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5-HT6R Viral Vector-Mediated Indirect Pathway Activation in the Dorsolateral Striatum: A Discussion on Basal Ganglia Habitual and Goal-Directed Circuits

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A Discussion on Basal Ganglia Habitual and Goal-Directed Circuits

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Abstract

Altering maladaptive behavioral tendencies is relevant for clinical interventions, making research on underlying mechanisms of habit essential. Mechanisms of habit are explored here with differential activation of the indirect pathway in the basal ganglia. Viral vector-mediated overexpression of the 5-hydroxytryptamine 6 (5-HT6) receptor in the indirect pathway of the dorsolateral striatum was used to increase indirect pathway activity. Subjects were trained such that control animals were expected to exhibit habitual behavior. We hypothesized increased activation of the indirect pathway would maintain goal-directed behavior. To test this hypothesis female rats were assigned to 5-HT6 receptor upregulation or control groups in a reward devaluation behavior paradigm to assess habitual behavior. Although our results do not show anticipated behavioral results following reward devaluation, a lack of statistical power due to small sample sizes does not allow conclusions to be reached.

Introduction

Understanding Habitual Behavior. Balancing habitual and goal-directed behaviors is beneficial for optimizing efficient environmental interactions (Balleine & O'Doherty, 2009; Shan, Christie, & Balleine, 2015). Novel interactions within an environment are understood to use goal-directed behavior; the subject is motivated to perform a certain behavior based on the availability and salience of a reward (Coutureau & Killcross, 2003; Shan et al., 2015). As interactions with the environment become more familiar, behavior is understood to likely transition into habit (Balleine & O'Doherty, 2009; Shan et al., 2015). In the research presented here habit is defined as the lack of sensitivity to reward devaluation (Adams & Dickinson, 1981). Exploring indirect

pathway upregulation techniques promoting goal-directed behavior is important for understanding the role of indirect pathway activity in habitual behavior.

Basal Ganglia and Dorsolateral Striatum. The Basal Ganglia (BG) is a series of behavior-refining nuclei implicated in decision making processes with favorable, targetable properties for research (Schmidt, Leventhal, Mallet, Chen, & Berke, 2013). Literature has shown how subnuclei of the BG participate in certain behaviors, with the dorsolateral striatum (DLS) for habitual behavior and the dorsomedial striatum (DMS) for goal-directed behavior (Gremel & Costa, 2013; Yin et al., 2004). The dorsolateral striatum is necessary for the formation of habitual behavior (Yin, Knowlton, & Balleine, 2004). Characterization of dorsal striatum subnuclei is relevant in discussions for psychopathologies like Huntington's disease, Tourette's Syndrome, and Parkinson's disease, as well as addiction research (Balleine, Delgado, & Hikosaka, 2007; Eskenazi, Brodsky, & Neumaier, 2015; Nelson & Killcross, 2013).

As mentioned, the DLS participates in the production of habitual behavior (Gremel & Costa, 2013; Yin et al., 2004). The landscape for habitual and goal-directed behavior research supports the concept of a threshold between habit and goal, at which point a shift in behavior occurs (Unpublished data, Toufexis Lab; Gremel & Costa, 2013). This research is primarily concerned with the DLS and behavioral flexibility resulting from increased activation of the indirect pathway.

Habitual and goal-directed behaviors employ both direct and indirect pathways (Eskenazi et al., 2015; O'Hare et al., 2016). Although research has shown surgical manipulation of a single pathway can change habitual behavior formation (Gremel & Costa, 2013; Yin et al., 2004),

research is moving away from the hypothesis that the indirect and direct pathways are naturally singularly activated and mutually antagonistic (O'Hare et al., 2016). Instead of the on/off approach to describe the activity of the direct or indirect pathways, a more inclusive explanation incorporates pathway timing. The research presented in this document explores how goal-directed behavior can be the result of greater indirect pathway strength than direct pathway strength.

Evidence for relative pathway activation in behavior formation includes research by O'Hare et al. (2016) where timing of direct and indirect pathway striatal projection neuron (dSPN and iSPN, respectively) connectivity shifted between habitual and goal-directed behavior. Habitual subjects showed dSPN firing before iSPN firing, and goal behavior subjects showed iSPN firing before dSPN firing, regardless of spike amplitude. O'Hare et al. (2016) suggested a new interpretation of pathway balance, prioritizing competitive pathway timing as a metric for pathway interaction producing either habitual or goal-directed behavior (O'Hare et al., 2016; Schmidt et al., 2013).

Sex Difference in Habit Formation. Data has shown female rats achieve habitual behavior with fewer reinforcers than male counterparts (Unpublished results, Toufexis Lab). Male subjects show habitual behavior at 240 reinforcers. In female Long Evans rats, research by Schoenberg et al. (Unpublished data) has determined the training threshold between habit and goal in female rats to be between 120 and 140 response-outcome pairings on a VI-30s schedule in an operant conditioning paradigm specified in methods below.

Research has shown 5-HT6 receptor upregulation in the indirect pathway is associated with behavioral flexibility in overtrained male rats (Eskenazi et al., 2015). Their work showed 5-HT6

receptor upregulation in the indirect pathway of the DLS produced a greater lever press rate difference between omission trained and yoked subjects than control subjects. These results have yet to be established in female subjects. This present research contributes and complements the canon of primarily male subject research in research studying the development of habitual behavior. This experiment utilized gonadally-intact female rats with viral vector methods described in work by Eskenazi et al. (2015) to combine indirect pathway activity increase with habit threshold research.

This present research contributes to the understanding of the strength of the 5-HT6 receptor for selective pathway activation as tested above threshold in a female subject model. Above-threshold testing is required experimentation to participate in the understanding that increased indirect pathway activation maintains goal-directed behavior.

Introduction to 5-HT6 Receptors as Modulators. In this experiment the infusion target was the indirect pathway and was isolated using a preproenkephalin-targeting viral vector for 5-HT6 receptors (pENK-5-HT6). The 5-HT6 receptor is expressed throughout the striatum across the indirect and direct pathways (Roberts et al., 2002; Ward & Dorsa, 1996). The 5-HT6 receptor is coupled to adenylyl cyclase/cAMP pathway with the Gαs protein (Masson, Emerit, Hamon, & Darmon, 2012).

The research presented here leveraged molecular differences between the indirect and direct pathway with the expression of enkephalin and dynorphin, respectively. Enkephalin and dynorphin signaling molecules are not expressed in significant concentrations in both pathways (Ward & Dorsa, 1996). Enkephalin is only expressed in the indirect pathway while dynorphin is

only expressed in the direct pathway (Ward & Dorsa, 1996). This provides a valuable endogenous distinguishing feature between the indirect and direct pathways. 5-HT6 receptor genetic information coupled to enkephalin promoter regions provides an opportunity to selectively activate the indirect pathway.

Hypothesis. We hypothesized viral vector-mediated overexpression of 5-HT6 receptors in the indirect pathway would maintain goal-directed behavior at a degree of training expected to produce habitual behavior.

Methods

Viral Vector. Herpes Simplex Viruses (HSV) containing 5-HT6 preproenkephalin (pENK) and GFP pENK genetic information were used (Neumaier Lab at The University of Washington). 5-HT6 receptors were modified to contain a double hemagluttinin (HA) tag differentiating introduced receptors from endogenous. Viruses were stored in 1.5 mL microcentrifuge vials at -80 degrees Celsius. Viruses arrived on September 2017 and were kept on ice until February 2018. Refreeze transactions were minimized, and when necessary viruses were pre-refrozen using liquid nitrogen.

Subjects. 12 female, ovary-intact, 75-90 day-old Long Evans rats from Charles River underwent a 7 day period of habituation upon arrival. Animals were weighed and handled daily. Following recovery from surgery all animals were maintained at 85% of their pre-surgery weights by food restriction. Food restriction was implemented to motivate subjects for appetitive reinforcement during conditioning (see Methods: Behavior). 6 subjects were assigned to the GFP-pENK control group and 6 were assigned to the 5-HT6-pENK experimental group.

Surgical Procedure. 5-HT6 receptor infusion surgeries began on January 26th and were completed by February 5th 2018. All control animals underwent infusion surgeries with GFP-pENK before experimental groups with 5-HT6-pENK.

Subjects were anesthetized with 2-4% isoflurane gas and 1.5 O_2 flow for the duration of surgery. The scalp was cleared using an electric razor. Once cleared, the subject was mounted into a stereotax and isofluorane gas was reduced to 2-2.5% flow through a sealed nose cone. Scalp was cleaned with betadine then EtOH and gauze, and eye goop (Puralube Vet Ointment) was applied to subject's eyes prior to incision. A curved scalpel was used to open scalp anterior to posterior along the midline, producing a 2 cm incision allowing visibility of bregma. Scalp was held using 4 bulldog clips at corners of incision. Once exposed, the skull surface was cleaned gently with sterile q-tips and mechanical force to separate any remaining layers of tissue covering skull. H_2O_2-dampened gauze was used to clean skull if mechanical force was insufficient to visualize bregma. To improve coordinate visibility, the skull was scrubbed with the end of a narrow spatula then polished with gauze. The bregma-lambda plane was made horizontal by adjusting the stereotax height of the mouth bar, and ear bars were centered as necessary. With a fine ink marker, bregma was marked and the stereotax was zeroed to this point. The DLS was targeted using the coordinates: A/P: +0.7 mm from bregma; M/L: +/-3.8 mm from midline; and D/V: -4.0 mm from brain surface (Eskenazi et al. 2015). A 0.75 mm spherical drill bit and mounted Dremel tool were used to produce bilateral vertical bore holes.

A 1 mL Hamilton syringe was prepared by dampening the plunger of the syringe in fresh PBS repeatedly, coating the interior of the chamber with PBS to create suction. The entire syringe chamber was flushed with PBS before plunger insertion using a 10 microliter pipette to ensure

maximum suction. The syringe and plunger were mounted into a Harvard Apparatus Pump 11 Elite and 5 microliters of virus were drawn into the syringe. Syringe needle tip was lowered to the surface of the brain where the dorsal/ventral syringe height was recorded. From these coordinates the needle was lowered 4 mm ventrally. 2 minutes elapsed between completion of needle depth and infusion commencement to allow tissue accommodation before infusion. The infusion program delivered 2 microliters of virus over 10 minutes. After infusion completion, 5 minutes were allowed for backflow, then the syringe needle was slowly withdrawn from brain using the stereotax arm.

This infusion procedure was repeated for the contralateral bore hole and bore holes were monitored for bleeding throughout. Following infusion, a wax plug was applied to each bore hole, puttied flush with surface of skull using the end of a narrow spatula. The scalp was brought together along midline and veterinary sutures were used to stitch, then Vetbond by 3M was applied to sutures after closure. The subject was dismounted from stereotax, and carprofen and ringer's saline were administered.

Behavior Paradigm. Animals were trained to perform nose pokes for sucrose pellet rewards. Nose poking was recorded to quantify habit formation, with half of the animals pairing their presentation with lithium chloride (LiCl), a nauseating agent. LiCl for reward devaluation has been established as an effective method for assessing habitual behavior (Adams, 1982). Animals in habit should be insensitive to devaluation of the reinforcer and show no significant decrement in responding following LiCl pairings (Adams & Dickinson, 1981).

The training apparatus was comprised of six standard rat operant chambers (Med Associates, St. Albans, VT) kept within individual noise-attenuating cabinets ventilated by low-noise fans. In the center of the right-facing chamber wall was a head-entry port into which a hopper delivered a 45-mg sucrose pellet (Bio-Serv). To the right of the head entry was a nose-poke device (ENV-114, Med Associates) which emitted an infrared beam; when animals performed a nose-poke, this beam was disrupted, and nose-poke entries were recorded. All data from the operant boxes was monitored and collected by MED-PC software (Med Associates). At the onset of training, all animals were assigned to a specific operant chamber in which they received all subsequent conditioning and testing for the duration for the experiment, to reduce context switch effects. The data collected from each animal's behavior provided the foundation for these analyses and conclusions. The behavior paradigm is outlined here, beginning with Magazine Training and completing with Reacquisition Test.

Magazine Training: Subjects were first exposed to the operant chambers in two 30-minute sessions. Nose-poke holes were physically blocked during these sessions, and sucrose pellets were freely delivered on a random time (RT) 60-second schedule. These sessions were designed to build associative cues between the sound of a sucrose pellet reinforcer dropped in the hopper and the salient reward of the pellet. Pellet consumption was recorded at this phase.

Free Response: Following magazine training, animals underwent two free response sessions in which animals could nose-poke for sucrose pellets. Pellets were delivered on a continuous reinforcement schedule until 25 pellets were earned. This phase of behavior reinforces sucrose-seeking behavior directly, helping subjects encode that the nose-poke behavior results in reinforcement. Both nose pokes and sugar pellet consumption were recorded during this phase.

Variable Interval-30: following free response training, animals were given three daily acquisition sessions in which nose-poke responses were reinforced on a variable interval-30-second (VI-30) schedule (DeRusso et al., 2010). Each session terminated after animals had earned 50 reinforcers, for a total of 150 reinforcers earned during VI-30 acquisition. Data suggests female Long Evans rats show habitual behavior at 140 response-outcome pairings on a VI-30s schedule. These three days of VI-30 training are referred to as acquisition in the results of this research. Nose pokes and pellets consumed were recorded at this stage.

Reward Devaluation: Half of the subjects were randomly assigned to the reward devaluation paired group and their cage mates assigned to the unpaired group. All subjects experienced a reinforcer devaluation paradigm which proceeded until criterion was me (i.e. all animals in the paired group had ceased all consumption of sucrose pellets). It was crucial to drive consumption to zero in the paired group during this procedure to dissociate responding at test from any operant motivation for the reinforcer. During each session of reward devaluation nose-poke responses were prevented by removal of the nose-poke holes from the operant chambers. Animals were freely delivered pellets on a VT 30-s schedule.

On odd-numbered days all subjects experienced operant boxes although only subjects assigned to the paired group received sucrose pellets starting with a total of 40 pellets on Day 1. The unpaired subject was yoked in these sessions to the paired subject in the neighboring operant box; their sessions were terminated at the same time when the paired subject received all pellets. Upon the completion of odd-numbered sessions, all rats were immediately removed from the operant chambers and injected intraperitoneally with a 10 ml/kg dose of .15 M lithium chloride (LiCl) to induce nausea.

On even-numbered days subjects assigned to the unpaired group received sucrose reinforcers, while paired subjects were placed in the operant chambers for the same duration as their yoked counterparts without receiving sucrose. The paired subject was yoked in these sessions to the unpaired subject in the neighboring operant box; their sessions were terminated at the same time when the unpaired subject received all pellets. Immediately following the termination of these sessions, all animals received an intraperitoneal injection of 0.9% physiological saline of equivalent size to the LiCl injections. In this way, all rats experienced the same amount of time in the operant chamber and the same number of injections of both LiCl and saline. As devaluation continued, paired animals consumed increasingly fewer pellets during their sessions, and the average number of pellets consumed would be presented the following day to the unpaired animals in their sessions.

Extinction Test: Following RD, habitual behavior was evaluated under extinction conditions: the nose-poke holes were made available but responses were not reinforced. Because habit was operationalized as an insensitivity to devaluation of a reinforcer, habitual animals in the paired group should have demonstrated no significant differences in responding from their unpaired counterparts. If animals remained goal-directed, the devaluation of the sucrose reinforcer should have led to paired animals performing significantly fewer responses than their unpaired counterparts, because in the paired group the outcome is no longer motivating. Nose pokes were recorded.

Consumption Test: On the day following the extinction test, a consumption test was conducted. Nose-poke holes were removed from the operant chambers and animals were freely delivered 20 sucrose pellets. Consumption for both groups was recorded. This test confirmed the success

of the devaluation of sucrose in paired animals who were expected to reject all delivered pellets. This allowed for the interpretation that responding by paired animals in the extinction test was a result of habitual behavior and not any remaining operant motivation for the reinforcer.

Reacquisition Test: The success of the reinforcer devaluation paradigm was confirmed with a 30-minute reacquisition test. Nose-poke holes were made available in the operant chambers, and animals could earn sucrose reinforcers on a VI-30s schedule. When re-exposed to the sucrose reinforcer, rats for which an aversion was successfully conditioned (paired group) were expected to exhibit decreased nose-poking for the sucrose.

Viral Vector Verification. Following behavior experimentation, subjects were perfused with 4% paraformaldehyde. Brains were extracted and allowed to post-fix in 4% paraformaldehyde for 2 hours then cryoprotected in 30% sucrose. Sections were taken at 25µm thickness and stored in wells loaded with PBS and sodium azide.

In preparation of immonolabeling verification, floating tissue sections (25µm) were washed with 0.1M PBS for ten minutes, two times. Sections were then blocked for 60 minutes (PBS 0.1 M; 0.1% BSA; 0.2% Triton X-100; 2% serum). Sections were incubated in the primary antibodies, 2% rabbit anti-enkephalin (Immunostar) and 2% chicken anti-HA (Abcam) at 4 degrees Celsius for 48 hours. Sections were then washed with 0.1M PBS for ten minutes, four times. Secondary antibody, species-specific Alexafluor 488 (green) and 568 (red; Invitrogen), was allowed to incubate sections for 60 minutes. Following secondary antibody incubation, sections were rinsed with 0.1M PBS for ten minutes, two times. Sections were mounted onto slides and coverslipped using Vectashield (Vector laboratories).

Statistical Analyses. Average nose-pokes per minute were recorded for all phases of the experiment. Recorded data was analyzed using IBM's SPSS statistical analysis software, and GraphPad Prism software was used for graphical presentation of the data.

Results

Verification of Expression. At the time of this writing full verification of vector expression was in progress. We anticipate anti-HA colocalization with anti-ENK in indirect medium spiny neurons (Eskenazi et al., 2015). Additionally, we anticipate anti-HA will not be colocalized with markers for direct medium spiny neurons. While current verification is incomplete, preliminary evidence from pilot research shows successful single immunolabelling for the HA tag (Figure 1). Verification immunolabeling will continue while sections are available.

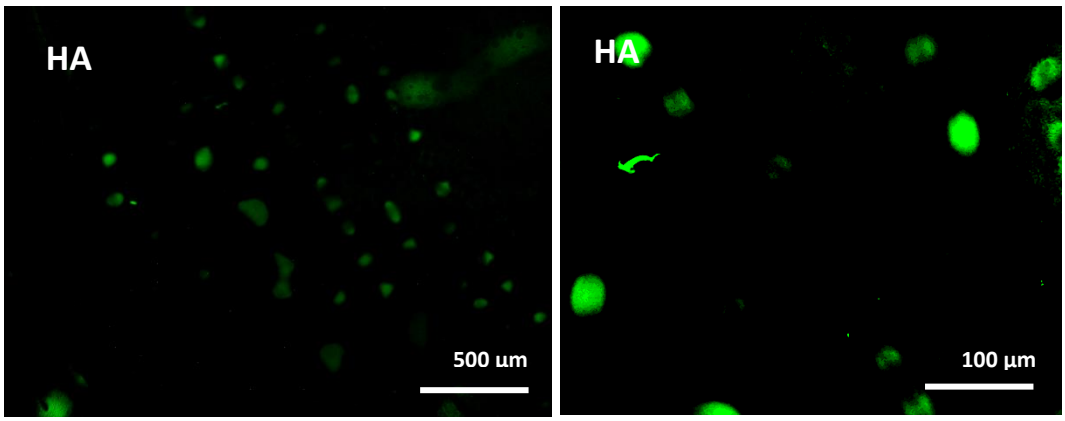

***Figure 1**: Pilot research single immunolabeling showing successful anti-HA visibility*

Behavior.

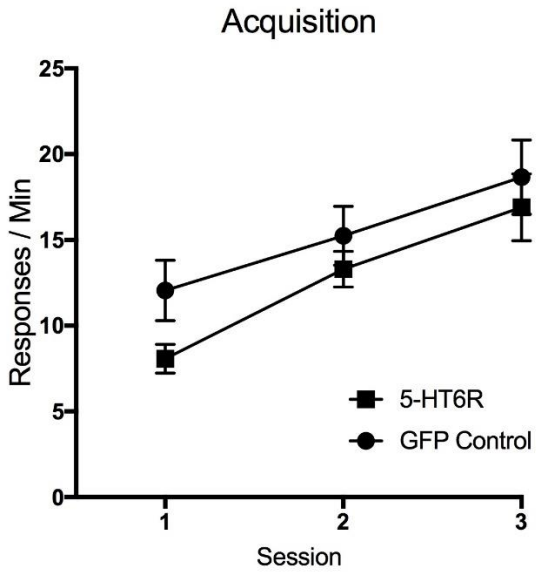

***Figure 2**: Mean responses per minute in acquisition.*

Acquisition: A 2 (Virus Group: 5-HT6R, GFP) x 2 (Pairing Group: Paired, Unpaired) x 3 (Training Session: 1, 2, 3) repeated measures ANOVA revealed a significant main effect of session ($F(2,16 = 32.42$, $p < .001$), indicating that all animals acquired nose-poking for sucrose (Figure 2). Additionally, there appeared to be no significant differences between virus groups or anticipated pairing group in acquisition: there was no significant main effect of Virus Group ($F(1,8) = 1.41$, $p = .269$), anticipated Pairing Group ($F(1,8) = 1.16$, $p = .313$), and no significant Virus group x Pairing group interaction ($F(1,8) = .23$, $p = .644$).

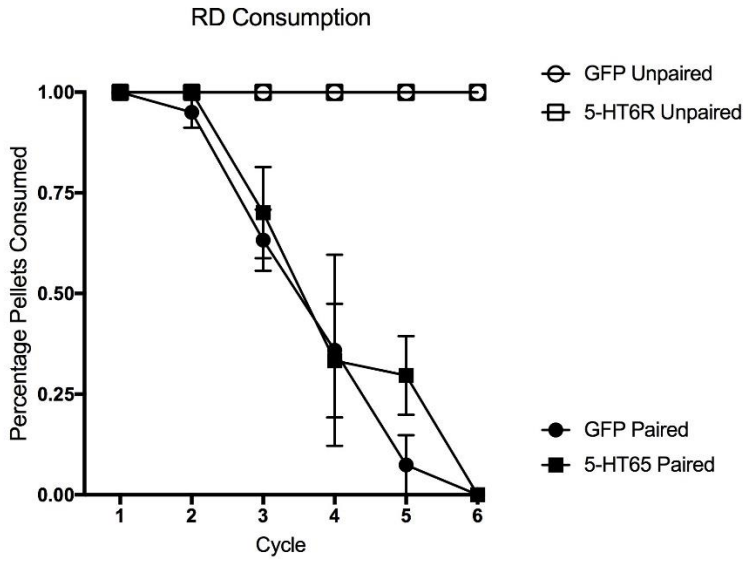

***Figure 3**: Consumption of sucrose pellets in paired and unpaired animals across sessions of RD.*

Reward Devaluation and Extinction: All animals in the paired group reached criterion (zero pellets consumed) by the end of reward devaluation, and all unpaired animals consumed all delivered pellets (Figure 3). A 2 (Virus Group: 5-HT6R, GFP) x 2 (Pairing Group: Paired, Unpaired) factorial ANOVA revealed a lack of significant main effects of pairing ($F(1,8) = .473$, $p = .511$) or virus group ($F(1,8) = .583$, $p = .467$). Additionally, there was no significant Virus Group x Pairing Group interaction ($F(1,8) = .011$, $p = .921$). Further, pairwise comparisons with estimated marginal means revealed no significant difference between nose-poke response rate between 5-HT6R Group Paired ($M = .900$, $SEM = 2.177$) and Unpaired ($M = -.900$, $SEM = 2.177$; $p = .690$), or between GFP Group Paired ($M = 1.217$, $SEM = 2.177$) and Unpaired ($M = -1.2177$, $SEM = 2.177$; $p = .592$; see Figure 4).

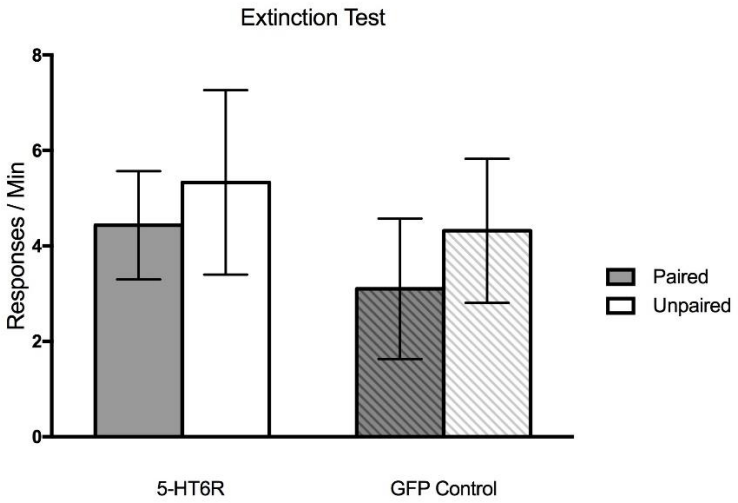

Figure 4. *Mean nose-pokes per minute during the extinction test.*

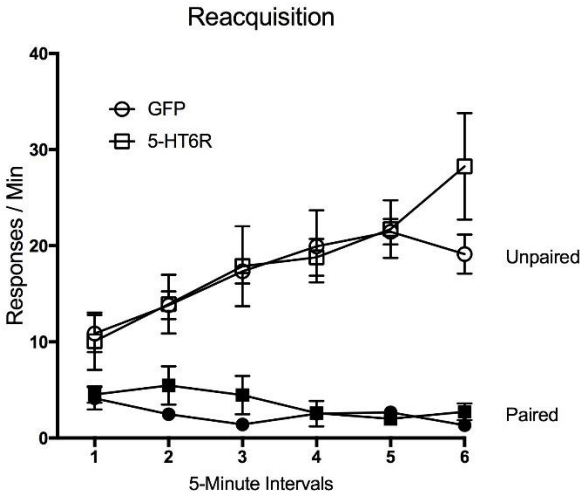

Figure 5*: Mean responses per minute in reacquisition.*

Consumption and Reacquisition: The consumption test confirmed successful devaluation: on average, paired subjects rejected all delivered sucrose pellets and unpaired subjects consumed all delivered pellets. Responding in reacquisition was analyzed using a 2 (Virus Group: 5-HT6R, GFP) x 2 (Pairing Group: Paired, Unpaired) x 6 (Time: 6 five-minute bins) repeated measures ANOVA. Results of this analysis revealed significant main effects of time ($F(5,40)=5.97$, $p<.001$)

and Pairing ($F(1,8)=77.65$, $p<.001$), and a significant time x pairing group interaction ($F(5,40)=12.65$, $p<.001$), indicating that, collapsed across viral groups, paired and unpaired animals responded significantly differently from one another. Paired animals significantly increased responding across the test and unpaired animals significantly decreased responding (Figure 5).

Discussion

This research hypothesized indirect pathway activation would increase behavioral flexibility. We predicted 5-HT6 receptor-mediated indirect pathway activation would maintain goal-directed behavior at reinforcer levels previously associated with habitual behavior in female subjects. Although our results did not show significant behavioral changes following reward devaluation, a lack of statistical power due to small sample size does not allow conclusions to be reached.

Our hypothesis was tested using subjects assigned to 5-HT6 receptor upregulation or control groups and assessed in a behavior paradigm characterized by reward devaluation sessions to distinguish habitual behavior. Together, exploring goal-directed behavior past the predetermined reinforcer threshold for habit was important for understanding the role of indirect pathway activation in goal-directed behavior promotion. This research has implications for the treatment of behaviors associated with indirect pathway imbalances, such as behaviorally rigid and habitual states, and addiction behavior.

Technique Selection. Selective pathway activation research participates in the growing understanding of habitual behavior formation in the mammalian brain (Schmidt et al., 2013). Many techniques can produce pathway-specific activation; however, few techniques employ

endogenous neurotransmitter concentrations or receptors for more naturalistic pathway modulation (Brodsky, Gibson, Smirnov, Nair, & Neumaier, 2016; Eskenazi et al., 2015; Neumaier et al., 2010).

The research presented here combines the technique potential of pathway specific peptides plus 5-HT6 receptors in a viral vector designed to fulfill three requirements: i) visibility, ii) specificity, and iii) modulation. These three criteria provide the most applicable and accurate results to understand habitual and goal-directed behavior in a rat model. Viral vectors emerged as the optimal technique for 5-HT6 receptor upregulation in the indirect pathway for the desired scope of this experiment.

Optogenetics, knockin and knockout, and electrode stimulation techniques are techniques shown to activate pathways for on/off activity (Hall, Limaye, & Kulkarni, 2009; Mei & Zhang, 2012; Surmeier, Ding, Day, Wang, & Shen, 2007). Optogenetics offer the opportunity to increase or decrease pathway activation in the DLS with advantageous visibility, excellent temporal resolution of stimulation, and the opportunity to use customized receptors (Mei & Zhang, 2012). Optogenetic techniques have not been used to produce a continuum of activation, rather optogenetic techniques produce an on/off activation in target regions (Eleftheriou, Cesca, Maragliano, Benfenati, & Maya-Vetencourt, 2017; Mei & Zhang, 2012). Similarly, knockout and knockin techniques were not chosen in this experiment for their insensitivity to modulation, irreversibility, and potential off-target compensatory effects (Hall et al., 2009). Lastly, electrode stimulation of medium spiny neurons have not shown successful results, therefore electrode stimulation lacked the modulation necessary for realistic pathway activation (Surmeier et al., 2007).

Viral vectors as delivery tools for 5-HT6 receptors emerged as a promising technique in this experiment. Viral vectors provided advantages over competitor selectivity techniques for visibility, specificity, and modulation. For visibility, coupling 5-HT6 receptor and green fluorescent protein (GFP) genetic information with preproenkephalin promoter region ensured indirect pathway targeting and allowed for target verification. For specificity, viral vector-mediated receptor upregulation provided a solution to the pathway selection challenge when viral vectors target some of the few inherent chemical differences in the direct and indirect pathway – enkephalin and dynorphin. And for modulation, the 5-HT6 receptors are endogenously present in the striatum, leaving modulatory effects of endogenous neurotransmitters intact. Serotonin receptor family expression is present in the enteric and central nervous systems, except for the 5-HT6 receptor (Woolley, Marsden, & Fone, 2004). Unlike all other serotonin receptors, the 5-HT6 receptor is expressed in negligible quantities in the gut but shows high concentrations in the BG (Woolley, Marsden, & Fone, 2004) providing an additional specificity advantage.

Considering delivery tools, herpes simplex viruses (HSV) have well-documented efficacy for transmitting customized payloads of genetic information with confidence in fidelity of genetic information transfer (Eskenazi et al., 2015). HSV is a powerful delivery tool for influencing the genome of nondividing cells, unlike adenovirus or retroviral counterparts which are effective in dividing cells (Fink, Deluca, Goins, & Glorioso, 1996). An additional advantage of HSV is the induced inability to reactivate, a characteristic allowing researchers to intracranially infuse a substance-carrying HSV without concern of continuous reactivation or latent storage of the original viral genome (Fink et al., 1996).

Drug Addiction and Habit. It is necessary for the research community to recognize how drug addiction to psychostimulants and analgesics is increasing in the United States (Hedegaard, Chen, & Warner, 2015). Individuals experiencing drug addiction perform habitual drug-seeking behavior characterized by repeated attempts to acquire and administer drugs. The BG circuit is implicated in the reward value system and reinforcer-seeking behavior, making pathway modulation of particular interest in behavioral neuroscience research (Balleine et al., 2007; Brodsky et al., 2016). If direct and indirect striatal projection neurons can be better characterized in relation to habit- or goal-directed behavior then drug addiction research will have a better understanding of the timing, magnitude, and plasticity of the connections formed when an individual presents with drug addiction tendencies.

This research was founded on work showing gonadally-intact female Long Evans rats demonstrate habit above 140 reinforcers and goal-directed behavior below 120 reinforcers. These results differ from male rats showing goal-directed behavior at 240 reinforcer exposures (Unpublished Results, Toufexis Lab). These particular findings have implications for treatments for behaviorally rigid states in women. Estrogen and methamphetamine are understood to be accelerators of habitual behavior, as women exposed to these compounds show reduced training time to reach addiction, as well as increased relapse frequency (Becker & Hu, 2009). Combining these results, future research could conduct behavior training in the presence of psychostimulants in conjunction with an indirect pathway upregulator, such as 5-HT6R, to elucidate the influence of habit promoters and habit suppressors simultaneously. Another research opportunity could combine both 5-HT6 receptor introduction in the indirect pathway with estrogen replacement in ovariectomized female subjects.

One limitation of the present research includes verification of BG targets. Pathway selectivity of the viral vector is in the process of immunocytochemical verification, with results anticipated to show colocalization of enkephalin and 5-HT6 in the indirect pathway. These immunocytochemical results are necessary to relate behavioral data to the indirect pathway.

Conclusions

The methods presented here establish a protocol for selective activation in the indirect pathway. We hypothesize that 5-HT6 receptor upregulation for selective pathway activation will alter habitual behavior. Together, this pilot research contributes to the literature understanding habitual behavior to take steps toward better therapies for individuals experiencing a range of psychopathologies in which habitual behavior participates.

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Supplemental Materials

Acquisition SPSS Output.

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F
time_bin	Sphericity Assumed	240.936	5	48.187	5.966
	Greenhouse-Geisser	240.936	2.261	106.550	5.966
	Huynh-Feldt	240.936	4.380	55.010	5.966
	Lower-bound	240.936	1.000	240.936	5.966
time_bin * virus_group	Sphericity Assumed	71.745	5	14.349	1.777
	Greenhouse-Geisser	71.745	2.261	31.728	1.777
	Huynh-Feldt	71.745	4.380	16.381	1.777
	Lower-bound	71.745	1.000	71.745	1.777
time_bin * pairing	Sphericity Assumed	510.656	5	102.131	12.645
	Greenhouse-Geisser	510.656	2.261	225.830	12.645
	Huynh-Feldt	510.656	4.380	116.592	12.645
	Lower-bound	510.656	1.000	510.656	12.645
time_bin * virus_group * pairing	Sphericity Assumed	58.549	5	11.710	1.450
	Greenhouse-Geisser	58.549	2.261	25.893	1.450
	Huynh-Feldt	58.549	4.380	13.368	1.450
	Lower-bound	58.549	1.000	58.549	1.450
Error(time_bin)	Sphericity Assumed	323.067	40	8.077	

	Greenhouse-Geisser	323.067	18.090	17.859	
	Huynh-Feldt	323.067	35.039	9.220	
	Lower-bound	323.067	8.000	40.383	

Tests of Between-Subjects Effects

Measure: MEASURE_1

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	7783.361	1	7783.361	154.554	.000
virus_group	29.645	1	29.645	.589	.465
pairing	3910.227	1	3910.227	77.645	.000
virus_group * pairing	.094	1	.094	.002	.967
Error	402.880	8	50.360		

Extinction SPSS Output.

Tests of Between-Subjects Effects

Dependent Variable: avgpokepermin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7.577[a]	3	2.526	.355	.787
Intercept	221.450	1	221.450	31.152	.001
pairing	3.360	1	3.360	.473	.511
Viralgroup	4.142	1	4.142	.583	.467
pairing * Viralgroup	.075	1	.075	.011	.921

Error	56.870	8	7.109		
Total	285.898	12			
Corrected Total	64.447	11			

Estimated Marginal Means

1. pairing * Viralgroup

Pairwise Comparisons

Dependent Variable: avgpokepermin

Viralgroup	(I) pairing	(J) pairing	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound
gfp control	unpaired	paired	1.217	2.177	.592	-3.803
	paired	unpaired	-1.217	2.177	.592	-6.237
enkephalin virus	unpaired	paired	.900	2.177	.690	-4.120
	paired	unpaired	-.900	2.177	.690	-5.920

Pairwise Comparisons

Dependent Variable: avgpokepermin

Viralgroup	(I) pairing	(J) pairing	95% Confidence Interval for Difference Upper Bound
gfp control	unpaired	paired	6.237
	paired	unpaired	3.803

enkephalin virus	unpaired	paired	5.920
	paired	unpaired	4.120

Pairwise Comparisons

Dependent Variable: avgpokepermin

pairing	(I) Viralgroup	(J) Viralgroup	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound
unpaired	gfp control	enkephalin virus	-1.017	2.177	.653	-6.037
	enkephalin virus	gfp control	1.017	2.177	.653	-4.003
paired	gfp control	enkephalin virus	-1.333	2.177	.557	-6.353
	enkephalin virus	gfp control	1.333	2.177	.557	-3.687

Pairwise Comparisons

Dependent Variable: avgpokepermin

pairing	(I) Viralgroup	(J) Viralgroup	95% Confidence Interval for Difference Upper Bound
unpaired	gfp control	enkephalin virus	4.003
	enkephalin virus	gfp control	6.037
paired	gfp control	enkephalin virus	3.687
	enkephalin virus	gfp control	6.353

Reacquisition SPSS Output.

Tests of Within-Subjects Effects

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