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Chapter 6

HYBRIDIZING EXPERIMENTAL PARADIGMS TO INCREASE HIGH THROUGHPUT OF NEUROBEHAVIORAL DATA

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ABSTRACT

Although traditional (relatively simple and mostly single-domain) animal models of brain disorders continue to dominate biological psychiatry, combining experimental paradigms in an intelligent manner can save time, minimize the use of resources, and increase throughput of neurobehavioral data. This approach, also termed "hybridization of models," not only enables innovative modeling of neuropsychiatric disorders (through broad and thorough investigation of complex phenotypical characteristics) but contributes to the improvement of research ethics by following two of the 3R principles – *Reduction* [of animal numbers] and *Refinement* [of the research process by employing less distressful procedures]. This chapter will discuss methodological aspects and multiple benefits of using "hybrid" experimental paradigms in neurobehavioral research.

Keywords: animal (experimental) models, behavioral paradigms, neuropsychiatric disorders, 3R Principles.

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INTRODUCTION: THE EXISTING CHALLENGES

Animal studies are widely used for screening psychotropic drugs, finding candidate genes, and developing valid translational models for brain disorders and dysfunctions [1-3]. The growing use of animals in neurobehavioral research necessitates adherence to certain principles which both protect the welfare of the animals used in research, and ensure valid and reliable data acquisition. These ethical principles of biomedical research are outlined in the 3Rs declaration [4], which aims to *Replace*, *Reduce* and *Refine* animal use for scientific studies. In accordance with these aims, the use of experimental models that follow the 3Rs principle is becoming critically important.

Using batteries of single-domain assessments for behavioral analysis is a common research strategy employed in order to achieve increased data density and explore different behavioral domains. However, this approach requires overcoming several methodological challenges in order to keep pace with the need for appropriate animal models for newly appreciated clinical phenomena [5, 6]. In their effort to minimize laboratory/animal resource use while still maximizing test information density, many researchers subject animals to intensive batteries of single-domain behavioral assessment trials [7, 8]. At the same time, a confounding effect of these assessment batteries is that environment and prior test history may modify an animal's behavioral performance [9, 10], therefore influencing data validity and reliability [11-13]. Furthermore, these factors contribute to the common problem of being unable to correctly dissect animal phenotypes in behavioral experiments [5, 14], and parallel them with highly variable, complex, and co-morbid clinical phenotypes [15-18].

However, it is also clear that the neurobehavioral field needs fast, low-cost, high-throughput behavioral screens (figures 1, 2) that appropriately advance their research [7, 19, 20]. Likewise, animal models are increasingly needed to examine the "continuum" nature of brain pathogenesis as well as for integrative versus disorder-specific modeling of brain pathogenesis [21-23].

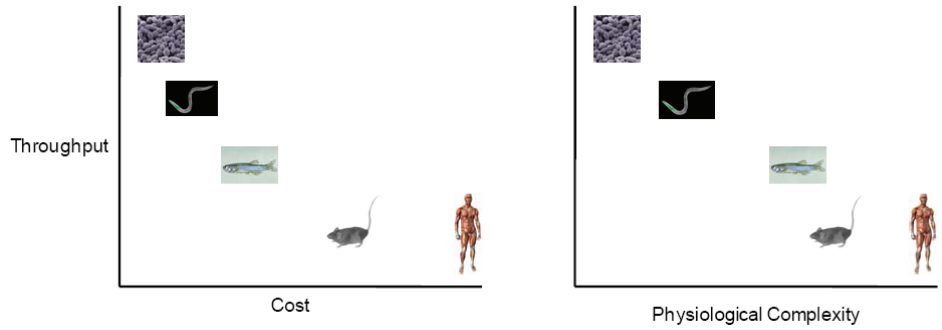

Figure 1. Current challenges in phenotyping research; the right panel is modified from [41].

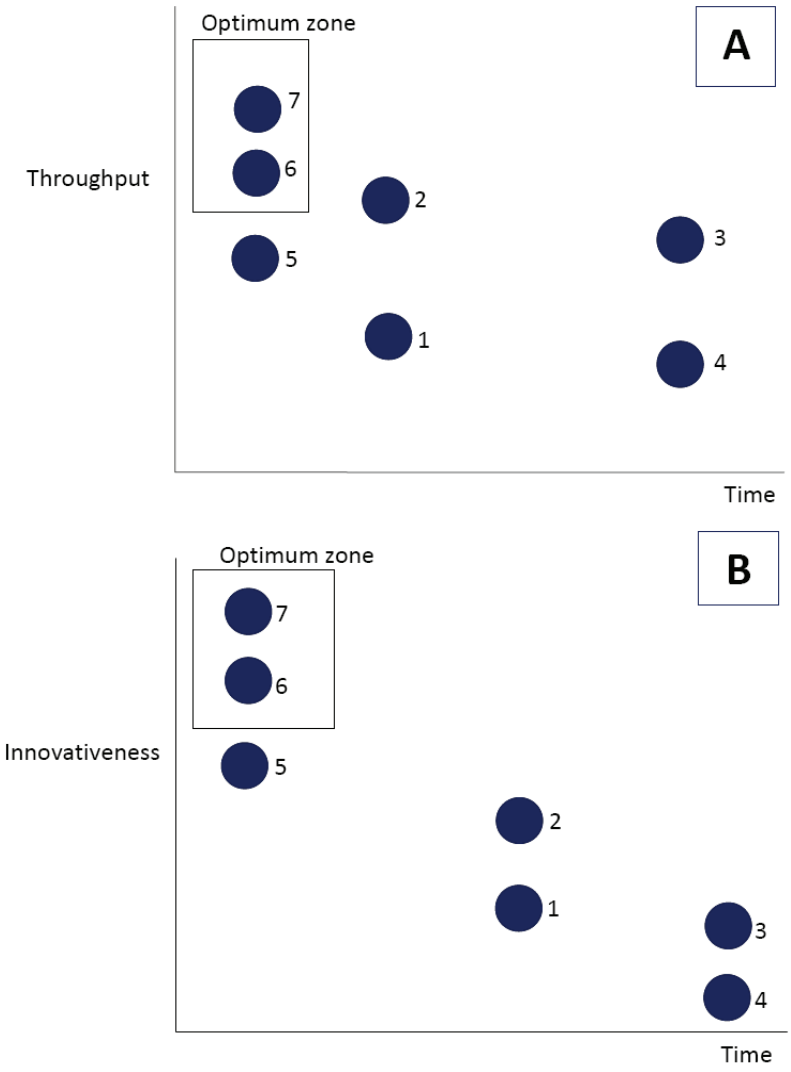

Figure 2. Throughput, innovativeness and time-costs of different experimental strategies: 1 -"Smart" battery of standard tests; 2 - "Smart" battery of standard tests with additional endpoints added; 3 - Test battery of standard tests with additional endpoints added; 4 - Test battery of standard tests; 5 - Combining several tests within one apparatus (physical combination); 6 - Combining several tests within one apparatus with several additional endpoints added; 7 - "Conceptual" combination of several different paradigms to asses both "traditional" domains and newly appreciated brain phenomena.

In general, these current challenges to appropriate neurobehavioral modeling can potentially be overcome through either increasing throughput of the existing research tools, or by applying alternative strategies of experimental design. As we will argue here, a wide use of hybrid models that comprehensively assess multiple behavioral domains may be instrumental in surmounting these obstacles and achieving more valid data, while simultaneously improving the state of animal treatment within the field [24].

The implementation of experimental protocols that assess multiple domains in parallel offer clear benefits for neurobehavioral research. Logistically, this is accomplished by combining experimental paradigms in an intelligent manner which allows for the study of multiple phenotypes in a single cohort (table 1, figure 2). Additionally, and perhaps most importantly, hybrid models in general require far fewer stress exposures (than single-domain batteries) and have the ability to assess more domains per experiment. Consequently, hybrid models adhere to the 3Rs principle by virtue of reducing animal numbers and minimizing stress. Furthermore, a particularly well-designed hybrid model can better implement the 3Rs, since the ability to assess more domains per experiment allows the researcher to utilize more behavioral endpoints per experiment, saving time and reducing the quantity of laboratory resources needed. For these reasons, hybrid models become efficient, high-throughput, and less expensive behavioral paradigms.

Table 1. Examples of hybridizing strategies in neurobehavioral research

Research strategies	Examples	References	Potential advantages	Potential disadvantages
Combining several models in a "smart" battery	Swimming tests (including pre- and post-swimming behaviors)	See review [24]	Optimizes test battery stress, saves time and minimizes animal experimental stress	May introduce some additional conceptual complexity, increased test battery effect
Combining several models physically	"Open field-elevated plus maze-light/dark box" combined apparatus -Modified hole board	[37] [38]	Minimizes test battery effects, saves time, Minimizes animal stress, tests multiple unconditioned behavioral endpoints	Measures the same standard constructs as the traditional models. May not be optimal in terms of lab space occupancy
Combining several models conceptually	Elevated T-maze Suok Test	[39] [24]	Minimizes test battery effects, saves time. May target some additional subtypes of brain disorders	May be differentially sensitive to different constructs; may require further validation
Measuring additional endpoints within "traditional" models	Spinning, hind leg clasping assessment during the tail suspension test	[28]	Minimizes test battery effects, saves time, reduces animal stress and suffering	None (but may require additional training of research personnel)

3Rs and Behavioral Research

The 3Rs principle was introduced to the biomedical field in order to set a standard for the ethical treatment of animals as sentient beings and is aimed towards improving protection and respect for the welfare of animals involved in all sectors of research (Russell and Burch, 1959). The 3Rs are particularly relevant to behavioral research as it is one of the main areas that uses animal models to examine phenotype expression and numerous clinical phenomena, and is often based on using stressful and/or painful manipulations. In order for neuroscientists

to apply the 3Rs principle to their research effectively, they must aim to meet two specific goals, namely creating effective models for neurobehavioral research while maintaining the ethical values of the 3Rs and developing alternative approaches to animal research.

The standards of treatment for animals are taken seriously in many countries. In the United States, treatment and welfare of all animals is regulated in part by the Animal Welfare Act as well as by the governing bodies for laboratory research. The principles of the Animal Welfare Act ensure that all animals intended for use in research facilities are provided humane care and treatment. Further regulation pertaining to treatment and adjustment of principles applicable to animal welfare is governed by the Office of Laboratory Animal Welfare and the National Institute of Health. The ideals of the 3Rs are covered in depth and application of their principles is emphasized in some training modules, especially those on detecting and minimizing animal pain and suffering. Another module that could potentially benefit all researchers would be one pertaining to experimental design. In line with this, hybrid protocols may be emphasized within this module due to their ability to assess multiple behavioral endpoints, provide a high throughput of data and adhere to the 3Rs principle. Clearly, more awareness among the research community of hybrid protocols could further the capacity to refine the research process so fewer animals are involved in research while still ensuring that animals are treated humanely.

BENEFITS OF USING HYBRIDIZING APPROACHES: BEYOND THE 3RS

From a methodological perspective, it is crucial to consider how behaviors can be affected by the previous testing experience of the animal, and to determine what measures should be undertaken to ensure that the data's validity is not compromised as a result. For example, timing seems to be an important point of contention, as some studies indicate that mice respond differently when tested in a battery rather than in individual tests alone [10], thereby demonstrating that some behavioral tests are more susceptible to the previous experience of the animal than others. While this phenomenon can complicate behavioral interpretation, the fact that one test alters the behavior in another, does not disqualify that test from further use. Indeed, one can consider the notion that the combination of the tests may provide qualitatively new opportunities for eliciting clinically relevant behaviors that could not be achieved with either test alone. This possibility forms the methodological basis for the hybrid (or smart) battery approach for neurobehavioral research [24].

Hybrid battery designs comprised of fewer, but more extensive, behavioral assessments may serve to reduce the impact of prior stress on subsequent behaviors (some of which are potentially conditionable), providing less confounded and more valid behavioral data for analysis. Hybrid models can focus on simultaneous yet distinct domains (such as anxiety and depression) which enable researchers to mimic clinically relevant phenomena (e.g., co-morbidly) that are difficult or impossible to target in standard single-domain models. In addition, by examining a wider spectrum of behavioral phenomena, hybrid models are more likely to elucidate novel and/or complex phenotypes. This becomes particularly evident when examining mutant animals with unknown or unclear profiles, as well as allowing for a more

accurate focus on the newly appreciated "continuum" nature of brain pathogenesis [21, 23, 25].

An additional aspect to consider is that the nature of behavioral tests *per se* may sometimes preclude them from being able to form a battery due to their environmental constructs. For example, the traditional use of various swim tests does not allow investigation of depression (assessed by immobility in the Porsolt's forced swim test [14]), neuromuscular abilities (assessed by the ability to swim in the water tank [26]) or spatial (hippocampal) memory (assessed in the Morris water maze [27]) in the same cohort of animals due to their habituation to the swimming environment. Consequently, assessment of these domains individually requires at least three separate cohorts of animals and a considerable amount of testing time. The hybridizing approach on the other hand, offers a conceptually different perspective, which is based on a specific fusion protocol. These protocols are designed to either assess several different domains simultaneously, or logically combine several single-domain tests in a specific and special way in order to maximize the number of phenotypes or domains that are collectively measured by the smart battery (table 1).

EXAMPLES OF "HYBRID MODELS"

A hybrid study design can be created through a number of means, some requiring a great deal of ingenuity on the part of the researcher, while others require only some open-mindedness regarding established experimental protocols. Sometimes, simply by realizing that multiple behavioral endpoints could be assessed during a single behavioral test, a researcher may be capable of creating an elegant and high-throughput hybrid protocol requiring very few modifications from the original. For example, generally the tail suspension test (TST) is used only to assess depression-like phenotype [28]. However, a number of other additional behavioral endpoints can also be measured during this test without any major modification of the procedure *per se*. These include an assessment of general coat state, checking for the presence of barbered patches, the presence of aberrant tail-climbing or hind leg clasping behavior, as well as noting the presence of aberrant spinning (indicative of a vestibular disorder). Furthermore, by combining the TST with an open field test or a stress-induced hyperthermia test (prior to or after the TST) and forming a battery, a researcher can also measure the animal's baseline and "potentiated" stress and anxiety levels.

Another hybrid protocol involves the intelligent combination of several neurobehavioral tests to assess multiple domains in parallel. Step 1 begins with a short pre-swim open field test allowing researchers to assess baseline anxiety and activity/motor phenotypes [29], novelty-evoked grooming behavior [30], within-trial habituation (spatial working memory) and potential behavioral perseverations, such as meandering/turning or stereotypic circling [31]. Step two of this "smart" battery would include an acquisition trial of the Morris water maze, a necessary step in this model, used as a Porsolt's forced swim test [32, 33]. Examination of depression-related immobility during the swim test enables a parallel assessment of depression-like behaviors without affecting the Morris water maze procedure (which is performed later). In addition to assessing these behavioral domains, analysis of per-minute distribution of the animal's activity enables the assessment of their within-trial habituation (spatial working memory). Also, poor swimming during this trial will be

indicative of motor/neuromuscular problems, whereas frequent circling and sinking, if present, may suggest vestibular deficiency phenotype in these animals [24, 26]. Aberrant turning/navigating and meandering in this situation may suggest altered spatial strategies. Finally, swim stress-evoked ultrasonic vocalizations [34], if noted in this test, may be measured as stress-related indices.

Generally, after these behavioral tests have been completed, a researcher would normally remove the animals from the water tank, dry them off, and return them to their home-cages [24]. However, several additional domains may be assessed at Step 3 using the "smart" battery approach. By placing the animal in an observation cylinder for 5 min immediately following the swim test a researcher can assess a different type of grooming behavior – the "artificial" swim-induced grooming [29]. In the event that the animal's grooming phenotype has been affected by an experimental treatment, this test may eventually lead to interesting findings about sequential organization of animal grooming. By comparing activity and sequencing of pre-swim "spontaneous" grooming with the "artificial" swim-induced grooming, changes in grooming patterns and sequencing can be elucidated. Furthermore, as recently demonstrated [29], by comparing pre- and post-swim behavioral activity levels some conclusions may be made about animal fatigueability- another important domain that merits scrutiny in neurophenotyping research. Lastly, Step 4 of this battery includes subsequent trials of the Morris water maze that, according to the traditional protocols, assesses the animal's spatial memory [35, 36]. This example offers a particularly compelling demonstration of the benefits of utilizing a hybridization approach over a traditional approach.

Researchers have also attempted to combine experimental protocols either physically or in a more conceptual manner (table 1). In response to the common problem of being unable to correctly assess the full measure of an animal's emotional reactivity and emotional profile while limiting test battery effects some researchers have offered to physically combine the three most widely used behavioral tests (the open field, elevated plus maze, and light/dark box) into one apparatus [37]. While this combination saves time and clearly minimizes potential test battery effects, it is limited in its ability to assess behavioral endpoints beyond the standard constructs measured by the traditional models from which it is based. Also, this combination of apparati takes up a sizable amount of laboratory space, which can be problematic (although the models can be disassembled and stored separately to minimize the storage space requirements). It does, however, provide a method for measuring, in a relatively straight forward manner, a wide range of unconditioned exploratory behaviors in just one trial [37].

Another experimental paradigm derived from the physical combination of multiple apparati is the modified hole board. In this construct, which combines the hole board and open field, a researcher is able to measure two behavioral domains in one test, namely anxiety and social behaviors. Behavioral endpoints measured include: anxiety-related behavior, risk assessment, exploration, locomotor activity, arousal, and social affinity. It also provides a measure of unconditioned innate anxiety with face, predictive and construct validity [38]. An additional benefit of this experimental paradigm is that it minimizes animal stress by allowing the animals to maintain social contact with its group mates during the test [38]. For these reasons this paradigm meets the aims of the 3Rs quite effectively by minimizing animal stress and enabling the measurement of multiple behavioral endpoints in one test. Again, while the physical combination of traditional experimental paradigms is beneficial in many ways it is

still limited conceptually in that it only measures the same endpoints as traditional tests, albeit in a much more efficient and less stressful (to the animal) manner.

The conceptual combination of experimental paradigms offers the advantages of minimizing test battery effects, saving time and, most importantly, has the potential to elucidate additional (and possibly new) subtypes of brain disorders. Relevant examples of these types of conceptual combinations include the Suok test and the elevated T-maze experimental paradigms [39, 40]. In these paradigms, an intelligent combination of behavioral assessment tests are used in order to examine multiple behavioral domains within one test as well as serving as potential models for new phenomena. For example, the elevated T-maze, a combination of the elevated plus maze and the standard T-maze, allows for the simultaneous assessment of an animal's spatial memory and anxiety related phenotypes (table 1).

Likewise, in the Suok test, an elevated horizontal rod is used in combination with a light-dark modification [40]. The animals balancing phenotype is observed as well as their place preference (light/dark aversion). This allows for the simultaneous assessment of anxiety, activity (exploration), and neurological (motor coordination, motor-vestibular anomalies, anxiety-induced motor impairments) phenotypes. Clearly, conceptual combinations of experimental paradigms advance the field of neurobehavioral research by creating a test that can measure multiple behavioral domains in parallel and has the potential to be useful as a test of newly appreciated brain disorders. However, due to their nature, conceptual models may be differentially sensitive to different constructs and thus may require further validation before they can be widely used.

CONCLUSION

The implementation and wider use of hybrid approaches serves several goals in the field of neurobehavioral research. Most apparently, hybrid-based models allow for adherence to and continued progression in furthering the ideals of the 3Rs by reducing the number of animals involved in research as well as refining procedures to minimize animal pain and distress. At the same time, by examining certain phenotypes in parallel (while also being able to more accurately focus on the continuum nature of brain pathogenesis), hybrid models allow scientists to examine many more behavioral phenomena (phenotypes, genes x environment interactions, etc) in a more accurate and well-designed manner.

The use of hybrid protocols demands a significant innovation on the part of the researcher, as batteries require the creative fusion of standard behavioral assessment tests in a way that allows for multiple behavioral phenotypes to be observed without confounding each other. Admittedly, this approach may not work for every protocol and may also have several potential (but manageable) disadvantages (table 1). However, there is always a future potential for researchers to creatively develop new hybrid protocols which will continue to advance the field. For example, the protocols which seemed to be un-combinable today may later form an interesting hybrid battery. Thus, hybrid models are not only beneficial in furthering the understanding of behavior, but also stand as a way for neuroscientists to constantly improve the very process by which research is performed.

The ability of hybrid models to asses multiple domains also provides much more data than traditional single model study designs, which has the advantage of being acquired in a

comparable or shorter time frame. This aspect of hybrid designs is beneficial in increasing the throughput of neurobehavioral data acquisition and analysis (figure 2). Importantly, not only is more data collected, but the interactions of each element of the study design can be observed. Clearly, these highlighted characteristics of hybrid models have the potential to foster the field of ethical and high-throughput neurobehavioral research.

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