented in laboratory research with strains and mutations in flies, worms (*C. elegans*), and mice, and examples with humans are also well established for several mutations. One lesson from this body of research is that the specific features of the environment that are most influential in altering the consequences of a genetic variant depend strongly on the gene in question. The exquisite specificity of the gene–environment interaction is related to the nature of gene expression at the molecular level. It is therefore necessary that we gain a deeper understanding of this relation through molecular analysis. Perhaps in this way we can also discover more effective means to alter the course of development and devise better therapies for a wide range of mental and behavioral disorders. Thus, the demonstration of G×E interaction with classical methods for studying global effects of differences in heredity forms the foundation for a new direction of research in neurobehavioral genetics.

1.5.1 The Regulation of Gene Expression

Genetic and molecular biological approaches using model organisms such as the fruit fly, *Drosophila melanogaster*, and the mouse have provided a basis for unraveling the complex hierarchical interactions between genes, their RNA, and proteins in certain aspects of development.[73] More recently, nervous system development and function have also become the subjects of genetic and molecular analyses.[49] To make this chapter accessible to a broad audience, we include a brief summary of how genes work and illustrate how the environment may modulate the action of genes

(taken in part from Kandel[74]). Genes are comprised of long strands of DNA and every cell in the body (aside from germ cells) has the same complement of DNA. What makes cells different from each other is that only a small (<20%) subset of genes is expressed in a given cell type. The actual DNA sequences that are transmitted intact from parents to offspring through the generations are not directly responsive to environmental regulation. Rather it is the expression of these genes that is regulated. Gene expression can be regulated by transcriptional control that determines (1) whether or not a gene is transcribed and, if so, (2) the rate at which it is transcribed. Transcription involves the synthesis of RNA from DNA. It is initiated when RNA polymerase binds to the DNA in the promoter region so that nuclear RNA can be made from the DNA. This RNA is then processed and modified into cytoplasmic messenger RNA (mRNA), which is then translated into a protein. Differential protein modification (a posttranslational process) determines which proteins will be retained and function (via activation) in the cell.

Transcriptional control occurs through transcription factors that bind sites in the promoter called promoter elements. Transcription factors can be cell specific or ubiquitous. In some cases transcriptional regulation is thought to proceed when transcription factors form a hierarchy. This results in a cascade of expression of hierarchically arranged transcription factors. For example, studies in development have shown that only a few genes that code for transcription factors can have crucial effects on the expression of many other genes in development.[73]

Other regulatory elements or sequences in the genome are enhancer and response (silencer) elements. These elements can be found either upstream or downstream of the promoter. They contain sequences that bind specific proteins and they are involved in the tissue-specific control of gene expression. When an enhancer-protein complex is formed, it then interacts with the promoter. As a result, proteins involved in multiple signaling pathways can act on a transcription factor bound to a promoter. Signals such as hormones can act on these regulatory elements when, for example, an enhancer binds a hormone responsive transcription factor. Both intracellular and extracellular signals can be environmentally responsive and join with enhancer or response elements to act on the gene's promoter.

The level of transcription of a gene results from the net effect of the factors described above: enhancers, response elements, tissue-specific proteins, and extracellular regulators. This system of gene regulation provides organisms with a versatile approach that enables gene transcription to be superbly sensitive to environmental stimuli.[74] These environmental stimuli can include such complex factors as different learning paradigms and social experiences as well as more easily defined environmental factors such as the pattern of light/dark cycles in circadian rhythms.

1.5.2 Detecting and Localizing RNA

In this section, we focus on techniques that can be used to quantify RNA abundance with particular focus on techniques that can measure differences in RNA expression. Northern Blot Analysis has been the molecular workhorse in providing measures of RNA abundance. It is the only method that provides information about mRNA size and alternative splicing. In Northern analysis, similar levels of total RNA or mRNA

are loaded on a gel, the RNA is transferred to a membrane, and a labeled probe from the gene of interest is applied to the membrane. The abundance of the RNA in each lane is then visualized on film or on a phosphoimaging device. To obtain an estimate of the total RNA loaded and transferred in each lane, the membrane is also treated with a control probe usually taken from a ubiquitous, "housekeeping" type gene (e.g., a ribosomal protein such as *rp49* in *Drosophila*[75]). Good control probes are best obtained from genes expressed at a constant level during development and throughout the organism. The RNA abundance in the sample of interest is then adjusted by its loading control.

Northern blots have been extensively used in analyses of the cycling in RNA of genes involved in circadian rhythms in *Drosophila* and other organisms (for review see Dunlap[76]). The sensitivity limit of Northern hybridization is 1–5 pg of RNA target molecule,[77] and in some instances the sensitivity of Northern blots is not sufficient to detect RNA. This occurs when the amount of tissue sampled is limited and/or the RNA abundance of a particular gene is very low. This might occur when a small subset of tissue such as a brain region is used or in the case of organisms carrying null mutants of a vital gene where early mortality limits the number of samples available.

The localization of RNA transcripts in tissue (whole mount or sections) is done using *in situ* hybridization.[78] However, it is not always useful for quantification of differences in RNA levels between samples. The relative difference in the level of a signal between mutant and wild-type or treated and untreated animals can sometimes be visualized using this technique, but differences in abundance of RNA must be relatively large to be able to quantify these differences. Specifically, problems arise with the insensitive and inaccurate quantification of mRNA expressed at low levels. RNase protection assays[79] enable one to map the transcript initiation and termination sites and intron/exon boundaries and to discriminate among related mRNA of similar size that migrate to similar places on the Northern blot. All of these techniques suffer from low sensitivity.

1.5.3 Real-Time RT-PCR

The reverse-transcription polymerase chain reaction (RT-PCR) has been used to overcome many of the aforementioned problems because RNA of low abundance can be detected in small amounts of tissue. However, RT-PCR is a complex process and as a quantitative technique it suffers from the problems inherent to PCR. These problems include questions about the technique's true sensitivity, its reproducibility, and its specificity. The reproducibility problems that result are difficult to interpret because it is not possible to process controls for every PCR reaction.

A promising technology was recently developed to overcome these difficulties. It is a fluorescence-based kinetic RT-PCR procedure known as Quantitative Real Time PCR. The principle of TaqMan real-time detection is based on the fluorogenic 5′ nuclease assay that allows simple and rapid quantification of a target sequence during the extension phase of PCR amplification. The web page (http://www.applied-biosystems.com/techsupp/tools.html) provides detailed protocols and advice on probe design for this technology. Advantages of this technique are that (a) little

tissue is required, (b) controls (often a housekeeping gene) can be run for each reaction, (c) optimization of the reaction is relatively easy, (d) the technique uses two-gene specific primers and a gene-specific probe that lies within the primers, making the technique highly sequence specific, and (e) with some technologies (the Roche thermal cycler — http://biochem.boehringer-mannheim.com/lightcycler/), the ongoing reaction kinetics can be visualized graphically. Bustin[80] provides an excellent review of the technical aspects of this technique, comparing the conventional and real-time RT-PCR approaches for quantifying gene expression and comparing the different systems commercially available for real-time PCR. The disadvantage of real time RT-PCR has been the high cost, but it is decreasing. In addition, RT-PCR cannot be used to identify differences in expression patterns in unknown genes because it is done using primers from known genes. The Molecular Tools web page at http://www.nlv.ch/Molbiotoolsrtpcr.html#PE compares the various technologies available for real-time PCR.

To our knowledge, analyses of G×E interactions on complex behavior have not yet been published using real-time PCR. In the last year, several studies in a variety of systems have used this technique successfully. These include the analysis of brain homogenates of adult Wistar rats for mRNA expression of the genes *bc1-2* and *bax*, both involved in chemical preconditioning in ischemia,[81] quantification of multiple human potassium-channel genes at the single-cell level,[82] gene expression of neuronal nitric oxide synthase and adrenomedullin in human neuroblastoma,[83] and analysis of gene expression of the D2 receptor in regions of the human brain.[84]

Proper experimental design including replication is crucial for accurately quantifying the relative differences in RNA using real-time RT-PCR. The experiments are designed as in Figure 1.2. It is important to run all G×E treatments and their replicates simultaneously in one randomized block representing one full replication of the experiment. Four independent mRNA extractions for all treatments and replicates comprise the four experimental blocks. This design produces highly reproducible results amenable to statistical analysis. This design mimics our behavioral analyses that test G×E interactions (see Figure 1.2) and enables both sets of data (behavioral and RNA expression data) to be analyzed statistically with analysis of variance.

1.5.4 DNA Microarray Technology

A microarray contains DNA sequences (full or partial cDNA) from both known and unknown genes. This DNA is spotted onto a solid support, usually nylon membranes or glass slides. The array is then hybridized with RNA isolated from different experimental conditions (e.g., mutant vs. wild-type; an environmental treatment vs. a control; drug treatment vs. placebo; experience vs. no experience; immature vs. mature). The expression of large numbers of genes (thousands of genes and in some cases entire genomes) is simultaneously analyzed for each experimental condition so that the expression of each gene in both conditions can be compared. Some genes will be up regulated, others will be down regulated, and still others will not be affected by the treatment. The data are visualized using a reader to detect many fluorescent spots in a grid pattern. Each spot represents one of the DNA clones initially put on the chip. The brightness of the spot gives an indication of the

magnitude of the change in expression and the color of the spot, usually red or green, gives an idea about whether the expression of that gene has been up or down regulated by the experimental treatment. It is important to design microarray experiments and replicate them so that the number of false positives can be minimized, because it can take an inordinate amount of time to sift through these false positives. All positive clones (and often there are hundreds of them) need to be confirmed using an independent technique such as Northern analysis or real time RT-PCR. The sensitivity of the microarray technique is similar to that of Northern Blot Analysis; it is difficult to reliably detect gene expression changes less than 2- to 3-fold on average. This limitation should change as the technology improves. The technique is still very expensive and requires good knowledge of the technology. On the other hand, the DNA microarray technology provides us with the possibility of finding many of the genes and processes involved in the phenomenon of interest.

1.5.5 Microarrays: Experimental Design Issues

The particular design chosen for the microarray experiment is crucial to its success. Advice given in Section 1.3.1 and Figure 1.2 are directly applicable here. If genes are being manipulated, then the genetic background of the strains to be compared should be identical or else many differences in expression will be detected that are not related to the phenotype of interest. For example, if mutant and wild-type are to be compared, the strains should be co-isogenic; this means that allelic variation between the strains should only be in the locus of interest. Similarly, if a transgenic strain is being compared to a mutant or wild-type strain, then the transgene should be on an identical genetic background to the strain of interest. Strains should be reared in an identical fashion, and animals of the same sex and age should be compared so as not to cause gene expression to vary due to uncontrolled environmental factors. Dissections and RNA extraction must also be done under identical conditions. If the design involves an environmental treatment, then it is critical that there be no genetic variation within and between the strains used (as described in Section 1.3.1 above). The ideal situation is to treat the same clone (or group of highly inbred isogenic animals) with the environmental or pharmacological treatment. G×E interactions could be tested on microarrays by using for example the two-way design shown in Figure 1.2.

For instance, in one laboratory we could choose two natural strains of Drosophila flies called rover and sitter that differ only in their allelic composition at the *foraging* gene.[50] We could give each strain one of two treatments (food and water vs. water only) 3 h prior to their RNA extraction. This would give us four groups: rover fed, rover unfed, sitter fed, and sitter unfed. This experiment would be replicated several times so that there are at least three replicates for each array for a total of 12 arrays. The pattern that the four arrays produced could be analyzed for a strain effect, a feeding effect, or an interaction. The interaction would suggest that different strains (rover or sitter) respond differently to the feeding treatment. The response is measured as changes in the patterns of gene expression. For example, rovers may significantly up regulate genes a, b, and d, whereas sitters may down regulate c and d but upregulate b. This approach would uncover the molecular underpinnings of

G×E interactions on food search behavior. It is important to note that in this design DNA microarrays only examine short-term changes in gene expression. It is conceivable that a gene is important for the development of a structure or system that is crucial to the performance of the adult behavior but that this gene is not expressed in the adult stage of development. The role of such a gene in the development of adult behavior would remain undiscovered in the microarray experiment.

Microarray experiments designed to measure gene–environment interactions and changes in gene expression during development require statistical analysis which can handle this type and quantity of data. One decision to be made is what constitutes a significant change in gene expression — a 0.5-fold, 1-fold, or 2-fold change? Obviously, a lower cut-off yields more false positives. On the other hand, some genes that play crucial roles in the process of interest may only show a relatively small fold change, and by setting the cut-off too high, these genes would be missed. Another problem with analysis of microarray data stems from the newness of this technique. Software that enables exploration and statistical analysis of microarray data has been lacking (see Chapters 7 and 8 of this volume). Tools are required that can analyze the expression of individual genes, gene families, and gene clusters, compare expression patterns, and directly access other genomic databases for clones of interest.

A number of very recent studies successfully used DNA microarray analysis to identify changes in gene expression of known and novel genes. As was the case for real-time PCR, there is a paucity of studies that use microarrays to address issues of complex behavior and G×E interactions. The first comprehensive genome scan examined the response of the yeast genome to aerobic and anerobic fermentation conditions.[85] High-density DNA microarrays containing several thousand *Drosophila melanogaster* gene sequences were used to study changes in gene expression during a developmental stage called metamorphosis known to involve an integrated set of developmental processes controlled by a transcriptional hierarchy that affects hundreds of genes.[86] Of the differentially expressed genes found in this study, many could be assigned to developmental pathways known to play a role in metamorphosis, while others were involved in pathways not previously known to play a role in metamorphosis. Still other genes that were identified were novel and had previously unknown functions. Another study found that brains of aging mice showed parallels with human neurodegenerative disorders at the transcriptional level and that caloric restriction, which retards the aging process in mammals, selectively diminished the age-associated induction of genes encoding inflammatory and stress responses.[87] DNA microarrays have also been used to identify differentially expressed genes in purified follicle cells, demonstrating that the technique can be used for cell type-specific developmental analyses.[88] Changes in the expression patterns of >2,000 *Arabidopsis* genes after inoculation with or without a fungal pathogen or after treatment with plant-defense signaling molecules resulted in molecular evidence for coordinated defense responses,[89] suggesting multiple overlapping signal transduction pathways in plant defense mechanisms. The ability to detect interactions between different expression patterns in plant defense mechanisms shows promise for analysis of pathways involved in complex behavior patterns. DNA microarrays have also been used to study expression profiles in multiple sclerosis lesions and

in Alzheimer's disease tangle-bearing CA1 neurons.[90,91] The technologies available for high throughput analysis of gene expression in the human brain are reviewed by Colantuoni et al.[92]

Dubnau and Tully (unpublished data) are using microarrays to unravel changes in gene expression associated with learning in *Drosophila*. They use (a) a genetic manipulation — comparing gene expression in isogenic populations which differ at a single gene that affects learning, (b) an environmental manipulation — comparing gene expression in one homozygous population which has been trained using different learning paradigms, and (c) a pharmacological manipulation — comparing gene expression in one homozygous population where half of the individuals have been treated with a chemical known to alter learning scores. The expectations from their experiment are that: (1) some of the genes and signal transduction pathways identified will be shared in common between all of the treatments whereas others will differ, (2) changes in the expression of genes known to be involved in learning will be identified along with known genes and pathways not previously thought to be involved in learning, and (3) previously unidentified novel genes will be associated with one or several of the treatments. This type of experimental design could in theory be applied to any behavior of interest using a genetically malleable organism.

1.6 SUMMARY

The relations between genes and behavior currently are studied in two ways: differences in behavior between (conspecific) individuals are associated with genotypic differences, and changes in the behavior of an individual are associated with changes in gene expression in the brain. Because these two approaches have historically proceeded independently, there is a major gap in our knowledge of precisely how genes and the environment interact to regulate behavior. Our challenge is to use the new technologies along with the data from the genome projects to unravel the molecular mechanisms underlying G×E interactions involved in the development and functioning of complex behavior.

The abundance, developmental timing, and localization of gene products can influence the probability of a behavior being performed. A predisposition to perform a behavior can be thought of as giving the adult organism a certain probability of performing a behavior under a certain set of environmental circumstances. However, there is a subtle interplay during development between predisposition and experience. Hence, one needs to consider the environment during development that influences gene expression and the environment during adulthood that affects the expression of the behavior of interest. We have discussed statistical and molecular techniques that enable the analysis of G×E interactions. For gene-brain-behavior relationships, however, ongoing feedback from the interaction of the organism with the environment often affects how the brain develops and functions. Performing the behavior itself can cause changes in gene expression and the function of nerve cells.[93] For example, when free-ranging sparrows hear a conspecific's song, this changes the level of ZENK, a transcriptional regulator thought to play a role in song learning.[94] Interactions between mothers and their infants are reflected in changes in brain

neurochemistry during development and across generations.[95] Social modulation of amine responsiveness at particular synaptic sites occurs during lobster aggressive interactions.[96] Species-specific patterns of oxytocin and vasopressin receptor expression in the brain are associated with monogamous vs. nonmonogamous social structure in voles.[97] These complex environmental effects combined with the complexity of the genetic millieu contribute to the tremendous challenge ahead in addressing questions of the molecular underpinnings of gene–environment interactions during the development and functioning of complex behaviors.

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