Principles for interpreting interactions among the multiple systems that influence food intake

RANDY J. SEELEY AND TIMOTHY H. MORAN

1Department of Psychiatry, University of Cincinnati, Cincinnati, Ohio 45267-0559; and 2Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Seeley, Randy J., and Timothy H. Moran. Principles for interpreting interactions among the multiple systems that influence food intake. Am J Physiol Regulatory Integrative Comp Physiol 283: R46–R53, 2002; 10.1152/ajpregu.00021.2002.—The widespread use of molecular biological tools has led to a pronounced increase in the number of signals that are now implicated in the controls of food intake and body weight. However, a complete understanding of the roles of these multiple signals requires that we assess how they interact with one another to alter ingestive behavior. Although many such experiments are being conducted, the methods employed to delineate these interactions are often fraught with interpretive difficulties. The purpose of this article is to explore these difficulties and offer practical advice for minimizing these issues in experiments that seek to explore the important interactions among these signals. In particular, the issues of additive vs. nonadditive results, the use of sub- or suprathreshold dose combinations, and the choosing of multiple-dose analyses are all addressed. Furthermore, the possibilities of using intake measures other than cumulative intake and complementary nonbehavioral endpoints are encouraged.

dose-effect curve; leptin; multiple-dose combination; obesity; peptide

WE HAVE ENTERED AN IMPORTANT ERA of research on the physiological controls of food intake. The revolution of molecular biology has brought us an embarrassment of riches by identifying dozens of potential controllers of food intake. Recent advances in high-throughput genomics and bioinformatics are likely to turn this torrent of new hormones and transmitters into a veritable flood. It is tempting to think that the mere act of adding a new protein to the list of things that either suppress or enhance food intake means that we understand more about how food intake is controlled than we did before. Although adding to the list is vital information, the mere addition should not be confused with understanding. To understand how food intake is controlled, not only must we know about the potential proteins but we must also know how these proteins interact with one another to influence complicated and diverse sets of behaviors that control the ingestion of food. Most researchers understand this issue either implicitly or explicitly, and hence more and more studies try to delineate the overlap between systems in a variety of ways. However, such experiments are fraught with interpretive difficulties that can result from poorly chosen experimental designs. In this article we will attempt to lay out some of the more common difficulties inherent in studying the interaction of different controllers of food intake. The issues raised for the control of food intake are of course common to many systems that have multiple controls. However, we have specifically chosen to address this issue for food intake because of the rapidly growing body of literature that centers on this important clinical issue.

Although no single experimental approach can answer every issue, we hope to give researchers some practical advice about experimental designs that minimize some of these interpretive difficulties. A few general rules for increasing interpretative power can be stated up front. Although frequently used, data from experimental designs involving single-dose combina-
tions are particularly difficult to interpret. Examining how the addition of one agent modifies the feeding dose-effect curve of another significantly improves the interpretability of the data arising from such interactions. The use of ingestion testing paradigms with multiple dependent variables significantly facilitates interpretation. Finally, the addition of electrophysiological or other indexes of cellular activation to measurements of changes in food intake can greatly aid in the interpretation of how (and where) signaling systems involved in the controls of food intake may interact with one another.

Interpretation of drug interactions has long been an issue in pharmacology (2, 9, 11, 13). Although progress has been made in identifying ways of presenting, analyzing, and interpreting data from experiments involving multiple compounds (13) or single compounds with multiple sites of action (12), this continues to be an area of some confusion and controversy. Different investigators have used different terminology, and the models used have assumptions that may not generalize to all situations. However, much of this work has relevance for the present discussion, and we will refer to this work where appropriate.

DOSE COMBINATIONS

The first generic example is one in which the experimenter uses single doses and one dependent variable, a design too frequently employed. If a researcher was interested in the potential interaction of system A with system B, one approach might be to administer an agonist for system A and combine it with an agonist of system B. For argument’s sake, let’s imagine that exogenous administration of agonist A results in decreased food intake while agonist B results in increased food intake. A common way to test the hypothesis that these two systems interact in the control of food intake has been to give a dose of agonist A that suppressed food intake and see if that effect can be reversed by agonist B. Hypothetical results of such an experiment are shown in Fig. 1. If agonist B either partially or completely reversed the effect of agonist A, it is tempting to conclude that systems A and B must interact in the control of food intake.

The ability to draw a meaningful conclusion to this experiment, however, is limited by several issues. The first issue is whether the results represent additivity or nonadditivity of the effects of the two systems (1). While it is literally true that system A and system B must interact in the control of food intake, it need not be true other than at a trivial level. Both agonist A and agonist B ultimately produce changes in ingestive behavior and therefore both must influence output motor pathways that control ingestive behavior. Consequently, they have to interact at this level, but this conclusion could have been drawn simply by knowing that the two compounds influence food intake. The real issue here is that the independent variable being measured (food intake) is a product of a number of processes that converge on the act of ingestion. That act is limited by the fact that organisms have a single mouth. Hence, when cumulative food intake is the measure and one signal tells the animal to eat less and another, nonrelated signal tells the animal to eat more, there is only one way for the animal to reflect those two influences and that is to consume an intermediate amount of food. Consequently, the default expectation for two such treatments should be that these independent effects would be additive, and such a result therefore provides very little in the way of new information about how systems A and B might interact.

The potentially informative outcome of this experiment then is not additivity but rather nonadditivity. This could happen in one of two ways shown in Fig. 2. Agonist B could have little or no effect on the ability of agonist A to suppress food intake (Fig. 2A) or the ability of agonist B to increase food intake may have superseded the effect of agonist A to reduce food intake (Fig. 2B). Such an outcome would violate additivity and provide some clues about the relationship between systems A and B. As hopefully is clear from this example, interpreting these experiments is counterintuitive. At first blush, the results depicted in Fig. 1 would seem to indicate that systems A and B interact. Rather, it is the situation in Fig. 2 where it looks like the presence of agonist A has no impact on agonist B (or visa versa) when additivity is violated. The logical conclusion of such results is that the presence of agonist B actually prevents (or enhances) the ability of agonist A to influence food intake over and above the expected additive effect of the two treatments, and that represents “interaction” in a way that cannot be explained merely by two opposing actions that impact the same motor pathway. Such results may imply that one system is downstream from the other. Modulating the activity of the upstream system loses its effectiveness when the downstream system is independently manipulated. As a practical manner, trying to statistically prove whether two suprathreshold effects were additive or nonadditive is extremely difficult. For example, in Fig. 2A, one would need to demonstrate that the difference between the control condition and the combined treatment condition was larger than the algebraic sum of the difference between the control condition and agonist A and the difference between the control condition

![Fig. 1. Theoretical results from an experiment using a single dose of 2 compounds and the combination: agonist A that decreases food intake and agonist B that increases food intake. Results depicted are strictly additive.](http://ajpregu.physiology.org/Downloadedfrom)
and agonist B. Such calculations involve combining several measures each with their own associated variance. As a result, the derived measures have even larger variances, greatly limiting the power of the comparisons and making the null hypothesis of additivity a difficult one to reject under most experimental conditions. One strategy that has been commonly used to get around this issue has been to use a dose for one of the two treatments that is subthreshold and consequently has no effect on the amount of food consumed. This design has the advantage that to prove there is “nonadditivity” may be statistically easier because one treatment has no effect. Consequently, if the effect of agonist A is different from the effect of the combined agonists A and B, it would provide evidence for a significant interaction between these two systems, meaning that rejecting the hypothesis of additivity might be just a simple statistical comparison between two groups (see Fig. 3).

Although outcomes that show that two treatment effects on food intake are nonadditive are potentially the most informative, there are a number of reasons why for any given experiment the results might be nonadditive but do not indicate an actual interaction between the two systems. The first potential confound are ceiling and floor effects. For example, if two treatments are used that both suppress intake, it could be that strict additivity would result in negative intake (see Fig. 4A). Because it is unlikely for the subject to give back food that has already been consumed, this situation is likely to yield nonadditive results that do not reflect an interaction between the two systems. Although this is the most trivial and obvious example of such ceiling and floor effects, they appear in other circumstances as well. There are numerous conditions when the lower limit an animal might eat under a given set of circumstances is not zero. Hence, the floor for intake and the potential for getting nonadditive results may not be at zero intake. Likewise, when...
dealing with treatments that increase food intake, there may be both physical and physiological constraints that limit food intake from going above a certain level. As a consequence, results may appear to be nonadditive that do not reflect interaction among the systems.

One way to limit the possibility for ceiling and floor effects is to carefully choose doses (or other parameters of the manipulation being used) such that the effect on food intake of each treatment is small. The obvious extension of this experimental strategy is the subthreshold design discussed above. By choosing a dose of one of the treatments that has no effect on its own and the dose of the other treatment that has a small but reliable effect, one lessens the possibility that potential nonadditive effects are the result of ceiling or floor limitations. However, to choose such appropriate doses requires extensive pretesting. Such pretesting can be quite laborious because dose-effect curves for food intake are notoriously dependent on a number of factors, including the efficacy of the specific batch of compound being used and small changes in age, body weight, temperature, humidity, handling, and delivery of the treatment. Consequently, such dose-effect curves often have to be reestablished for each group of animals before the formal interaction test. This is particularly true for the subthreshold dose because even nonsignificant changes in food intake can make clean interpretations difficult. Moreover, it is possible that the dose used is so low as to result in no change of the effect of the other treatment. Hence, there is a strong need for substantial dose-effect curves to identify the highest dose that is still clearly subthreshold.

Even when the potential confound of ceiling and floor effects is eliminated, there are still other issues even when using the subthreshold dose. In our hypothetical example, it is still possible that the effect of treatment A to decrease food intake alters the baseline intake and that from this new baseline, the dose-effect curve for agonist B is different from what it was when the dose was determined to be subthreshold. Looking at Fig. 3, it may simply be that the chosen dose of agonist B to stimulate food intake is subthreshold when the baseline intake is 10 but that it is not a subthreshold dose when the baseline intake is 5. After all, it is not hard to imagine that it is more difficult for the same dose of agonist B to increase food intake from 10 g to 15 g than it is to increase food intake from 5 g to 10 g. Although both situations involve an absolute increase in intake of 5 g, to add that 5 g on top of a baseline of 10 g involves overcoming significantly higher levels of a variety of negative feedback signals than increasing intake 5 g on a baseline of 5 g.

The bottom line is that even nonadditive results using a subthreshold dose still leave open an alternative interpretation. One way to address this issue is to show that the dose of agonist B is still subthreshold if baseline intakes are somehow reduced to levels that are equivalent to what occurs under agonist A (10). One method for lowering the baseline might be to change the paradigm (i.e., from using fasted animals to fed animals or changing the food to something less palatable) such that the baseline intakes are now lower. Changing the parameters of such behavioral experiments, however, runs the risk of changing the nature of the interaction between systems A and B. It is possible that the interaction between the two systems is more important under some circumstances than it is under others.

The safer path is to use a third treatment that the investigator has reason to believe does not interact with system B. As depicted in Fig. 4B, if one chooses a dose of agonist C that suppresses food intake equivalently to agonist A but whose effect cannot be reversed by the same dose of agonist B that reversed the effect of agonist A, it makes a strong case that the ability of a subthreshold dose of agonist B to alter the effect of agonist A is a result of an important interaction between systems A and B and not merely the result of baselines shifting the dose-effect curve for agonist B. This result also speaks importantly to the issue of specificity. The demonstration that agonist B does not interact with system C means that system B does not interact with everything that potentially influences food intake, and therefore the interaction between systems A and B is not merely a result of the additivity encumbered by having only one mouth.

Although some of the above suggestions may increase the probability of correct interpretation from single-dose combinations, examinations of the results of individual dose combinations run into the general problem that dose-response curves in biological systems are rarely linear. The standard dose-response relationship is S shaped. There are doses below which no effect is obtained and doses above which no further effect is seen. Above threshold, increasing dose has only a minimal effect at first, larger more linear effects in the midrange, and smaller effects again near the dose maximum. (For further background on dose-effect relationships, see Ref. 14.) The predictions from adding doses of two compounds depend in part on where on the dose-effect curve such doses are. The simplest way to think about this issue is predicting the results of adding two doses of the same compound. Two doses from the lower tail of the dose-effect curve when added would have an effect greater than the simple sum. Two doses from the upper end would be expected to have less of an effect than their sum. With such a complication from adding the effects of two doses of a single compound, the interpretation from adding two doses of different compounds acting on different systems is even more complex.

Analyses involving how one agent changes the dose-response function of another are more informative. Getting back to our original example, one can examine how the presence of agonist B (typically at the EC_{50} or IC_{50} dose), which decreases food intake, modifies the ability of a dose range of agonist A to increase intake. Potential outcomes of such an experiment are shown in Fig. 5. Data are graphed as percent maximal response to agent A across a dose range. The line labeled “A only” is the dose relationship in the absence of agonist B. The
addition of a dose of agonist B can have a number of possible effects. The simplest is when there is no effect and the dose-response relationship for agonist B is unchanged. Such a result might again imply that the actions of agonist B are downstream from those of agonist A. There is also the possibility that agonist A acts as if it were a direct antagonist for the actions of agonist B. Such a result would produce a linear shift of the dose-response relationship to the right as shown with line 1. A more complicated outcome is shown in line 2 in which the presence of agonist A has different effects depending on the dose of agonist B. Similar patterns of results can be obtained with the use of two agents that both reduce (or increase) food intake. The presence of one agent may shift the dose-response curve for the other to the left so that lower doses of agonist B are more effective. Again, the effect of compound A may depend on the dose of agonist B.

Such experiments do involve more work. Multiple doses need to be assessed, meaning repeated testing or the use of multiple groups of animals.Repeated testing may become an issue for injections directly into brain tissue where there tends to be accumulating tissue damage with multiple injections. It is also possible that some types of tachyphylaxis can develop such that repeated testing of the same compound at the same dose may not yield the same effect. However, looking at how one agent affects the dose-response function of another does get around many of the interpretational difficulties that plague single-dose combinations and provides a much more accurate assessment of how agents acting on separate pathways may interact.

The most confident interpretations can be made from experiments involving multiple doses of both agents. A model for interpreting the results of such experiments has been developed and involves the use of the isobolographic technique (7). An example of this can be given using two agents that both inhibit food intake. In such experiments, individual dose-response relationships for the two agents are generated. As demonstrated in Fig. 6, the doses of each agent are plotted against each other, and a line is drawn between the doses of each that produce a set magnitude of response, usually that dose that produces 50% of the maximal response (the ED50). The predictive interaction of the two compounds is defined by this line, and the degree to which various dose combinations conform to that line is assessed. This assessment involves finding the dose of one compound that produces 50% of the maximal response at a given dose of the other agent. Combinations that are infra-additive fall to the right of the line. Combinations that are supra-additive fall to the left of the line. Traditionally, this technique has been used to assess whether two compounds were likely interacting with the same receptor. With two agonists acting at the same receptor, additivity would be the anticipated result. Thus, when additivity is obtained, it is as if the two compounds were different forms of the same substance, and the result has been interpreted as supportive evidence for at least a similar mechanism of action. Departures from additivity have been taken to imply that the two compounds are acting by different mechanisms (14).

For studies addressing interactions among substances known to have different sites and mechanisms of action, isobolographic analyses still have considerable utility. They allow the nature and direction of the interactions to be specified and, by using broad dose ranges of both compounds, provide increased confidence in the interpretation of the results. Naturally, as above, the disadvantage is that use of this technique involves a considerable experimental investment. Complete individual dose-response curves need to be evaluated before the effects of dose combinations can be assessed. Assessments of combinations involve finding the dose of one agent that, in combination with set doses of the other, produces a 50% response. This is often not practical.

The above discussions have focused on interactions between different agents that affect food intake, but the same principles hold for interactions between the effects of the same compound given at two sites. There are multiple examples of feeding-related peptides that have both central and peripheral actions. How admin-

![Fig. 5. Theoretical results of dose-effect curves for treatment A alone (solid line) and in combination with a single dose of treatment B (broken lines 1 and 2). The addition of treatment B may affect treatment A equivalently at all treatment A dose levels (line 1) or may have differing effects depending on dose level of treatment A (line 2).](image)

![Fig. 6. Graphic representation of isobolographic data. a–b is the theoretical line for the addition of doses that produce 50% of the maximal effect. Combinations that require more than the theoretical levels to produce 50% of maximal response are termed infra-additive (point A). Combinations that require less of either compound are termed supra-additive. (point B).](image)
istration of such a compound at one site affects the efficacy of the same compound given at another raises the same range of issues. Interactions may be expressed as additive influences on a common mediating pathway, or actions at one site may be downstream or mediate the behavioral effects of actions at another site. Single-dose comparisons will provide little information. Only by employing multiple-dose comparisons will we be able to appropriately interpret how signals interact.

MEASURING MULTIPLE FEEDING PARAMETERS

Another way of improving the interpretability of dose combinations is to measure more than just cumulative food intake. Although the total amount consumed provides both quantitative and qualitative information, there are multiple ways in which overall changes in food intake can be accomplished. Identification of specific changes in the patterning of food intake can provide significant information about potential mechanisms. Two commonly used methods are licking microstructural and meal pattern analyses. The choice of method depends in part on the duration of the effect of the agents of interest. For examinations of effects in single meal situations, microstructural analyses of the patterns of licking of liquid diets can provide extensive information. As recently reviewed by Davis (3), rates of licking during different parts of the meals appear to be under the influence of different control systems. That is, altering properties of the ingestant itself or how the organism can respond to the ingestant can have very different effects on lick rates during the meal. For example, the initial rate of licking of a liquid diet is greatly affected by the diet “palatability” or the organism’s responses to palatability changes. Thus increasing the sucrose concentration or positively modifying endogenous systems mediating reinforcement mechanisms increases the initial ingestion rate (Fig. 7). Altering the preabsorptive ingestive consequences results in changes in the way at which lick rate decays during a meal. For example, adding mannitol to a solution retards intestinal absorption and results in an increased negative-feedback signal to ongoing ingestion and a more rapid decay in lick rate. Thus the size of a meal can be altered in multiple ways, and examinations of the pattern of licking within that meal provide significant information about the nature of the mechanisms leading to smaller meal sizes.

Getting back to interpreting the effects of a simple dose combination, examining the microstructure of licking during the test situation can provide significantly more information about the nature of the agents’ interactions. The two agents may affect different aspects of the lick rate function yet result in the same change in cumulative intake. For example, agonist A may reduce the size of the meal by reducing the initial lick rate, a result that would imply an alteration in the perceived palatability of the ingestant, while agonist B may reduce the meal size by accelerating the rate of decline lick rate, a result that would imply a magnification of the ingestant-induced gastrointestinal negative feedback. The two agents in combination may continue to have these results and produce an additive effect on ingestion, but they would have done so independently with little or no interaction. Only by examining effects on the rate of licking during a meal would such an interpretation be possible.

For manipulations that have longer-term effects on food intake, examinations of meal patterns can provide similar kinds of information. Thus some agents or manipulations can have specific effects on meal size, while others may more specifically affect the duration of the intermeal interval. The addition of such measures to a simple index of 24-h intake greatly increases the ability to interpret how dose combinations produce changes in overall intake. Again, two agents that reduce food intake may have additive consequences on total consumption but may have each affected an aspect of ingestion pattern independently.

ADDITION OF NONINGESTIVE DEPENDENT VARIABLES

The interpretation of how two agents that affect food intake interact is also greatly facilitated when there are additional dependent measures beyond those related to food consumed. One recent strategy used by a number of investigators has been to examine patterns of neural activation in response to individual agents and their combination (4, 6, 8). This can be done with electrophysiological techniques or with other measures of neural activation such as identifying the expression or presence of immediate early genes such as c-Fos. Both techniques have relative advantages and disadvantages. Electrophysiological measurements are time intensive and require that an appropriate neural location for a supposed interaction be identified. The advantages are clear. Both positive and negative interactions can be studied at the level of single cells, providing great interpretational power as to the nature of such interactions. C-Fos techniques allow explora-

![Fig. 7. Rate of licking per minute during an ingestive bout for either 0.95 M (solid line) or 0.8 M (dotted line) sucrose (adapted from Ref. 3). The figure demonstrates that while the lick rates at the termination of the bout are the same, the initial lick rates are lower for the lower sucrose solution. Thus the initial lick rate provides information about the perceived palatability of the sucrose solution in the absence of postingestive feedback.](http://ajpregu.physiology.org/Downloadedfrom)
tion of multiple brain sites within individual experiments. Although interactions can be quantitatively identified in terms of the number of cells expressing the c-Fos protein, the level of activation within individual neurons cannot be identified. Thus this technique can provide information about the overall level of activation within a brain region. It has the additional caveats that it cannot identify instances in which the level of activation within an individual neuron may be decreased from baseline, and not all cells express c-Fos so that areas of activation in response to the treatments can be missed.

The data produced by these techniques can greatly add to the interpretability of feeding data. For example, let us propose a situation where agonist A, at a dose that has no effect by itself, greatly increases the degree to which agonist B reduces food intake (Fig. 8A). A usual interpretation of these data might be that agent A in some way modifies the animal’s response to agent B. However, with just the feeding data, little more can be concluded. The addition of c-Fos data could provide information about both the site and the nature of interaction. For example, c-Fos data from the nucleus of the solitary tract (NTS) may show that, unlike in the feeding situation, both agent A and agent B produced a degree of cellular activation and that the combination was additive (Fig. 8B). Alternatively, agent A may not have produced activation by itself but increased the degree of activation produced by agent B (Fig. 8C). Although the feeding data may be identical for these two situations, the addition of the c-Fos data would allow very different sets of interpretations. In the first situation, the neural activation data demonstrate that there is actually an additive interaction that the behavior does not directly reflect. It may be the case that the additional complexity of the behavioral output does not directly reflect the neural activation produced by the two treatments. The degree or the site of activation produced by agent A may not have been sufficient to effect a behavioral change. In the latter case, in which the behavioral effect mirrors the c-Fos result, agent A may be said to have altered the responsiveness of the affected cells to agent B, and the outcome of this altered responsibility is reflected in the behavioral change. The latter case represents what has been shown for the ability of leptin to alter responsivity to within-meal signals such as the brain gut peptide CCK or an intragastric nutrient load (4, 6). Leptin alone at the doses used has no effect on short-term intake and does not induce c-Fos in NTS neurons. Leptin does, however, magnify the degree to which CCK or gastric load inhibits intake and induces neural activation at this important hindbrain site. In the absence of the c-Fos data, identifying the nature or site of leptin’s interactions would not have been possible.

The issue of where specific proteins act to alter food intake deserves some additional comment. Exogenous administration of these compounds rarely can simulate their endogenous secretion pattern. Although clearly true in the periphery, this issue is even more difficult within the central nervous system where neurotransmitters can be secreted at multiple locations but not necessarily at all of the locations of the specified receptor. Exogenous administration (peripheral, via the ventricular system or even into specific neural locations) cannot simulate these secretion patterns. Thus another interpretive issue that infuses all of these experiments is that the pharmacological action of exogenous compounds may or may not reflect the biological activity of the endogenous system.

SUMMARY

The study of how food intake is controlled continues to be a vital arena for research as the burden of obesity...
across the developed world escalates. To translate the growing list of potential systems that play a role in the control of food intake into viable therapeutic strategies, however, will require a thorough understanding of how and where these signals interact with one another to influence energy intake. As should be obvious from the issues raised here, an accurate depiction of these interactions depends on numerous issues of experimental design. As the number of identified systems that influence food intake increases, the need for additional research that addresses their potential interactions will continue to increase at an even higher rate.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-54080, DK-54890, and DK-19302 and by funds from the Procter & Gamble Co.

REFERENCES