



Review

The use of norms of reaction to analyze genotypic and environmental influences on behavior in mice and rats

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Abstract

Norms of reaction (NoRs) represent the phenotypic values of genotypes as functions of environmental parameters and permit the visualization of differences in phenotypic response of different genotypes. NoR graphs can be used to analyze interactions between genotypic and environmental factors during development to produce phenotypes in inbred strains of rats and mice. We describe the main features of NoRs, the history of their use in this context, and discuss several applications in behavioral neuroscience. In addition, we give a test for determining whether distinct strains have different NoRs.

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Keywords: Norm of reaction; Reaction norm; Strain differences; Mice; Rats; Inbred strains; ANOVA

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1. Introduction

Inbred rodent lines differ over a range of phenotypes. Some of these strain differences are in the arena of behavioral neuroscience. For example, commercially available mouse strains vary with respect to nociception (Mogil et al., 1996; Kest et al., 1999); the speed and duration of forced exercise (Lerman et al., 2002); the neuroregulatory role of steroids (Phan et al., 2002); the effect of anxiety on learning (Dockstader and van der Kooy, 2001); the anxiolytic effects of benzodiazepines (Griebel et al., 2000), etc. In rats, strain differences have been found in responses to anxiogenic stimuli, as measured by the emergence test (Pare et al., 2002), the open field test (Ramos et al., 2002), and the plus maze test (Ramos et al., 2002). Strain differences also have been reported in the effects of neonatal handling (Aguilar et al., 2002) and environmental enrichment (Fernandez-Teruel et al., 1997) on exploratory behavior; and in prepulse inhibition (PPI), the amount that an auditory prepulse reduces startle magnitude (Swerdlow et al., 2000; Swerdlow et al., 2001; Faraday, 2002). If data such as these are to be used profitably, new tools must be developed for analyzing and visualizing strain differences (Crawley et al., 1997; Anagnostopoulos et al., 2001; Abbott, 2002; Abbott and Knight, 2002). The norm of reaction (NoR), a graphical method that has long been used by researchers without being analyzed systematically (but see Platt and Sanislow, 1988), can serve as one such tool.

We believe that the inferential power of NoRs continues to be underappreciated in behavioral biology in general and behavioral neuroscience in particular. Though we focus on rodent data from which NoRs can be constructed published since Platt and Sanislow's (1988) review, we mention an earlier experiment that illustrates the role of NoRs (van Oortmerssen et al., 1987) but was not discussed in that review. The genotypes we will consider here are inbred rodent (mouse and rat) strains that were produced by 15 or more generations of sibling matings; different alleles are assumed to be fixed in each strain (Silver, 1995).

This review first outlines the history of NoRs with an emphasis on mouse and rat research (see below). We next show how to test the hypothesis that different genotypes have distinct NoRs. This method is relevant to all contexts in which NoRs are used, and is not limited to rodent genetics. We then show how NoRs can be used to evaluate qualitatively the sensitivity of a phenotype to environmental manipulations and genetic factors. We suggest ways in which NoRs may be of use in behavioral neuroscience, from the study of differences between inbred strains and transgenics to research on the effects of androgens and estrogens.

2. The norm of reaction

Norm of reaction (NoR) graphs are two-dimensional graphs depicting data on phenotypes (morphological or behavioral traits) collected in experimental studies, those in which the investigators manipulate the level of the independent variable (Glantz, 2002). A NoR graph shows several curves, each of which represents the response of a particular genotype to an environmental treatment. We call the curves NoRs and the graph containing the curves the NoR graph. The shapes of the NoRs, for instance, whether they are parallel or intersect, can be used to infer important information about genotype-environment ($G \times E$) interactions. Thus, NoRs provide a method for studying the relative importance of genes, environmental factors, and $G \times E$ interactions during individual development.

NoR graphs have five general features that are relevant to the analysis presented here (Fig. 1):

- (i) The x -axis of the graph measures the environmental parameter to which the organism is exposed. This parameter can be categorical in nature such as repetitions of a task. (As experience accumulates, the subject may react differently to iterations of a test.) It may also be an ordinal unit such as the dosage of a drug. Most commonly, it is an environmental factor that can be varied continuously.
- (ii) The y -axis of a NoR graph shows the value of the phenotype subjects display in response to each environmental manipulation.
- (iii) The slope of a NoR shows the strength of the phenotypic response (of a genotype) to a change in an environmental parameter, with greater slopes representing enhanced sensitivity.
- (iv) Analyses based on NoRs require at least three, and preferably more, experimentally determined values for the phenotype. Unless there at least three points, the question as to whether a NoR is linear becomes vacuous. If the phenotypic response is not linear, it can be approximated by a linear NoR.
- (v) To use a NoR graph for inferential purposes, the responses of at least two genotypes must be plotted in the graph, so as to compare their phenotypic reactions to the different experimental regimes. Each data point on the curve represents the mean phenotypic value of a group of animals of the same genotype tested at that level of the environmental manipulation. Different data points that make up the curve representing one genotype may have different error bars because phenotypic sensitivities at different levels of the experimental parameter are likely to vary.

In a previous review, Platt and Sanislow (1988) compared the NoR to a related concept, the 'reaction range'. Sinnott et al. (1950) designated the 'reaction range' as 'the potentially possible or actually realizable phenotypes

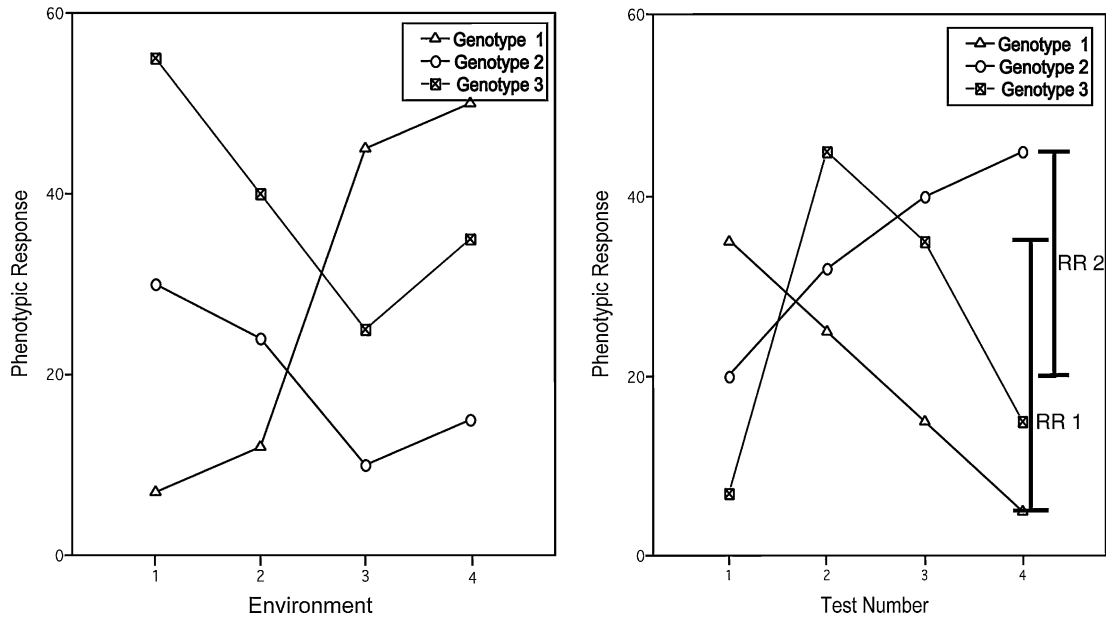


Fig. 1. An example of a norm of reaction (NoR). *x*-axis, environmental manipulation; *y*-axis, phenotypic response. The responses of three genotypes to four levels of an environmental manipulation are plotted in the same graph so that the phenotypes exhibited by different genotypes may be compared. Data points represent the mean phenotypic response of a group of animals with the same genotype tested at one level of the environmental manipulation, not the performance of a single animal. For the right side of the figure, the environmental manipulation is the test number, which measures the effect of experience as animals undergo repeated trials of the same test. Vertical bars (RR) depict the 'reaction range' of genotypes 1 and 2 (see text for details).

of a given genotype' (p. 22). This broad sense 'reaction range' makes it identical to a NoR (Sarkar, 1999). However, in a discussion of the influence of environmental factors on intelligence quotient in humans, Gottesman (1963) used 'reaction range' in a narrower sense, defining it as the difference in the mean phenotypes a genotype manifests in two environments. The reaction range sensu Gottesman ('narrow-sense' reaction range) is a property of the NoR and can be represented as a vertical line alongside the line that delimits the range of phenotypes that a genotype exhibits in response to an environmental regime (Fig. 1). Whereas NoRs simply describe phenotypes that genotypes exhibit over a range of environments, Gottesman viewed the reaction range as a genetic constraint that restricts the phenotypes that a given genotype can develop (Gottlieb, 1995).

Gottesman's diagram of the reaction range was widely reproduced in textbooks and Platt and Sanislow review the use of the concept by psychologists ca. 1950–1988. Our approach will differ from that of Platt and Sanislow in a number of respects. First, we will restrict our discussion to NoRs and ignore narrow-sense reaction ranges. However, unlike Platt and Sanislow, we will not restrict ourselves to studies in which the phenotype being investigated is a measure of memory or learning. In addition, Platt and Sanislow identified methodological problems with some studies that purported to have produced NoRs for rodent strains. Our review will only discuss studies that we think have produced authentic NoRs based on the above criteria. Finally, unlike Platt and Sanislow, we will not speculate

about what NoRs constructed for animal systems might tell us about influence of the environment on human intelligence.

3. History of the NoR concept

Since their introduction in 1909, NoRs have been systematically used to study a variety of plant and animal species. NoRs are particularly easy to construct if the organism reproduces clonally as all individuals have identical genotypes. In the case of sexually reproducing species, the same result is approximated as far as possible through inbreeding across multiple generations to create pure lines.

Historically, the interaction between the genotype and the environment was a principal research concern at the beginning of classical Mendelian genetics. In this context, Woltereck (1909) introduced the NoR technique in a study of morphological responses to environmental differences by different parthenogenetic pure lines of *Daphnia* and *Hyalodaphnia* species from German lakes. He found that various quantitative traits were affected by some environmental factors (e.g. nutrient levels), were independent of others (e.g. ambient temperature) and varied cyclically with others (e.g. seasonality). Though Woltereck's experiments were widely discussed, at least in Germany (e.g. Baur, 1922; Goldschmidt, 1920; Goldschmidt, 1928), the NoR was generally ignored in the genetical literature of the West until 1950. In 1920 Krafka published what are among the first

graphical depiction of NoRs with his studies of eye facet number in *Drosophila melanogaster* and its dependence on temperature for different genotypes (Krafka, 1920). Ten years later, Hersh (1930) used data from Krafka and many other sources to produce several graphical representations of NoRs for nine genotypes (and the same phenotype that Krafka had used). He also tried to provide a mathematical description of the curves (see also Hersh, 1934). Driver (1931) produced similar figures. Neither Hersh nor Driver mentioned Woltereck or referred to NoR graphs.

Using Krafka's data, Hogben (1933) noted that the NoRs for the eye facet number were not parallel, arguing for the 'interdependence' of nature and nurture. This argument effectively shows that phenotypic variation (as measured by variance) cannot be additively decomposed into genotypic and environmental parts because there is a variable interaction between the genotype and the environment. Hogben was arguing against a facile genetic reductionism, a belief that phenogenesis can be entirely explained from a genotypic basis (Sarkar, 1998). While Hogben criticized eugenic proposals to improve allegedly desirable human phenotypes by genetic intervention through selective breeding, the NoR has seen frequent use in debates over the origin of complex human traits such as IQ (Lewontin, 1974).

Meanwhile, in 1918, Fisher (1918) introduced the analysis of variance to decompose the phenotypic variability of a population into genotypic and environmental components, and their interaction. Soon afterwards, and independent of Fisher, Wright (Wright, 1921) distinguished between three genotypic components of variability of a continuous trait: (i) additive effects of alleles at all loci; (ii) effects of dominance at each locus; and (iii) the result of interaction between loci (epistasis). Though Wright had implicitly been using a concept of heritability since the 1920s in analyzing breeding options in various animals (especially guinea pigs), Lush (1943) finally explicitly defined and distinguished between 'narrow-sense' and 'broad-sense' heritability (for a history, see Bell, 1977). The former was the ratio of the component of phenotypic variance (in a population exposed to a specific range of environments) due to additive effects of alleles to the total phenotypic variance. The latter was that ratio for the total genotypic variance.

Meanwhile, the NoR was adopted as part of a conceptual framework of genetics developed in the Soviet Union in the 1920s (Sarkar, 1999). This framework was much less deterministic than its Western counterpart and much more sensitive to the complexities of individual development. That program collapsed with the advent of the Lysenko era but Dobzhansky brought some of its insights to the West when he emigrated to the United States in the 1930s. Thanks to Dobzhansky's influence, starting in the 1960s, the etiology of NoRs became a locus of active research in evolutionary biology. Since then, the most disputed question has been whether there are specific plasticity

genes or whether NoRs acquire their characteristic shapes due to selection on mean trait values (Pigliucci, 2001). Initially, molecular biology, following the example of classical Western genetics, overlooked the $G \times E$ interaction but recently attention has shifted away from an extreme gene-centered view that obscures the developmental biology of the organism.

The earliest rodent experiment of which we are aware that included a NoR graph, though it was not so labeled, was Falconer's analysis of strain differences in lactation (Falconer, 1947). Using his own experimental data and results from earlier studies, Falconer constructed a NoR graph for three inbred strains in which the environmental manipulation was litter size and the phenotypic response litter weight at twelve days of age. The NoR graph suggested that the inbred strains reached an upper limit of milk yield at nine pups per litter because their NoRs approached a horizontal asymptote. A NoR plotted for an outbred strain did not show this pattern. In another early study that constructed NoRs for mice, Lindzey et al. examined the effects of infantile trauma (a loud noise at four days of age) on four inbred strains (Lindzey et al., 1963). From one hundred to 110 days of age, the open-field behaviors of treated mice were compared with those of controls. A NoR graph plotted from these data (x -axis: test day; y -axis: incidence of defecation and urination in the open field) shows that during the initial days of testing the treated animals showed greater emotionality than the controls to the extent that the slopes of the former's NoRs were steeper than those of the latter. Lindzey et al. note that this pattern was not detected by ANOVA because the null hypothesis that the treated and control mice had the same average emotionality scores could not be rejected. Other early experiments with mice for which of NoR graphs were constructed include Thiessen and McClearn (1965) and Fuller (1966).

4. ANOVA vs. NoR

In an experiment involving two factors, A and B, such that A has m levels and B has n levels, two-way analysis of variance tests the following three sets of hypotheses (Sheskin, 1997):

- (1) $H_0 : \mu_{A_1} = \mu_{A_2} = \dots = \mu_{A_m}$
- (2) $H_0 : \mu_{B_1} = \mu_{B_2} = \dots = \mu_{B_n}$
- (3) H_0 : there is no interaction between Factor A and Factor B

If the two factors are genotype and environment, hypotheses (1)–(3) are interpreted as follows. (1) states that the mean phenotypic value of individuals with genotype one is the same the mean phenotypic value of individuals with genotypes two through m . (2) states that the mean phenotypic value exhibited by individuals in environment one is the same

as the mean phenotype exhibited by individuals in environments two through n . (3) states that there is no $G \times E$ interaction. Hypothesis (3) is rejected provided that F_{AB} , the F value for the interaction, is greater than the critical value of F distribution corresponding the significance level (e.g. 0.05). Increasing n and m decreases F_{AB} . Thus, as the number of genotypes (n) and environmental treatments (m) increase, it becomes more difficult to detect $G \times E$ interaction.

For this reason, even when $G \times E$ interaction is patent in the data, two-way ANOVA may fail to reject the null hypothesis of no $G \times E$ interaction. Suppose (3) is not rejected but upon constructing a NoR graph for the data set we notice that the NoRs of different genotypes cross one another or that differences among the NoRs depend on the environment to the extent that the NoRs of distinct genotypes are close to one another at some levels of the environmental treatment but far apart at other levels. In such cases, a model for predicting the phenotype needs to incorporate significant effects of the genotype, the environment, and their interaction, although two-way ANOVA states that the interaction is insignificant. This suggests the following algorithm for detecting $G \times E$ interactions:

- (i) Conduct a two-way ANOVA. If null hypothesis (3) is rejected, stop. Otherwise, go to (ii).
- (ii) Construct a NoR graph so as to provide a quick qualitative analysis of the relationships among the genotypes. If the NoR curves of the distinct genotypes run parallel and do not cross, stop. Otherwise, go to (iii).
- (iii) Test for differences among the NoR curves (see below). If they differ in a statistically significant manner, reject hypothesis (3).

In the following section, we explain how to quantify differences among NoRs in step (iii) and analyze a data set in which the NoR algorithm identifies $G \times E$ interactions not detected by two-way ANOVA.

5. Measuring differences amongst NoRs

A set of NoRs can be said to differ if (i) two or more NoRs in the NoR graph are nonparallel (Pigliucci, 2001) or (ii) at one or more levels of the environmental treatment, the confidence intervals of the NoRs do not overlap. However, neither measure provides a probability that the observed difference between the NoRs results from chance alone. We used a randomization test (Manly, 1997) on a dataset from Swerdlow et al. (2001) to test the hypothesis that differences amongst NoRs representing four rat genotypes were significant. As part of an experiment comparing drug sensitivity in Sprague–Dawley rats from different breeding facilities, rats from each strain were injected with a vehicle and their motor responses in the absence of startle

stimulus were recorded following the protocol of Geyer and Swerdlow (1998) (Fig. 2). We analyzed the movement phenotype using a repeated measures ANOVA (Koch et al., 1988) for movement period. We modeled the movement of rat l with genotype i in trial j as follows: $\text{movement}_{ijl} = \mu_{ij} + s_{i,l} + e_{ijl}$, where μ is the mean phenotype exhibited by the genotype, s is a within-subject factor that records variation in rat l 's performance across trials, and e is an error term. All tests were done with the ANOVA procedure in Intercooled Stata 8.2 (Stata Corporation, 2003). ANOVA revealed a significant effect of genotype ($F_{3,28} = 5.14$, $P = 0.0059$) but no interaction between genotype and trial number ($F_{12,84} < 1$, $P > 0.05$).

The NoR graph constructed from these data reveals a complex relationship between movement and experience, the environmental variable (Fig. 2). The NoR curves of the SDHi, SDHsd, SDHt, and LEH genotypes are not parallel. In particular, the SDHi and SDHsd genotypes vary little in movement across trials whereas the SDHt and LEH rats show more movement with experience up to a threshold (trial three), then moved much less in trial four. This is unexpected because the SDHt strain is related more closely to SDHsd and SDHi than to LEH. Even though the $G \times E$ term in the ANOVA is not significant, these patterns indicate that a type of $G \times E$ interaction is taking place. To predict the magnitude of a rat's movement in Swerdlow et al.'s data set, we need to know: (i) its genotype, (ii) the level of the environmental treatment, and (iii) how its genotype reacts to the current level of the environmental treatment. (iii) represents $G \times E$ interaction, though it is not the same as the definition of the $G \times E$ term in two-way ANOVA. Suppose only (i) and (ii) were used to predict movement in the LEH strain. In this case, the predicted movement phenotype would probably overshoot the actual value because the LEH genotype responds to the final trial by moving significantly less (Fig. 2). Since (iii) as well as (i) and (ii) is needed to estimate the behavior of the LEH rats, a model for predicting movement in this strain needs to incorporate significant $G \times E$ interaction. However, since the $G \times E$ term in two-way ANOVA is not significant, we need an alternative test to measure the interaction.

To quantify the type of $G \times E$ interaction that the NoR graph identifies in Swerdlow et al.'s data set (Swerdlow et al., 2001), we adapted a method developed by Barot et al. (2004) for comparing the NoRs of male and female Atlantic cod. Barot et al.'s procedure tests the null hypothesis that the NoRs of distinct genotypes are the same. In the first stage of the procedure, a shuffling routine (Knuth, 1998; Austern, 1999; Matsumoto and Nishimura, 1998) reassigns genotypes to individuals at random. For each of 10 000 artificial data sets so generated, we carried out the ANOVA procedure and recorded the frequency with which the F statistic associated with the genotype term in the model was greater than in the observed data. Since this was the case less than five percent of the time ($P = 0.0028$), we inferred that there was an effect of genotype in the observed data

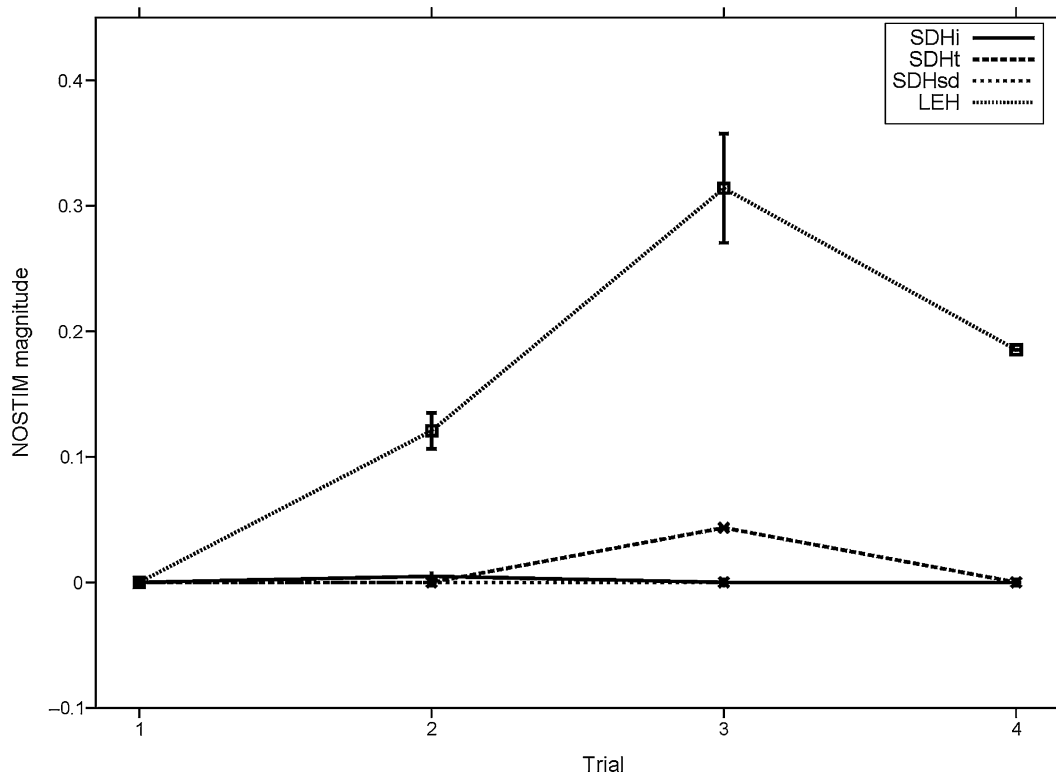


Fig. 2. Effect of trial number on NOSTIM magnitude. The data are from N. Swerdlow, personal communication, based on Swerdlow et al. (2001); x-axis, NOSTIM block; y-axis, NOSTIM magnitude (see text for details). Error bars show bias-corrected 95% confidence intervals based on 10,000 bootstrapped replicates.

and that differences amongst the NoRs were statistically significant. Barot et al.'s test fills in step (iii) of the algorithm described in the previous section. If, as in Swerdlow et al.'s data set, the $G \times E$ term in the ANOVA is not significant but NoR graph suggests that $G \times E$ interaction is taking place, we can employ Barot et al.'s procedure as an alternative means of quantifying that interaction.

6. Environment and phenotype

While it is conceptually incorrect to assume that the genotype and the environment contribute additively to phenotype in such a way that the relative contribution of each can be quantitatively identified (Platt and Sanislow, 1988; Sarkar, 1998; Wahlsten, 1990), in some instances it is appropriate to claim that the environment is the more important influence on the phenotype than the genotype. We first describe two simple examples and then use a NoR graph to analyze a third, more complicated, one.

The effect of diet on body weight in mice provides the first example. The New Zealand Obese (NZO) strain exhibits juvenile-onset obesity (Thorburn et al., 2000) due to increased hypertension and insulin resistance (Ortlepp et al., 2000). The Nonobese Nondiabetic (NON) strain is

glucose intolerant but usually not obese (Hasegawa et al., 1992). However, nutritional cues can reverse the body weight phenotypes typical of these strains. If NON pups are fostered to an obese lactating (NON \times NZO) F1 female, they become obese, probably because of the foster mother's milk (Reifsnnyder et al., 2000). Similarly, NZOs can be made lean by the dietary supplement CL (Koza et al., 2004). Intracerebroventricular infusion of leptin also reduces weight in NZOs and A^y , another genetically obese strain, though the latter require a 100-fold higher leptin dosage than NZOs (Halaas et al., 1997). In these cases, the developmental history of environmental factors, such as the milk ingested by the neonate, influences the body weight phenotype more strongly than does the genotype.

Phenotypes that one might assume to be 'innate' in mice and rats, such as mating behaviors, are strongly influenced by environmental cues and can be altered by conditioning (Domjan and Holloway, 1998). Similarly, in rats the quantity and quality of the mother's care has profound effects on the behavior and underlying neurochemical mechanisms of her pups when they reach adulthood. Thus, the pups of a dam that licks and grooms her offspring with a low frequency and rarely arches her back while nursing (low LG-ABN dam) behave similarly toward their own offspring (see Meaney, 2001 for a review). Pups of low LG-ABN dams are also more fearful and show greater hypothalamic–pituitary–adrenal

(HPA) stress reactivity than the offspring of high LG-ABN mothers. However, if the pups of a low LG-ABN dam are handled postnatally or fostered to a high LG-ABN dam, they will lick, groom, and arched-back nurse their own pups with the same frequency as the offspring of a high LG-ABN mother. The LG-ABN phenotype, rather than being determined exclusively by the genotype, is sensitive to environmental influences such as the behavior of the mother to which a female pup is fostered.

A third example a phenotype being significantly affected by environmental manipulations is provided by an experiment of van Oortmerssen et al. (1987) who studied the effects of testosterone (T) implants of various lengths on the weight of the seminal vesicles (SV) in castrated male mice of two strains, SAL and C. The C or 'control' line consisted of wildtype house mice whereas the SAL group was produced through 16 generations of artificial selection for aggression, measured as latency to attack an intruder male. van Oortmerssen et al. found that when both groups receive comparable T dosages, SAL males attack intruders more quickly, suggesting that genetic differences between the strains explain differences in aggression. However, the SAL and C strains do not differ in terms of all the phenotypes investigated. A NoR graph drawn from van Oortmerssen et al.'s data shows that the strains' mean SV weight is about the same when they are administered similar levels of T because the NoRs representing the two genotypes almost overlap (see Fig. 3). With respect to absolute SV weight, the influence of genetic factors proves to be less significant than that of environmental factors such as castration and T replacement in the determination of the phenotype.

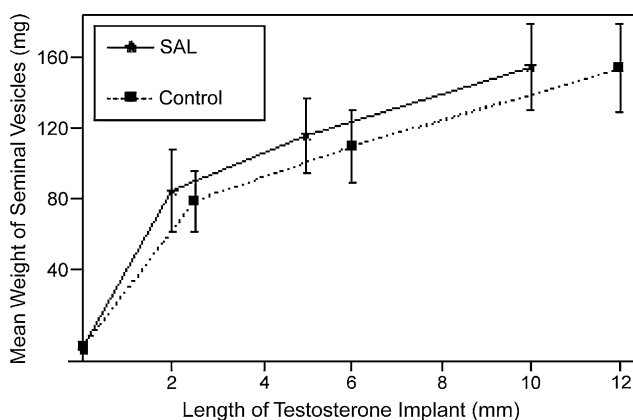


Fig. 3. NoR in which the influence of the environment is qualitatively greater than the influence of the phenotype. The data are from van Oortmerssen et al. (1987), (p.144, Table 2); x-axis, length of testosterone implant; y-axis, mean weight of the seminal vesicles. C and SL males received implants of different lengths because the strains differed in body weight so that the capsules placed in C males needed to be twenty percent longer than those implanted in SL males in order for both groups to receive the same amounts of T (van Oortmerssen et al., 1987, p. 141). In this and all subsequent graphs, the error bars are standard errors of the mean.

7. Strain differences

The traditional model of strain differences holds that differences between animals of the same inbred strain are caused by environmental factors, whereas differences between animals of different strains are caused by genetic factors (Mogil et al., 1996; Belknap et al., 1998). According to this model, if two inbred strains exhibit different phenotypes in the same environment, then the phenotype is under genetic, not environmental, control. NoRs identify several shortcomings of this model. First, a trait may differ between inbred strains in one environment but not in another. If, due to sampling bias, the inbred strains were observed when they were in an environment in which the trait differed between them, it might be concluded that the trait was controlled by genetic factors. For example, two genotypes might exhibit similar phenotypes at one temperature but different phenotypes at all other temperatures (Lewontin, 1974). Analyzing the effects of LSD on back muscle contractions in male rats, Ouagazzal et al. (2001) determined that it is only at the highest dosage of the drug (0.3 mg/kg body weight) that the SD and Wistar genotypes differ significantly in the number of contractions. If the two strains are observed at only the 0.3 mg dosage, it might be erroneously concluded that the muscle contraction phenotype is controlled exclusively by genetic factors, with the environment playing no contributory role. NoRs can be used to visualize the response of each genotype to each level of drug treatment and to specify the exact dosage at which two strains react differently to the drug (Fig. 4).

NoRs also point out a second flaw in the traditional model. A phenotype may differ between two inbred strains in all environments, but the extent to which it differs may depend on the environment. In such a case, it would be appropriate to say that the trait is partially genetically determined, insofar as it differs between inbred strains. But such a trait is also under environmental control, since the degree to which it differs between the strains varies across environments. To determine strain differences in the anxiolytic effects of benzodiazepines (BZs), Bert et al. (2001) administered diazepam to male Fischer rats and Wistar rats and then subjected the animals to an anxiogenic task, the elevated plus maze (Fig. 5). Among the behaviors investigated was the number of times animals from each strain reared up in a plus maze, which serves as a measure of novelty-seeking and exploratory behavior (Fernandez-Teruel et al., 1997). Bert et al. (2001) found that the threshold dosage at which rats receiving diazepam rear less than control animals depends on genotype; for instance, Fischer rats from the Harlan supplier rear less than control animals from the Fischer strain at the 2.0 mg/kg dosage whereas Harlan Wistars rear less than controls at 3.0 mg/kg. The strains' responses to diazepam can be further characterized by preparing a NoR from the dataset (Fig. 5). The NoR demonstrates that at three dosages of the chemical, Fischer rats differ from Wistar rats from two suppliers (Harlan Labs

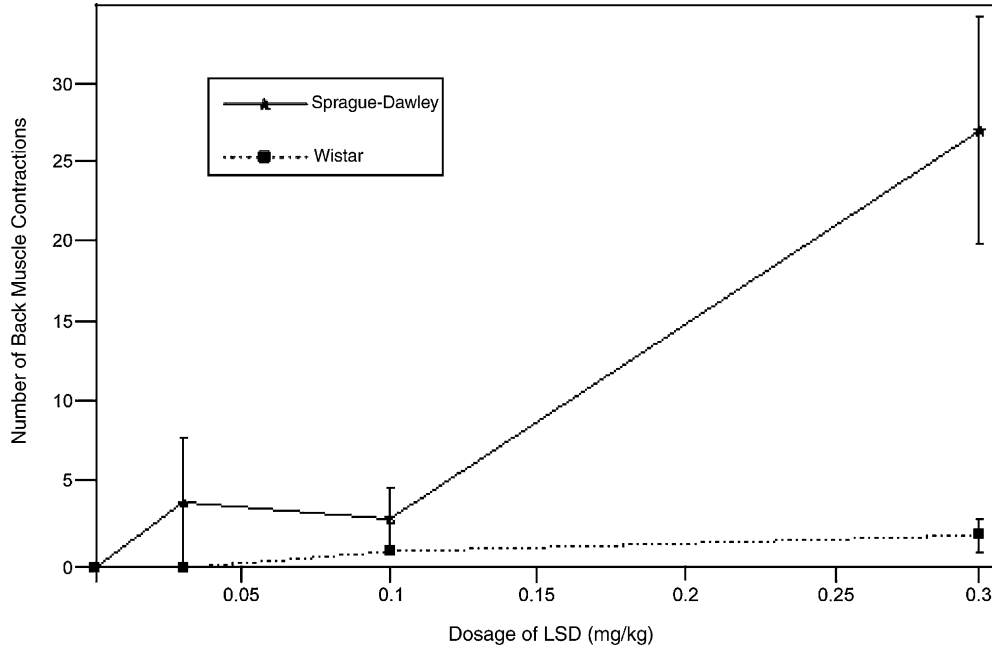


Fig. 4. Effect of LSD on back muscle contractions in male Sprague–Dawley (SD) and Wistar rats. The data are from Ouagazzal et al. (2001); x-axis, dosage of LSD; y-axis, number of back muscle contractions. The phenotypes that the two genotypes exhibit only differ significantly at the highest dose (0.3 mg/kg), demonstrating that different strains’ responses to the same environmental treatment cannot be assessed accurately unless the phenotype exhibited by each genotype is recorded at several levels of the treatment.

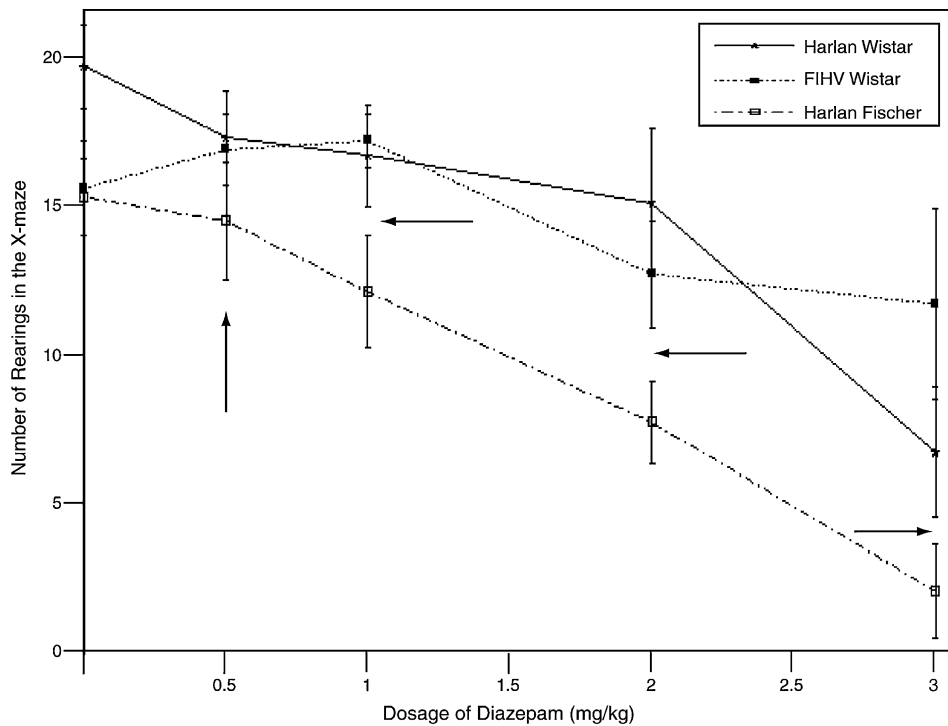


Fig. 5. NoR in which a phenotype varies between strains to different extents in different environments. The data are from Bert et al. (2001); x-axis, dosage of diazepam; y-axis, number of times the animal reared up in the X-maze; Male Fischer 344 rats from Harlan labs (Harlan Fischer) differ significantly in the number of rearings in an X-maze in comparison to Harlan Wistar males and FIHV Wistar males at three dosages of diazepam (horizontal arrows). However, at the 0.5 mg/kg body weight dosage, the number of rearings of the Wistar and Fischer animals do not differ because the confidence intervals of the lines representing the Fischer, Harlan Wistar, and FIHV Wistar strains overlap (vertical arrow).

and FIHV labs) in frequency of rearing in the plus maze, since the confidence intervals of the line representing the Fischer genotype do not overlap with the confidence intervals of the lines representing the Wistar populations. But the NoR also shows that at two other dosages of diazepam, Fischer rats either do not differ from the Wistars, or differ from Wistars from one supplier but not the other. Thus, while genetically distinct strains differ with respect to the rearing phenotype in most of the environments investigated in the study, the extent to which the strains differ depends on the level of the experimental manipulation. NoRs such as this one show that the view that genetically determined traits are those that differ between inbred strains (Mogil et al., 1996; Belknap et al., 1998) needs to be supplemented by an understanding of the differential expression of traits across environments.

NoRs can also be used to classify strains into qualitative classes and identify the strains whose responses to the environmental manipulation differ the most. Belknap et al. (1998) studied the effects of morphine on open field activity and body temperature in 15 inbred strains so as to provide a more detailed understanding of the response of mice to opioids (most previous studies had measured these traits in only the C57BL/6J and DBA/2J strains). Another aim of the study was to increase the statistical power of heritability estimates, which become more sensitive at detecting genetic correlations when a large number of strains are tested. Morphine was found to have no effect on open field activity in C3H and CE mice, increase activity in BALB/c, 129, C57BL/6, PL, and CBA and depress activity in DBA/1, DBA/2, and A/He animals. By providing a graphic depiction of the relationship between morphine, the environmental manipulation, and open field activity, the phenotypic response, a NoR plotted from these data offers additional information about the relationships between the strains being investigated. For instance, genotypes that produce lines with similar slopes and shapes can be said to be responding similarly to the drug and, conversely, a genotype that generates a line with a large positive slope (e.g. 129/J) can be said to respond to morphine quite differently than a genotype that produces a line with a large negative slope (e.g. DBA1/J).

Strains often differ with respect to a phenotype in some environmental contexts but not in others. Cabib et al.'s (Cabib et al., 2000; Gerlai, 2000) investigation of the effect of a starvation diet on drug preferences provides an example. DBA/2J mice show an aversion to, whereas C57/BL6J exhibit a preference for, amphetamines when food is available ad libitum. But when food is restricted for eleven days, the DBA group exhibited an amphetamine preference like that of the other strain. Within the same strain, knockout, heterozygous, or wildtype individuals may exhibit similar phenotypes in some, but not all, environments. For example, estrogen receptor alpha α knockouts have been behaviorally characterized under the assumption that it is the gene's product, and not the environment in

which the individual develops, that is the sole determining factor of aggression and mounting behaviors. However, Crews et al. (2004) demonstrated recently that the postnatal environment plays a modulatory role and that behavioral differences were more sharply defined when litters were restricted to single sex and limited to only knockout and wildtype pups. When knockouts, heterozygotes, and wildtypes differ with respect to a phenotype in some environments but not in others, two patterns will be visible in the NoR graph. First, the NoR curves will have the same sign and the difference between the slopes of the NoR curves will be small along the intervals of the environmental treatment in which the distinct genotypes show similar phenotypes. Second, along the intervals of the environmental treatment in which different genotypes show different phenotypes, the difference in the slopes of the NoR curves will be large (if the slopes have the same sign) or the slopes will have different signs. These two patterns will also be present in NoR graphs representing different strains if the strains show different phenotypes under some environmental regimes but similar phenotypes along other intervals of the environmental treatment.

Finally, NoR graphs can describe physiological as well as behavioral traits. To explain the delayed response to axonal injury characteristic of Wallerian degeneration slow (*wld^s*) mice, Araki et al. (2004) constructed seven RNA knockdowns from a *wld^s* background. Each was deficient in a distinct protein deacetylase hypothesized to protect axons. Araki et al.'s NoR graph (environmental treatment: time; phenotype: remaining neurites) suggests that the protein SIRT-1 provides the most protection because the neurite loss was consistently greatest in the SIRT-1 knockdown. NoRs graphs may also be used to assess the effects of androgens and estrogens on inbred rodent strains, which differ with respect to brain steroid receptors and the sensitivity of the central nervous system to steroids (Phan et al., 2002; van Oortmerssen et al., 1987; Ogawa et al., 1996). Strain responses to testosterone are organ specific and depend on the castrated or intact status of the animal, such that the hormone may effect organ and tissue weight in one inbred line more extensively than it does in another. In a study designed to test the effects of different levels of exogenous estrogens on the male reproductive system, Spearow et al. (1999) administered various dosages of estradiol (E2) to mice of the inbred S15/JIs, C17/JIs and C57BL/6JB6 strains and the outbred CD-1 strain and found that males from strains selected for large litter size (S15JIs and CD-1) are less sensitive to exogenous E2 than males of other genotypes. A NoR included in Spearow et al.'s article indicates that while testes weight decreases monotonically with increasing dosage of E2 in all four strains, the change in the slope of the NoR representing the CD-1 genotype is much more gradual than the changes observed in the other groups, providing visual confirmation that CD-1 is least susceptible to endocrine disrupters. (Spearow et al.'s paper does not include the data needed to do redraw their NoR

here). Spearow et al.'s NoR demonstrates that the response to exogenous E2 depends on the genotype of the strain being studied and the dosage of the hormone, factors which, they argue, should be taken into consideration when animal systems are being selected to test the safety of products containing estrogen.

8. Conclusions

Despite their extensive applications in neuroscience, NoRs have seldom been examined theoretically within the field. Our goals have been to discuss examples of NoRs in neuroscience research since Platt and Sanislow's 1988 review and to propose further uses of the technique by examining behavioral measures such as aggression and sexuality that lend themselves to being analyzed by means of NoRs.

NoR graphs sometimes bring out patterns overlooked by ANOVA, such as the finding that treated mice initially show greater emotionality in the open field (see above). In our analysis of Swerdlow et al. (2001) data set, we argued that although the $G \times E$ term in two-way ANOVA was not significant, the NoR graph highlighted patterns in the responses of the genotypes to the environmental treatment that can be construed as $G \times E$ interactions in a broad sense. It might be objected that this broad-sense $G \times E$ interaction, since it is less precise than the definition of $G \times E$ interaction in ANOVA, does not justify the use of NoR graphs. However, even if NoR graphs are not used as an alternative to ANOVA for measuring $G \times E$ interaction, they can complement two-way ANOVA if the two methods are used together. The NoR curve for a genotype consists of points connected by lines. The x -coordinate of each point is the level of the environmental treatment. The point's y -coordinate is the mean phenotype that several individuals of that genotype show at one level of the environmental treatment. The lines that link the points are the most informative aspect of the NoR curve. The slopes of these lines report how the genotype responds to a range of environmental treatments (for instance, whether the phenotypic value increases monotonically with the environmental treatment). The lines also allow us to infer what phenotype the genotype would show at levels of the environmental treatment for which we do not have experimental data. Suppose we focus on a single level of the environmental treatment and look only at the points that lie above this value on the x -axis of the NoR graph. Then we can use two-way ANOVA to test the null hypothesis that all genotypes exhibit the same mean phenotype at the current level of the environmental treatment (as well as two other hypotheses listed above). For instance, Wei et al. (2004) constructed a NoR graph to analyze the effect of the antidepressant desipramine (DMI) on immobility in the forced swimming test, a behavioral measure of despair (Porsolt et al., 1978) (x -axis: DMI dosage, y -axis: seconds of immobility).

At each dosage of the chemical, two-way ANOVA was used to compare the average duration of immobility in wildtype mice and transgenics overexpressing the glucocorticoid receptor (GR); in each case, the null hypothesis of equal immobility was rejected. Used in this way, two-way ANOVA zeroes in on each level of the environmental treatment but does not report the pattern of phenotypic responses across a range of environmental manipulations. Wei et al.'s NoR graph shows that, over the range of environmental treatments, movement frequency increased with DMI dose in transgenics, whereas the wildtypes have a flat NoR curve. This suggests that mice overexpressing GR are hypersensitive to antidepressants. Only performing the two-way ANOVA would not reveal this pattern, but the ANOVA complements the NoR method by evaluating specific hypothesis at each point along the x -axis of the NoR graph.

NoR graphs are most informative when several strains are examined because the slopes and shapes of the NoR curves of multiple genotypes can be analyzed. However, in the neuroscience studies we have surveyed from which NoRs can be assembled, only two or three inbred strains were typically used. Though practical considerations restrict the number of genotypes and environmental manipulations that can be employed in any experiment, NoR graphs could be used more profitably in neuroscience if more genotypes were represented.

Whether the environmental manipulation being investigated is the effect of experience or that of a pharmaceutical agent, NoRs provide a novel way to visualize relationships between genotypes. With the proliferation of transgenic strains and techniques such as quantitative trait locus mapping (Bergeson et al., 2001) that will refine our understanding of strain differences, new uses will continually be found for this ubiquitous graphing approach.

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