

Using Viral-Mediated Gene Transfer to Study Depressive-Like Behavior

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Abstract

The use of viral-mediated gene transfer in animal behavioral experiments has become very popular over the last decade. Altering gene regulation and assessing subsequent behavior can be a useful tool in unraveling a specific gene's contributions to complex behavioral processes such as depressive-like behavior. However, these types of experiments require detailed planning and designs to avoid pitfalls associated with issues such as surgical procedure or the peak of viral expression. This chapter is intended to be a primer on the design of such experiments and aims to discuss factors that must be considered in the early phases of experimental planning.

Key words Viral-mediated gene transfer, Depressive-like behavior, Stress, Serotonin, 5-HT_{1B}

1 Introduction

The use of viral-mediated gene transfer in animal behavioral experiments has become very popular over the last decade. Compared to other pharmacological strategies, using viral vectors to manipulate receptor populations has a number of advantages that this chapter will examine. Several design considerations impact the usefulness of this approach in behavioral experiments, especially if the stress (hypothalamo–pituitary–adrenocortical) axis or stress-sensitive behaviors are being investigated. Therefore, our objective is to provide guidance for the use of viral vectors in behavioral experiments in general, and stress models in particular. We will cover three main issues (1) general considerations in the use of viral vectors for behavioral experiments, (2) choosing the best vector from available and characterized viral vectors and the procedures for injecting the vectors and evaluating transgene expression, and (3) planning and measuring the subsequent behavior. We will do the latter with two vignettes designed to illustrate how our lab teases apart complex behaviors using viral vectors.

2 General Safety Issues

2.1 Governmental Regulatory Bodies

Oversight of biosafety issues is becoming more complex with evolving guidelines from regulatory agencies in each country and the particular policies of each institution. In the USA, biosafety falls under the oversight of institutions that range from the Centers for Disease Control, the Food and Drug Administration, and even local county boards of health. Conducting research is further complicated by the fact that funding organizations have also established regulations associated with specific types of research. For example, the National Institutes of Health publishes guidelines for research involving recombinant DNA; these guidelines impact NIH-funded research programs that use viral vectors in experiments, and the guidelines also stipulate that all research at the funded institution follow the guidelines regardless of funding source.

2.2 Environmental Health and Safety

Because viral vectors involve recombinant DNA technology, gene transfer, and live animal experiments, they are regarded with great sensitivity by investigators and regulators alike. The arm of the NIH called the Office of Biotechnology Activities has mandated the creation of Institutional Biosafety Committees (IBCs). Funding institutions in the USA use these committees, composed of Principal Investigators, department chairs, biosafety officers, and members of the public, for institutional oversight of recombinant DNA research. Oftentimes, the IBC works in concert with the institutional environmental health and safety department, since safety and use of biologicals is assessed by each body. Deciding to use a viral vector in one's research will require a detailed biosafety plan that spells out how the specific vector is obtained or made, a safety assessment, and the procedures for its use in *in vitro* and *in vivo* applications. Furthermore, investigators are required to have detailed exposure and spill mediation plans. For example, our laboratory has used an amplicon-based Herpes Simplex Virus (HSV) vector system for more than a decade. Since this HSV vector system utilizes replication-deficient helper virus for packaging the viral particles in a specific cell line, we are required to use Biohazard Safety Level (BSL)-2 precautions. Afterward, we carefully confirm that each batch contains no revertants that have regained replication competency using a plaque assay, and then that particular batch of vector can be used under BSL-1 containment. In our institution and at others that we have collaborated with, these arrangements must be negotiated by the investigator and the IBC prior to initiating use of the viral vector. Finally, the rules regarding which types of personal protective equipment and procedures are required for each type of viral vector during preparation, *in vitro* experiments, and in animals depend on the type of virus and must be taken carefully into consideration.

In the USA, there are four levels of biosafety, and each is associated with specific rules geared toward safety issues and the reduction of personal contamination or infection. These are the biosafety levels:

- Level 1: This level covers well-characterized agents that do not consistently cause disease in immunocompetent adult humans. They pose minimal potential hazards to laboratory personnel and/or the environment. Examples of a BSL-1 vector are adeno-associated virus and canine adenovirus.
- Level 2: Level 2 BSL covers well-characterized agents that pose a moderate health hazard to laboratory personnel and/or the environment. For this class, preventative and therapeutic interventions are often available. Examples of BSL-2 vectors include adenovirus and lentivirus (non-HIV pseudotypes); HSV is considered BSL-2 by our institution until each batch prepared is tested and confirmed to not contain replication-competent revertants, after which we are allowed to use BSL-1 procedures.
- Level 3: This BSL covers indigenous or exotic agents that may cause serious or potentially lethal disease to humans through inhalation. Preventative or therapeutic interventions may be available. Examples of viruses that fall under this category include anthrax and severe acute respiratory syndrome (SARS) coronavirus.
- Level 4: Level 4 BSL agents are dangerous and exotic, with a high individual risk for life-threatening disease. Preventative and therapeutic interventions are not usually available for these viruses and these are not used for biobehavioral experiments.

2.3 Animal Care and Use Committees

Another important issue to consider is the use of viral vectors in vivo. The necessity to house animals treated with viral vectors for an extended period presents a number of unique problems in comparison to in vitro uses of viral vectors in a standard laboratory environment. In our experience, institutional animal care and use committees now examine experimental plans involving viral vectors much more closely than they did just a few years ago. As with biosafety issues, there must be detailed plans in place for how to handle animals that have received virus, since the animal itself may be a source of subsequent transfer of transgenes to other animals, the environment, or laboratory personnel via contact with the animal. Protocols for managing soiled bedding that may contain shed virus or injuries to other animals or personnel via bites or scratches must be carefully documented. In some cases, animals treated with viral vectors must be housed separately, especially with BSL-2 vectors. The soiled bedding may require sterilization prior to disposal and careful documentation of the type of vector used in each animal is generally required so that personnel in contact with the animals can readily determine the nature of their own exposure

to the vectors. This may require devising a system that alerts animal care personnel to the presence of virus, while maintaining blinded conditions and preserving the integrity of the experiment for those collecting data. In our lab, we have dealt with this by treating every cage in an experiment as if the animals have been exposed to virus (i.e., even if it was a sham or vehicle injection). These types of issues dictate where the animals live (special BSL-dedicated housing or in the general population), as well the procedure for disposing of soiled bedding. High expectations for detail extend beyond just animal care. Finally, just as each drug used in a behavioral experiment must be described in an approved protocol, the type of virus and the nature of the transgenes encoded by the vector must be detailed; this can become a regulatory burden for both the investigator and the institutional committees overseeing the research procedures.

3 Considerations in Choosing a Vector

3.1 Overview

There are several common viral vectors from which to choose, including Herpes Simplex Virus type 1 (HSV), lentivirus, adenovirus (AV), adeno-associated virus (AAV), and canine adenovirus-2 (CAV). Use of these vectors in the CNS has been extensively reviewed [1]. Many factors will dictate which vector is best to use, including safety for the user, the vector's ability to induce an inflammatory response, and cell type infection specificity. Many individuals choose a particular viral vector based on their familiarity with the tool, advice from colleagues, or the convenience of packaging. Each of these vectors has advantages and disadvantages, and in most cases, more than one will work well for a particular experimental plan. In the end, the virus that is the safest and most effective, for both laboratory personnel and animals, and that appropriately expresses in the model system should be used. Some vectors are highly versatile and adaptable to different needs and others are more restricted in their potential. Characteristics to consider are cellular infectivity (which cell types or stages can be targeted), titer, time course of transgene expression, "payload" capacity, chromosomal integration (if the gene becomes incorporated into the host genome), and for vectors that do not integrate, cytoplasmic stability (how long a nonintegrated gene is actively transcribed); also the size of the gene or genes that can be packaged for transmission can be an issue. Table 1 compares characteristics of the different viruses that are commonly used in behavioral neuroscience research.

We have been using HSV for a number of years and it has been reviewed previously; this vector system has been reviewed previously [2]. Some advantages of this vector include its relatively large payload capacity (at least 5 kb and potentially much more), BSL-1 containment practices after confirming that the particular batch of

Table 1
Characteristics of different viral vectors

	Lentivirus	AV	AAV	HSV
Inflammatory response	Mild	Strong	Mild	Moderate
Cell type specificity	Neurons and glia	Neurons and glia	Neurons and glia (depends on strain)	Neurons
Integration into host chromosome	Yes	No	Possibly	No
Predictable site of integration	No	N/A	Yes	N/A
Titers	Moderate	Very high	Very high	Low
Insert size	Can be large		Must be small	Can be large
Gene expression time course	Prolonged and stable	Prolonged and stable	Transient gene expression	Transient gene expression

vector is replication deficient, rapid onset of intense, albeit short-term expression when using the HSV promoter, neuron specificity, high affinity binding to its cellular recognition protein, and low levels of retrograde infection (i.e., infection of axon terminals at the site of vector infusion). Disadvantages include difficulty achieving high titers unless packaged with replication-deficient helper virus, expression dissipates after about 1 week (unless a non-HSV promoter is used, see Ferguson and Neumaier chapter), fairly small areas are transduced (usually less than 1 mm is infected per injection), and fewer neurons are infected than with other vectors. However, each of these problems can be used to the investigator's advantage. For example, the rapid onset and offset of transgene expression allowed us to test the effects of increased 5-HT_{1B} auto-receptor expression on the acquisition vs. expression of conditioned fear [3]. Furthermore, the high affinity binding and limited spread of HSV is ideal for transducing genes into small brain regions. Currently, AAV may be the most commonly used vector; it has the advantages of very high titers with helper virus-free packaging, long-term transgene expression, low inflammatory responses, and the availability of commercial packaging. On the other hand, only a rather small payload can be incorporated (usually less than 3 kb), different serotypes have varying efficiency in infecting neurons vs. glia, and lower affinity for cellular binding leads to larger areas of infection (but this can be an advantage as well). Some basic characteristics of several common viral vectors are summarized in Table 1.

Viral vectors offer a broad range of potential manipulations of brain function. These include overexpression of an endogenous protein such as a receptor, enzyme, or structural protein. In most

cases, the transgenic receptor can be activated by an endogenous signaling molecule such as a neurotransmitter; since neurotransmitter release is presumably unaltered by the overexpression of postsynaptic receptors, this means that transgenic receptor activation will be governed by the dynamics of endogenous neurotransmitter release. It is also possible to determine whether viral overexpression of a receptor in a specific brain region increases the potency of an agonist administered systemically.

One recent technology includes designed receptors that are exclusively activated by designer drugs (DREADDs; see [4, 5]). The benefits of this system are obvious—receptors can be directed to specific regions or cells, and turned off or with agents that have no off-target effects. This technique is discussed in detail in the Ferguson and Neumaier chapter. One can also “knockdown” expression of an endogenous protein using viral expression of shRNA to reduce the accumulation of newly synthesized proteins; the rate of disappearance of the targeted protein will depend in part on its half-life. In some cases, it may be advantageous to introduce a mutated form of a protein either into wild-type animals or knockout animals that lack the gene of interest. Gain of function experiments involving reintroduction of a protein into a knockout animal is a powerful strategy for establishing the necessity and sufficiency of a protein for a particular function. Since many proteins must dimerize in order to function, another strategy to reduce the function of a specific protein is to express a dominant negative mutant protein using a viral vector; this has been used to study the role of CREB in drug reward mechanisms, for example [6]. However, it must be kept in mind that it is possible that such a mutant may also interfere with the function of other related proteins. Thus, viral vector strategies offer not only regionally and temporally precise manipulations of brain function but they also allow innovative methods for identifying which protein candidates are responsible for a particular behavioral observation within that region.

Even though viral-mediated gene transfer is well tolerated, there are several important controls that should be considered. It is possible that the surgical procedure or nonspecific features of a viral vector may alter the function of a brain region; therefore, nonsurgical or sham surgical controls should be included whenever a new brain region is targeted or major shift in method is initiated. After that, we usually use fluorescent protein expression as a primary control group. We modified the amplicon-based HSV vector system to contain a separate transcriptional cassette containing CMV promoter and green fluorescent protein (GFP), while expression of the transgene of interest was controlled by the HSV promoter [7]. In most cases, we have expressed an epitope-tagged transgene, such as hemagglutinin-tagged 5-HT_{1B} receptors, which we confirmed did not alter the functionality of this serotonin receptor [7]. Our usual control group has been GFP alone, expressed

from either the CMV or the HSV promoter. We have consistently found that GFP control groups perform similarly to unoperated or sham operated animals in most [8–10] but not all cases [11]. In some cases, it could be argued that the expression of a protein might have unintended consequences, such as altering the efficiency of processing of off-target neuropeptides by endopeptidases. In this case, one might consider a nonfunctional protein control, such as expressing des-tyrosine enkephalin. However, expressing nonfunctional proteins can have unintended consequences as well, such as dominant negative interactions with functional endogenous proteins, so these must be used carefully. Finally, anatomical controls can be quite powerful in demonstrating the role of a transgene in a discrete brain region [9].

3.2 Regional Specificity

Traditional drug infusion strategies are a popular and powerful method for testing the role of a target protein, such as a receptor, in a discrete brain region. However, drug infusions have limitations related to uncertainty about the drug concentration after infusion into the brain region (potentially affecting the selectivity of the injected ligand) and the potential of drug diffusion and spread away from the intended target. Viral-mediated gene transfer has the advantage that the surgery and drug infusion can occur long before the behavioral experiment, so the stress of performing a brain infusion immediately prior to behavioral testing is avoided. Furthermore, the site of transgene expression can be precisely determined histologically, particularly when a fluorescent protein or epitope tagging strategy is used. We have found that coexpression of GFP from a separate transcriptional cassette allows for convenient and precise identification of injection sites after completion of the behavioral experiments; it is more accurate than inferring injection sites from cannula placements. Furthermore, we routinely observe that about threefold more infected neurons will be revealed by using an immunostaining procedure as compared to direct visualization of GFP, for example. It is also possible to identify off-site transgene expression, and we observe about 1–2 % as many GFP-positive neurons in ventral tegmental area after infusion of HSV viral particles into nucleus accumbens shell, suggesting that axon terminals projecting to the site of infusion are occasionally infected.

We have recently started to use promoters associated with specific phenotypes of neurons to further refine gene expression using HSV vectors [12]. We have tested three promoters associated with specific subtypes of neurons (serotonin transporter, dynorphin, and enkephalin), in each case using 2–3 kb of DNA preceding a gene associated with a specific neuron type in addition to the transgene of interest. Each of these promoters was previously analyzed to identify regions that were thought to be especially important in conferring cell-type-specific gene expression. These hybrid vectors are interesting because they behave quite

differently than typical HSV vectors by producing lower levels of transgene expression for a much longer interval (at least 1–2 months). We have noted that these vectors greatly improve specificity in expressing transgene in the neurons of interest, but they are not perfect and at least 5–10 % of expressing neurons are not of the desired phenotype. Nevertheless, these vectors are being used in HSV and lentiviral constructs routinely now in many labs (the additional payload requirements make it more difficult to use AAV, though).

4 Working with Viral Vectors in Animal Models

4.1 *Procedural Stress*

Stress exposure can alter the results of any behavioral testing, particularly when stress responses are being studied. Therefore, we carefully adhere to standardized approaches to reduce nonexperimental sources of stress on subject animals. For example, at least 1 week of habituation to our vivarium is allowed prior to initiating experimental procedures and we use carefully standardized animal husbandry procedures. Furthermore, the choice of anesthetic agent used during surgical manipulation is important. We generally prefer inhaled anesthetic agents since the depth of anesthesia can be easily adjusted and the animals recover quickly after the procedure. However, we also use ketamine/xylazine in some circumstances, but the animal will take longer to wake up afterward and it is not possible to make fine adjustments in the dosing during the surgical procedure. We usually opt for acute viral vector injections rather than implanting a guide cannula, but in some experimental designs, a cannula may be useful. In either case, the key to the best viral vector infusions is to deliver the virus smoothly and slowly using an electronic pump (e.g., 2 μ l over 10 min); the needle is left in place for at least 5 min before it is very slowly withdrawn. Withdrawing the needle too quickly may result in dragging virus back up the needle tract. Beyond the physical stress of surgery, different viral vectors will activate inflammatory pathways to varying extents (Table 1). Therefore, the time required to recover from both of these stresses should be built in to the experimental design. Also, the animals will need time to physically recover from surgery, and if extended recovery time is required, cannula for virus injection may be used (discussed later in the chapter). Each vector takes a different interval before sufficient transgenic protein has accumulated and been trafficked to its destination within the cell; it is important to confirm that the transgenic protein is distributed within the cell as intended, using immunohistochemistry.

One of the most important things to take into account is the timing of the behavioral training and testing in relation to viral expression. It is essential to have solid knowledge of the expression

time course of the vector. This should be checked with each individual promoter construct. In our hands, HSV promoter-driven gene expression peaks at around 4 days and is essentially gone by 7–10 days [13]. We have not closely examined how early the dynorphin, enkephalin, or serotonin transporter promoters initiate gene expression, but we have found that expression seems stable after about 1 week and lasts for several more weeks at least [12].

4.2 Stereotaxic Surgery

There are many ways to do stereotaxic injections, but we have found several procedures that enhance the success of transgene expression. For example, a simple one-time injection may be used with viruses that are stable expressers. For our injections, we use hubless dental needles attached with Tygon tubing to a microprocessor-driven pump. The smooth infusion delivered by such a pump is absolutely essential, as virus preparations can be sticky and clog the line without constant pressure. We observe less clogging with beveled needles than blunt infusion cannula. In some cases, we implant a guide cannula, especially if it becomes necessary to make subsequent drug infusions. Then, the viral vector can be infused at the ideal interval prior to the planned experiment, usually while the animal is lightly anesthetized to facilitate the infusion and minimize movement and distress.

The type of behavioral procedure can also influence the infusion method. For example, in the resident intruder paradigm in which animals scuffle, there is the potential for a guide cannula assembly to either hurt the aggressor male, become loosened or damaged, or for the skin around the cannula to be injured during a confrontation (however it is not impossible; for example, Covington and Miczek have successfully conducted social defeats with indwelling intravenous catheters [14]). Guide cannula sites may also be more vulnerable to contamination during procedures such as the forced swim test. Other relevant behaviors that could be easily conducted with cannula implants would be the open field test, elevated plus maze, probe burying, and saccharin preference test.

4.3 Evaluating the Accuracy of Injection

As per any stereotaxic injection, the infusion coordinates should be established empirically. We confirm the accuracy of injections in every animal by examining GFP expression whenever possible. In a few cases, we complete behavioral testing after transgene expression has been allowed to dissipate; in this case, we have used gliosis around needle tracks or cannula placement, but this is less reliable. The histological analysis should be performed in a blinded fashion and the sites and extent of infection documented on brain atlas illustrations. In our lab, we consider an injection to be accurate when more than 50 % of the transgene expression (usually indicated by GFP) is within the target region, and “hits” must be bilateral when both sides of the brain are targeted.

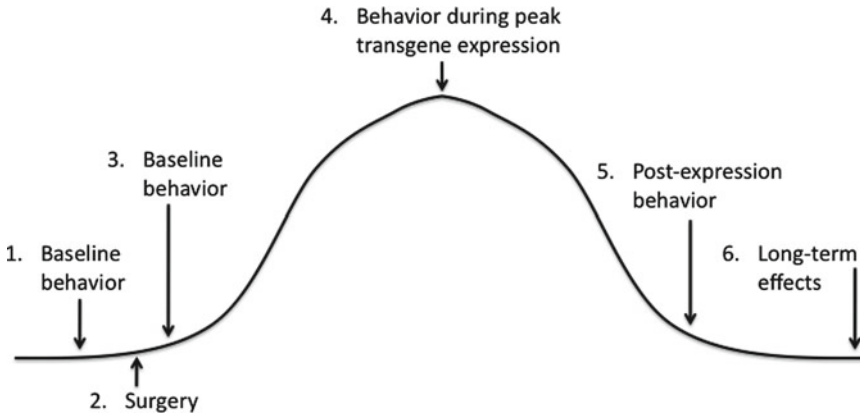


Fig. 1 Time course of HSV expression and opportunities for behavior analysis. (1) Behavior can be recorded before any manipulation takes place; this is the purest expression of baseline behavior; (2) surgery itself can affect animal behavior and recovery time must be allowed so assays must be planned accordingly; (3) baseline behavior can be recorded after surgery and will reflect the effects of the surgical procedure; (4) it is essential to determine the peak expression time of a viral vector, since expression time and intensity can be affected by promoter choice and other factors; (5) behavior can be assayed shortly after viral expression dissipates; this captures behavioral changes over short time scales; (6) long-term effects of viral expression can be assayed weeks or even months after viral expression dissipates

Vignettes

In order to illustrate the chain of events that leads us from experimental plan to data collection, we will present two vignettes. See Fig. 1 for a graphic representation of viral expression and design considerations.

Vignette #1

5-HT_{1B} receptors in the mesolimbic reward pathway, specifically those expressed on GABAergic projection neurons emanating from the striatum and terminating in the basal ganglia [15] have been shown to be regulated by cocaine, alcohol, and chronic social stress [16–18]. We postulated that this upregulation was due to compensatory adaptation (i.e., if these receptors in this pathway modulate the rewarding effects of drugs of abuse and they upregulate during chronic social stress, this might constitute a compensatory adaptation that diminishes the impact of aversive stimuli and enhances pleasurable experiences). Therefore, we planned to manipulate 5-HT_{1B} receptors at discrete time intervals during social stress exposure to determine if receptor expression altered behavioral outcomes after stress. In other words, to answer the question: could hedonic deficits that occur after stress be rescued? HSV is an ideal vector for this because it is transiently expressed, allowing us to upregulate 5-HT_{1B} receptor expression during specific intervals during stress exposure or behavioral testing. We hypothesized that enhancing 5-HT_{1B} receptor levels *during* chronic social stress would improve depressive-like behavioral

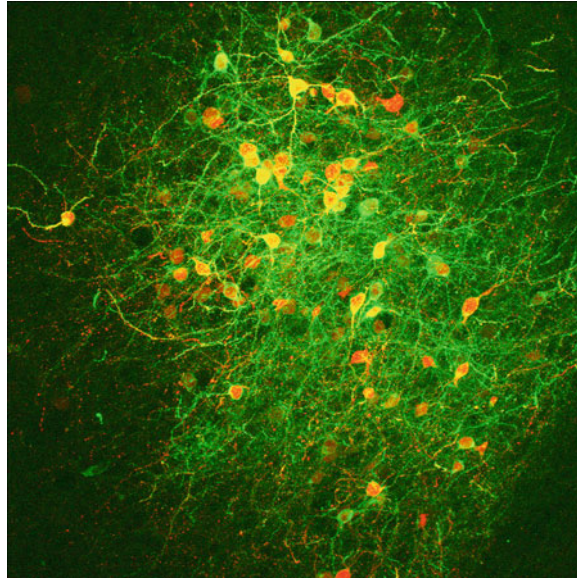


Fig. 2 Example of GFP coexpression with epitope-tagged transgene. Dual expressing HSV viral vector was infused into rat nucleus accumbens shell and was visualized microscopically 4 days later. Hemagglutinin-tagged 5-HT_{1B} receptor (*red*) consistently is localized in the same neurons as GFP (*green*); there is no expression in glial cells with this vector. The section was imaged using confocal microscopy at $\times 20$ magnification and the image stack was compressed in the z-axis

outcomes that follow the stress experience. Hedonic reactions were evaluated by measuring the preference for saccharin using a two bottle choice protocol; stressed animals display reduced preference for this sweetened solution [16] and this is widely interpreted as representing an anhedonic state, such as occurs in depressed individuals. Rats had two drink training exposures to a 0.1 % saccharin drink and then viral vector injections into the nucleus accumbens shell. Animals were allowed to recover for 2 days and then exposed to four sessions of social defeat (corresponding with the interval of greatest 5-HT_{1B} transgene expression) (Fig. 2). In our hands, we determined that four consecutive sessions of social defeat are sufficient to induce deficits in animals' consumption of a rewarding liquid such as saccharin [16]. Alternatively, we briefly considered using cannula so that we could allow the animals more recovery time, but decided against it because of the robust scuffles that are a feature of social defeat. After 4 consecutive days of social defeat, we tested the animals' hedonic responsiveness with the saccharin preference test again. This should be the time just after the peak of virus expression, so if increased 5-HT_{1B} expression in nucleus accumbens shell projection neurons was blunting the impact of the negative experiences and enhancing sensitivity to positive experiences and acting like an antidepressant treatment, then the stress+5-HT_{1B} group would have

a greater preference for saccharin. However, we found no effect of increased 5-HT_{1B} expression on social defeat stress-induced reduction in hedonic responses (Nair, submitted for publication). Thus, in the context of our larger body of work, we concluded that these 5-HT_{1B} receptors can alter artificial drug rewards but not natural rewards such as a sweetened solution.

Vignette #2

Previous work from our group and others has clearly demonstrated that distinct subregions of DRN have different afferent and efferent connections [19] and differential effects on different emotional behaviors such as fear, anxiety, and depression-like behavior. Since our previous work showed that the ventromedial DRN was important in anxiety-like behavior, and since the caudal DRN is stress sensitive, we wanted to determine the role of 5-HT_{1B} autoreceptors that expressed in this region and transported to axon terminals, and their role in mediating fear and anxiety. Once again, we took advantage of the transient expression levels of HSV vectors to express 5-HT_{1B} receptors in the DRN either during training and acquisition of conditioned fear or during expression of the learned behavior. We timed gene transfer (either 5-HT_{1B}/GFP or GFP alone to occur either 3 days prior to fear conditioning or 3 days prior to fear testing in two separate experiments). In the first experiment, we allowed enough time to elapse after fear conditioning so that transgenic 5-HT_{1B} overexpression had dissipated. We were able to show that receptor overexpression during acquisition of behavior did not alter the acquisition of conditioned fear behavior, whereas when increased 5-HT_{1B} autoreceptor expression occurred during expression of previously conditioned fear, the behavior was disrupted, suggesting that 5-HT_{1B} autoreceptors in the caudal DRN neurons projections regulate only the behavioral expression of conditioned fear.

5 Conclusion

In conclusion, working with viral vectors poses certain challenges that range from governmental and institutional oversight and regulations to experimental design considerations. Once biosafety practices are in place, careful designing of experiments can yield very useful information about complex animal behaviors that may eventually shed light onto complex human behaviors or enable us to treat certain dysfunctional aspects of behavior. Some of the issues that are most important to the success of a viral expression behavior experiment include paying attention to the timing, localization, and duration of gene expression in relation to key elements of the behavioral testing procedure. Further, optimizing stereotaxic procedures so that maximal transgene expression occurs at the key time points in the experiment, and histological confirmation of

accurate gene expression will greatly enhance the likelihood of success in stress experiments with viral vectors. The future of research using viral vectors is promising. There are hundreds of promoters that can be exploited to drive transgene expression in specific neurons or circuits, and most recently, the introduction of DREADDs that are activated by nonendogenous designer drugs allow the use of vectors without the confounding issues of endogenous ligands. Viral technologies afford a flexibility that will continue to inspire innovations in behavioral testing paradigms and the exploding number and combination of cells and circuits that can be studied are likely to yield answers to some of the most complex and perplexing questions in neuroscience.

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