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Developing Novel Methods to Investigate Real-Time \textit{in Vivo} Dopamine Dynamics in the Monogamous Prairie Vole

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Abstract

Humans are among a small percentage of mammals that form monogamous relationships. These relationships, along with other strong social bonds, are essential to human well-being. In the absence of strong social relationships, humans experience a variety of detrimental conditions such as depression, anxiety, and cardiovascular disease. Traditional barriers to studying social bonds stem from the use of lab rodents such as mice and rats which do not form selective social attachments. However, the prairie vole (*Microtus ochrogaster*) does form these selective social attachments. The robust social bonds – pair bonds – formed between adult prairie voles may be observed and therefore studied. An important element of these social bonds is the neurotransmitter dopamine which is well known to be involved in reward processes. GRAB<sub>DA</sub> is a synthetic dopamine type 2 receptor with an attached GFP, which upon dopamine binding, adopts the optimal conformation for fluorescence. Changes in fluorescence due to dopamine binding are observable in behaving animals via fiber photometry. I observed reliable increases in GRAB<sub>DA</sub> fluorescence upon sucrose consumption and these increases were largely blocked by the application of the dopamine antagonist, eticlopride hydrochloride. Together, these results suggest that GRAB<sub>DA</sub> is a suitable tool for measuring dopamine levels, although additional validation is ongoing. GRAB<sub>DA</sub> will help to elucidate the quantity and temporal changes in dopamine release in the nucleus accumbens when a prairie vole interacts with its partner or a stranger before and after forming a pair bond. For the first time ever, dopamine temporal dynamics are observable in real time in a behaving prairie vole.
Introduction

3-5% of mammal species, including humans, form monogamous relationships. While monogamy does not persist in every human culture, all humans experience social attachments which are crucial for both mental and physical health. The complex social bonds and attachments between mother and child, partners, and friends displayed by humans are fundamental aspects of human well-being. Because of the importance these interactions in humans, they must be better understood. Numerous studies have examined pieces of the neurological basis of social bonds, but many aspects of this field are still largely unknown.

Social attachments are of great interest to the neuroscience community due to their importance in overall human well-being. In the absence of healthy social bonds in humans, there is the potential for many detrimental conditions including depression, anxiety and schizophrenia. Additionally, mortality rates tend to be higher in individuals that experience a lack of social bonding. Conversely, healthy social bonding is known to correlate with increased recovery rates from illnesses and decreased disease rates. To better alleviate the effects of insufficient social attachment we must better understand how these bonds are formed and maintained.

Human research is unrealistic in the context of neuronal manipulation and behavioral testing. While understanding social attachment is a necessary piece of understanding overall human health, it is not easy, nor ethical to study these manipulations in humans. Traditional lab animals such as rats and mice do not display monogamy – characterized by observable behaviors such as preference to interact with one mating partner, affiliative behavior towards the partner, mate guarding, selective aggression towards non-partner voles, and biparental care of offspring. However, prairie voles (*Microtus ochrogaster*), are a socially monogamous rodent species that display this behavior in social attachments known as pair bonds. Previous work has shown
that mating or prolonged cohabitation are sufficient for prairie voles to form a pair bond\textsuperscript{8}. These bonds can be long lasting and tested using a behavioral test known as the partner preference test. The similarity in behaviors between prairie voles and humans provides an ideal model for the study of the neurobiology of social bonds – a behavior highly relevant to human social behavior.

Key to the formation and maintenance of social attachments is dopamine, a neurotransmitter that is released throughout the brain. Dopamine is released in response to rewarding stimuli which corresponds directly to one of its most well-known roles: reward learning. Previous work has shown that dopamine release in the nucleus accumbens during mating is necessary for the formation of pair bonds in voles\textsuperscript{9,10}. Specifically, it has been shown that administration of a DA type 2 receptor antagonist in the nucleus accumbens prior to mating impairs the formation of a pair bond indicating that dopamine activation of receptors is necessary for the formation of these bonds\textsuperscript{11}. Additionally, pair bonds can be induced in the absence of mating by administration of a dopamine agonist followed by a 6 hour cohabitation period\textsuperscript{10}. Due to the importance of dopamine release during these behaviors, understanding dopamine dynamics, in terms of both time and quantity, is critical in the search for a deeper understanding of the neurobiological mechanisms underlying these bonds.

Approaches used in previous work such as pharmacology, microdialysis, and fast scan cyclic voltammetry are insufficient for determining temporal resolution of dopamine dynamics. Pharmacological manipulations do not provide good temporal resolution and drugs used to perform these experiments often have off target effects. Microdialysis is a commonly used technique in which a cannula inserted into the head of an animal allows for sampling of dopamine levels up to about 1 sample per minute\textsuperscript{12}. Microdialysis has been used in prairie voles to examine dopamine levels in bonded voles versus non-bonded voles\textsuperscript{13}. This gives information
about the amount of dopamine in the brain region at one time, but temporal resolution is poor at this rate. Fast scan cyclic voltammetry (FSCV) detects dopamine with excellent temporal and spatial resolution by inducing electrical currents in brain tissue to stimulate oxidation of dopamine, which is detected via a voltammogram. FSCV is used \textit{in vitro} in freshly collected brain tissue incubated in a bath. This technique has been used to compare dopamine signaling in slices taken from bonded and non-bonded prairie voles \textsuperscript{14}. Alternatively, FSCV is also used \textit{in vivo}, but this is technically challenging in small, quickly moving, rodents and thus, has never been done in prairie voles.

While the necessity of dopamine release in the nucleus accumbens for reward, mating, and pair bond formation is understood, the quantity and temporal dynamics of dopamine release involved in the formation and maintenance of these pair bonds remains unknown \textsuperscript{15,16}. To overcome limitations of previous dopamine studies in social bonding I have successfully piloted the molecular tool GRAB\textsubscript{DA} to obtain real time, \textit{in vivo} quantification of dopamine release in the nucleus accumbens during specific behaviors of interest. GRAB\textsubscript{DA} has the potential to elucidate the temporal and quantitative dynamics of dopamine release in live, behaving voles.

GRAB\textsubscript{DA}, invented by the Li Lab, was successfully piloted in mice, frogs, and drosophila. Due to the biological differences between prairie voles and the other animals used to pilot GRAB\textsubscript{DA}, I aimed to verify the viability of this tool for use in prairie voles. I performed optimization experiments to determine the viral concentration best for infecting neurons in the prairie vole brain. In collaboration with Dr. David Root at CU Boulder, we performed preliminary optimization of fiber photometry use for detection of GRAB\textsubscript{DA} fluorescence changes in behaving prairie voles. Following optimization, of GRAB\textsubscript{DA} with fiber photometry, GRAB\textsubscript{DA} will be used experimentally to look at behaviors of interest in the prairie vole model, providing a
powerful tool for understanding the dopamine dynamics underlying social attachments. GRAB\textsubscript{DA} is a powerful tool for understanding the dopamine dynamics underlying social attachments. Following optimization, this tool will be used to provide greater insight into how changes in dopaminergic signaling correlate to social bonding.

The GRAB\textsubscript{DA} virus was packaged into an adeno-associated virus with serotype 1 chosen for its known ability to infect neurons in the vole brain. The GRAB\textsubscript{DA} virus (AAV1-hSyn-DA4.3 M205T) encodes a synthetic, membrane bound, dopamine type 2 receptor with an attached, circularly permutated, green fluorescent protein (GFP). When dopamine is not bound to the receptor, the GFP attached is in a non-optimal conformation so fluorescence is low. Unbound receptors emit low levels of fluorescence, which is referred to, in this study, as baseline fluorescence. When dopamine binds to the receptor, this causes a change in the conformation of the green fluorescent protein, allowing it to brightly fluoresce. This increase in fluorescence lasts only as long as dopamine is bound. A schematic of the GRAB\textsubscript{DA} system is shown in Figure 1\textsuperscript{15}. Using GRAB\textsubscript{DA}, dopamine release is detected by observing changes in fluorescence levels via fiber photometry.

Fiber photometry is a technique that utilizes optic fibers implanted into the brain of an animal\textsuperscript{17}. An optic fiber is a flexible, thin fiber with a glass core. Light is sent through an optic fiber without losing intensity. The fiber is implanted at the correct length and location to ensure
termination of the fiber at the brain region of interest. In this experiment, the optic fiber is used to deliver light to the nucleus accumbens from an LED and record the resulting light output. Once excited, if bound by dopamine, the GFP on the GRAB\textsubscript{DA} synthetic dopamine receptor emits a fluorescence of 509 nm – a different wavelength than that of the 465 nm wavelength that excited it. A wavelength of 405 nm is used as an isosbestic measurement to correct for motion artifacts and photobleaching. When used with GRAB\textsubscript{DA}, the timing and quantity of light emitted and recorded by fiber photometry corresponds to the timing and quantity of dopamine release \cite{18}. In initial GRAB\textsubscript{DA} experiments done by the Li Lab, changes from baseline fluorescence in response to stimuli were well established to be a representation of dopamine release \cite{15}.

In the future GRAB\textsubscript{DA} will be used to examine dopamine dynamics in response to a variety of stimuli including sucrose reward, mating, and bond maintenance to enhance our understanding of the dopaminergic signaling occurring during different phases of monogamous social bonds. Dopamine temporal dynamics will be observed during mating and subsequent interactions with a partner and novel vole. Additionally, dopamine signaling during these rewarding behaviors will be compared to that of the rewarding task of sucrose consumption. The information gathered from these three distinct behaviors will provide greater understanding of the dopamine dynamics involved in both reward responses and social bonding. Optimization and implementation of GRAB\textsubscript{DA} in prairie voles will begin to broaden our understanding of the neurobiological basis for monogamy and social bonding. This opens the door to future experimentation and knowledge that may provide great progress for understanding underlying determinants of mental health, physical health, and overall human well-being.

Until now, the timing and quantity of dopamine release in behaving prairie voles has been difficult to examine. Limitations in temporal resolution have made previous dopaminergic
investigations incomplete. The use of GRAB\textsubscript{DA} in behaving prairie voles allows us, for the first time, to observe real time \textit{in vivo} dopamine signaling during socially relevant behaviors. I piloted the viral transduction of the AAV1 encoding GRAB\textsubscript{DA} in the prairie vole brain. Additionally, I piloted the use of fiber photometry to observe changes in fluorescence due to dopamine binding to GRAB\textsubscript{DA} in the prairie vole model. This tool will allow for broad investigation into the dopaminergic signaling underlying social bonding in the monogamous prairie vole.

\textbf{Methods}

\textit{Prairie Vole Subjects}

The prairie vole colony was bred in house from two colonies, one from the University of California Davis and one from Emory University. Voles from both established colonies were bred together to establish a genetically diverse population for experimentation. Upon weaning at post-natal day 21, voles were housed with up to four same sex siblings. Voles were kept in a humidity-controlled room with a 14:10 light/dark schedule (lights on at 7am and off at 9pm). Food, water, and enrichment were provided \textit{ad libidum}. All male and female prairie vole subjects were at least sixty days old (P60) before beginning any experimental procedures. All protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado Boulder (protocol \#2435) and followed standard ethical guidelines set by the NIH.

\textit{GRAB\textsubscript{DA} Virus Production and Packaging}

The GRAB\textsubscript{DA} virus used for this experiment was produced from a plasmid provided by the Li Lab\textsuperscript{15} and packaged by Vigene Biosciences into an AAV1 serotype specifically chosen
for use in prairie voles. The AAV1 serotype was chosen because of the high rate of transduction in voles as shown in other experiments in the Donaldson Lab. Virus concentration and efficacy were tested as described below to determine the best working concentration for further studies and to confirm the ability of the virus to express as necessary for use in behavioral trials.

**Experiment 1: Optimization of GRAB\textsubscript{DA} virus transduction in prairie voles**

Due to the novel nature of GRAB\textsubscript{DA} use in prairie voles, the optimal virus concentration for use in the prairie vole model had not yet been determined. The amount of injected virus directly affects transduction of the virus and thus the number of neurons expressing the GRAB\textsubscript{DA} receptor. Correct expression of synthetic dopamine receptors is necessary for accurate fluorescent representation of dopamine binding. In neurons with too many receptors, the expression of too many receptors may be neurotoxic to the cell. In neurons with too few receptors, fluorescent signal is of too low intensity to acquire robust signal. Due to the important nature of optimal receptor expression levels, it was important I began with optimization of GRAB\textsubscript{DA} virus injection concentration. Four viral concentrations were tested in intracranial injections: undiluted GRAB\textsubscript{DA}, 1:5 GRAB\textsubscript{DA}: cortex buffer (an artificial cerebrospinal fluid), 1:10, and 1:20.

*Intracranial Surgeries for GRAB\textsubscript{DA} virus injection*

Test subjects received unilateral injections of the GRAB\textsubscript{DA} virus. Concentrations differed between subjects, but all injections were of the same volume (300 nl). Animals were initially anesthetized by 4% isoflurane with oxygen and placed into the stereotaxic instrument (Kopf) and on a heating pad. Isoflurane was then lowered to 1-2% and monitored throughout surgery. Voles
were given 300 nl unilateral injections of GRAB<sub>DA</sub> virus in the appropriate concentration using a Nanoject II (Drummond Scientific) and glass needle. Injections were targeted at the NAc using the stereotaxic coordinates given by Franklin and Paxinos Mouse Brain Atlas (AP: +1.7mm, ML: ±1.0mm, DV: -4.7mm, -4.6mm, and -4.5mm). Each of the 3 injection depths received 100nL of the total 300nL GRAB<sub>DA</sub> so that the volume of the virus was spread out over 0.3mm of brain tissue. Each test subject received a single concentration of virus chosen from the 4 concentrations tested: undiluted GRAB<sub>DA</sub>, GRAB<sub>DA</sub> diluted 1:5 in cortex buffer, GRAB<sub>DA</sub> diluted 1:10 in cortex buffer, or GRAB<sub>DA</sub> diluted 1:20 in cortex buffer. Following the intracranial virus injections, the glass needle remained in place at the -4.5mm D/V position for ten minutes as to avoid dorsal diffusion of the virus. Upon needle removal, animals received 2-3 stitches in the skin atop the injection point on their skull to close the surgical site. Stitches were covered with triple antibiotic ointment (Globe) and 4% lidocaine gel (Aspercreme). Animals were injected subcutaneously with 0.01 ml/g of the analgesic Meloxicam SR (Zoopharm, 4.0mg/kg) for pain management and 1ml sterile saline (Nurse Assist) for hydration following surgery and allowed to recover.

Post-operative care

Post-operative observations began immediately following surgery and occurred every five minutes for the first thirty minutes. Breathing, locomotion, and visual appearance were monitored. Following the 30-minute post-operative timepoint, observations continued once daily through 72 hours post op. A period of three weeks was allowed before perfusions and brain collections to allow for sufficient expression of the GRAB<sub>DA</sub> receptor.
Cardiac Perusions

Cardiac perfusions were performed to fix brain tissue. Animals were given a lethal injection of 0.15mL 1:2 ketamine/xylazine and perfusions were started upon an absence of response to reflex triggers. Perfusions were performed with 1X phosphate buffer saline (PBS) followed by 4% Paraformaldehyde in 1X PBS (PFA). Brains were extracted, collected, and submerged to sit in 4% PFA for 24 hours. Following a 24-hour soak in 4% PFA, brains were transferred to 30% sucrose and allowed to sit until saturated in sucrose (approximately three days).

Sample Preparations and Imaging

Upon sucrose saturation of the brain, brains were frozen and mounted on a microtome (Leica JungSM2000R) using Optimal cutting temperature embedding medium for frozen tissue specimens, known as O.C.T. Compound, (Fisher Healthcare) and frozen using dry ice. Coronal sections were taken at a thickness of 50 µm. Slices were put into 1% PBS and 0.05% azide (Thermo Fisher) in 24 well plates. Sectioned tissue was stained for GFP using immunohistochemistry. Sections were incubated on shakers at 4°C for 24 hours in the primary antibody (anti-GFP-Chicken (1:500) in 0.3% PBS-Tween/3% NDS) and at room temperature for 2 hours in the secondary antibody: (Cy2 conjugated Donkey anti-chicken (1:500) in 0.25% 1X PBS-Tween). Brain cross sectional slices were mounted on Superfrost Plus glass slides (Thermo Fisher) and cover slipped (Globe Scientific) using ProLong Diamond Antifade Mountant (Invitrogen by Thermo Fisher Scientific). After being allowed to dry for one day, slide edges were sealed with clear nail polish (Electron Microscopy Sciences) and stored at 20°C until imaging. Preliminary imaging was done on an Olymrs IX81 Inverted Widefield Microscope and
images were recorded from a Yokagawa Cell Voyager 1000 Confocal Scanner System (CU Boulder Light Microscopy Core Facility). Images were stitched using ImageJ and used for analysis.

*Image analysis and concentration selection*

For images taken in this experiment, the cell filling nature of GRAB\textsubscript{DA} receptors were observed. Concentrations with low levels of GFP on the neuronal membranes were deemed to be too low for optimal fluorescence recording during behavior. In cells with a limited number of receptors, the fluorescence signal will not be strong enough to be significantly detectible. Concentrations in which GRAB\textsubscript{DA} receptors completely filled the cells were deemed to be too high due to potential neurotoxicity of filling the neuron with receptors. Therefore, optimal concentration was determined to be the concentration in which neuronal membranes were highly populated with GFP, but no GFP was visible within the cell body of the neuron. Optimal concentration, based on the above stated parameters, along with verification from the Li Lab\textsuperscript{15}, was determined to be 1:5 GRAB\textsubscript{DA}: Cortex buffer. Experiments proceeded with this concentration for all viral injections of GRAB\textsubscript{DA}.

**Experiment 2: GRAB\textsubscript{DA} fiber photometry-based detection in behaving animals**

After determining the appropriate concentration, in collaboration with Dr. David Root at CU Boulder, fiber photometry detection of fluorescent changes in response to dopamine release was optimized for use in voles infected with GRAB\textsubscript{DA}.

*Intracranial Surgeries and Fiber Implants*
Test subjects received unilateral 300 nl GRAB\textsubscript{DA} virus diluted 1:5 in cortex buffer injections in the nucleus accumbens using the stereotaxic coordinates in reference to bregma (AP: +1.7mm, ML: ±1.0mm, DV: -4.7mm, -4.6mm, and -4.5mm) as described in experiment 1, in addition to fiber implants. Prior to injections, small screws were placed in three points on the skull to anchor the carbon powder and epoxy cap to the fiber optic implant and skull. After virus injections, fibers were implanted in the injection site at a depth of -4.6mm D/V. The depth, -4.6mm D/V was in the center of the injection site and was therefore chosen as the optimal location for capturing the greatest fluorescent changes from the greatest quantity of neurons infected with GRAB\textsubscript{DA}. Fibers were slowly inserted into the cortex as to avoid excessive pressure changes within the brain and neuronal damage. After slow insertion, fibers and screws were covered with a carbon powder and epoxy cap to cement and stabilize the implant. Carbon and epoxy caps were covered with triple antibiotic ointment (Globe) and 4% lidocaine (Aspercreme). Animals were injected subcutaneously with 0.01 ml/g of the analgesic Meloxicam SR (Zoopharm, 4.0mg/kg) for pain relief, 0.01 mg/g of the antibiotic enrofloxacin (0.5 mg/kg) to avoid infection, and 1 ml of sterile saline (Nurse Assist) for hydration and allowed to recover.

\textit{Post-operative care}

Post-operative observations began immediately following surgery and occurred every five minutes for the first thirty minutes. Breathing, motion, visual appearance, and weight loss were monitored. After the initial 30 minutes, observations continued once a day through 72 hours post op. Voles were weighed daily, given saline and enrofloxacin, and monitored for signs of infection or weight loss. A period of three weeks was allowed before behavioral testing began to ensure proper recovery and sufficient expression of the GRAB\textsubscript{DA} virus.
Post-Operative Water Restriction

Three weeks following surgery, voles were slowly adjusted to a water restricted diet. Water restriction increased motivation to consume sucrose water during behavioral trials, and in result, voles demonstrated more bouts of drinking. This water restriction protocol has been modified from a standard water restriction protocol for mice. 30 minutes a day is sufficient for mice to maintain above 85% body weight \(^{19}\), however voles require an hour of water a day to maintain 85% body weight. Animals were weighed daily and checked for evidence of normal behavior such as grooming, feeding, and a dislike for being handled. Any animals that lost more that 15% of their body weight or did not display normal behavior were removed from their home cage, moved to a new cage, and removed from the study. Water restriction began with 16 hours of water for the first 24 hours. During the next 24 hours, vole subjects were allowed 8 hours of access to water. Finally, they were allowed 1 hour of water over each 24-hour period. The 1-hour water restriction protocol persisted through the time required to perform behavioral testing with the voles.

Fiber Photometry with Reward Stimulus

Fiber photometry was used to observe changes in fluorescence from GRAB\textsubscript{DA} synthetic dopamine receptors upon binding of dopamine. Previous studies have shown via microdialysis that sucrose licking increases levels of dopamine in the nucleus accumbens \(^{20}\). We hypothesized a significant increase in dopamine release during the onset of sucrose licking, as sucrose elicits a reward response. Thus, we hypothesized a robust increase in fluorescence during the onset of licking.
Subjects began behavioral training after day 3 of water restriction. On day 1 and day 2 of training, subjects were placed in the test chamber and allowed to access to 8% sucrose water and habituate to the chamber for 1 hour each day. The purpose of training was to allow the vole to acclimate to the environment and to learn to lick the sucrose bottle. On day 3, voles were attached to Dr. Root’s fiber photometry system. A patch cord containing a glass fiber was connected to the LED and to the vole’s fiber implant. Voles were then placed again in the behavioral chamber. Upon the start of behavioral trials, a single sip bottle filled with sucrose was attached to the side of the behavioral test chamber and voles were allowed access *ad libidum* for one hour. The time and number of licks on the sucrose bottle were timestamped onto the fluorescence recording using a lickometer. GFP on the GRAB\textsubscript{DA} synthetic dopamine receptor was excited by light of a wavelength of 465 nm sent from an LED in the fiber photometry system, corresponding to blue light, and emitted a wavelength of 509nm, corresponding to green light. In addition to the blue LED, an isosbestic light output was utilized. Isosbestic light was emitted at a wavelength of 405 nm and was used to correct for motional artifacts, photobleaching, and fiber bending artifacts. The isosbestic light cannot excite GFP and therefore is a good measure of non GFP artifacts. Final fluorescence measurements were the result of subtracting the isosbestic fluorescence levels from the GFP fluorescence levels to eliminate fluorescence due to motion of the behaving animals. Following behavioral trials, voles were returned to their home cage and the water restriction protocol was continued.

48 hours following the initial trials, voles performed the same behavioral task of sucrose licking with an intraperitoneal injection of eticlopride hydrochloride (2mg/kg in saline). Eticlopride hydrochloride is a dopamine antagonist and was chosen due to its selectivity for D2 and D3 receptors. Because GRAB\textsubscript{DA} is a synthetic D2 receptor, we hypothesized that it should
block dopamine from binding to the GRAB\textsubscript{DA} receptor, therefore preventing fluorescent spikes as seen in previous tests two days prior. Test subjects were given eticlopride hydrochloride injections 5 minutes before being attached to the fiber photometry patch cable and placed into the same behavioral chamber equipped with a sucrose drinking bottle. Eticlopride hydrochloride was used to determine whether fluorescence caused by dopamine release could be inhibited by antagonists, serving as proof of concept that it is truly dopamine binding that causes GRAB\textsubscript{DA} to fluoresce.

\textit{Cardiac Perfusions}

Cardiac perfusions will be performed as in experiment 1 to fix brain tissue. Unlike in experiment 1, I will harvest full decapitated heads at the end of the perfusion and allow them to sit in 4\% PFA for 24 hours. The full head will be submerged to maintain an obvious optic fiber track for visualization upon imaging. Following a 24-hour soak in 4\% PFA, the optic fiber will be removed, brains will be extracted, transferred to 30\% sucrose, and allowed to sit until saturated in sucrose (approximately three days).

\textit{Sample Preparations and Imaging}

Sample preparations and imaging will be performed as described in experiment 1.

\textit{Injection target verification and image analysis}

Images acquired from experiment 2 will be used for verification of fluorescence results obtained through fiber photometry during behavioral trials. The location of injections in each subject will be compared to the location targeted for injections to ensure that injections were in
the correct place for proper quantification of results. Any disparities between location of injection and location of target injections including sub regions of the nucleus accumbens will be treated as such and separated for analysis purposes.

Results

I, alongside collaborators, have performed optimization work to establish GRABDA as a viable tool for use in the prairie vole model. Following optimization of viral transduction in prairie voles, sucrose reward was utilized as a simple, rewarding task to optimize fiber photometry for use in prairie voles with GRABDA. Both experiments were essential in piloting GRABDA for use in behaving prairie voles.

Experiment 1 Results: 1:5 concentration of GRABDA is optimal

Preliminary optimization suggested that a 1:5 concentration of GRABDA virus diluted in cortex buffer is the most appropriate for expression in the nucleus accumbens of the prairie vole brain shown in Figure 2. This concentration was

![Figure 2](image-url). 10X images taken from test subjects perfused 3 weeks post operation and fixed with PFA. A) Image of undiluted GRABDA virus. B) Image of 1:5 concentration of virus diluted in cortex buffer. C) Image of 1:10 virus dilution in cortex buffer. D) Image of 1:20 virus dilution in cortex buffer. Cell filling nature of GRABDA virus demonstrated by the perimeter of cells fluorescing without fluorescence in the center of the cells is witnessed in the 1:5 dilution. This criteria was used to select for the 1:5 dilution moving forward.
deemed more appropriate than other concentrations (undiluted, 1:10, and 1:20) in collaboration with the Li Lab. Optimal concentration was chosen by observing the cell membrane filling nature of the virus. To ensure accurate recording of fluorescence levels, GRAB<sub>DA</sub> receptors must be visibly fluorescent on neuronal membranes in the region of interest but not so concentrated they fill the neuron. In cells completely filled with fluorescence, overexpression can become neurotoxic to the cell. In cells without robust membrane expression of GFP, there might not be enough signal to accurately represent dopamine dynamics. The undiluted concentration produced cell filling quantities of GRAB<sub>DA</sub> receptors, therefore, this concentration was too high. The 1:10 and 1:20 dilutions did not produce a great enough number of GRAB<sub>DA</sub> receptors to produce robust fluorescence on the membrane indicating these concentrations were too low. Optimal concentration of virus was determined to be the concentration where GFP was visible on membranes but not within the cytoplasm and nucleus of neurons. Based on these criteria, 1:5 concentration diluted in cortex buffer was selected for use in all future experiments.

Experiment 2 Results: GRAB<sub>DA</sub> shows dopamine release in simple reward task

To determine whether changes in fluorescence resulted from dopamine release, we tested GRAB<sub>DA</sub> during a simple reward task known to elicit dopamine release in a behaving animal. Voles were allowed to lick 8% sucrose solution from a water bottle in the behavioral chamber while attached to the fiber photometry patch cable. The onset of each licking bout was time stamped onto fiber photometry fluorescence recording. An

![Figure 3. Graph of fluorescence levels resulting from dopamine release in the NAc during behavior. Green represents behavioral trials after i.p. injections of vehicle (n=5). Red represents behavioral trials after i.p. injections of D2 receptor antagonist eticlopride hydrochloride (n=4). Y axis indicates Z-scores above the mean baseline value taken from time -3 to -1. Time 0 represents licking sucrose solution. Dopamine signaling increased in response to licking in vehicle trials but not antagonist trials.](image)
average fluorescence collected from time -3 to -1 where the onset of a bout of licking was time 0 and used as the baseline level of fluorescence. Changes in fluorescence during licking bouts were compared to baseline level acquired before licking began. The emission from the isosbestic excitation was subtracted from the emission from the GFP excitation to normalize the changes in fluorescence observed due to motion artifacts instead of dopamine release. Based on observed increases in fluorescence from baseline, it was determined that licking a water bottle filled with a sucrose solution directly corresponded to a significant increase dopamine release.

In order to determine whether dopamine binding was the cause of fluorescence, a D2 receptor antagonist, eticlopride hydrochloride, was used to block GRABDA receptors. The presence of an antagonist during behavioral trials yielded results indicating that eticlopride hydrochloride reduced the large increase of fluorescence observed following sucrose licking as shown in Figure 3. After intraperitoneal administration of the antagonist, the change in fluorescence at the onset of licking was not significantly different from baseline. In addition, animals given eticlopride showed decreased number of licks, which may indicate a decreased reward or motivation for licking (data not shown). Due to dopamine’s known role in locomotion, it is possible eticlopride could have had effects on locomotion that were not measured, the limitations of which are discussed below. Regardless of the locomotive effects of eticlopride hydrochloride, it appears that the presence of an antagonist lessened the increase in fluorescence due to sucrose licking and therefore was successful in preventing dopamine from binding to the GRABDA synthetic DA type 2 receptors present in the NAc.
Discussion

In this study, I demonstrate that the synthetic D2 receptor, GRAB\textsubscript{DA}, is viable for use in the prairie vole model. With the help and guidance of my mentors and collaborators, Dr. Zoe Donaldson, Anne Pierce, and Dr. David Root, I show that dopamine dynamics are, for the first time, visualized in real time \textit{in vivo}, with excellent temporal resolution in behaving prairie voles.

Based on the results from experiment 1, the 1:5 concentration of GRAB\textsubscript{DA} diluted in cortex buffer was deemed appropriate because this dilution resulted in robust expression of GRAB\textsubscript{DA} receptors visualized by GFP only on the neuronal membrane and not within the cytoplasm and nucleus of neurons. This concentration was used for experiment 2 with the hopes of ensuring that GRAB\textsubscript{DA} expression was moderately consistent from one test subject to another. Concentration selection was verified by the Li Lab based on the same criteria \textsuperscript{15}. Experiment 2 demonstrated the viability GRAB\textsubscript{DA} for visualizing dopamine release in a behaving animal. Increases in fluorescent signal were successfully blocked in the presence of an antagonist suggesting that D2 type receptor antagonists successfully block dopamine from binding the GRAB\textsubscript{DA} receptors. These results imply that it is, in fact, dopamine binding to GRAB\textsubscript{DA} that induces the conformational shift responsible for increased fluorescence detected by fiber photometry. This validates the use of GRAB\textsubscript{DA} in the prairie vole model to examine dopamine signaling in any region during behaviors of interest.

One limitation of the sucrose licking optimization approach was that the eticlopride hydrochloride produced qualitative effects on licking patterns, reducing the rate of licking noticeably. This indicates that some of the results may be due observed to the differences in the number of licks that the animal displayed rather than simply having no or less dopamine bound. These effects are hypothesized to be the result of the antagonist impact on locomotion because
dopamine is known to be critical for both locomotion and reward behaviors. This effect, although not unexpected, makes for more difficult comparison between the two treatment groups. Optimization trials will move forward in future experiments without the behavioral confounds observed in experiment 2. The only way to do this is to remove behavior all together. Consequently, it has been decided that animals will be anesthetized for the next validation experiment (see future directions).

Thus far, GRAB\textsubscript{DA} stands out as a promising tool for observing dopamine release in the monogamous prairie vole. Further investigation is necessary to finalize optimization and observe behavioral dopamine release in the prairie vole. Results from future experiments will help us better understand dopamine signaling in the nucleus accumbens during social behaviors. This tool is now optimized for use in prairie voles and will be able to be used in experiments investigating dopamine dynamics in any region and during any behavior of interest in the prairie vole.

**Future Directions**

The two future directions experiments aim to further understanding of dopamine dynamics underlying social attachments with the novel molecular tool GRAB\textsubscript{DA}. The use of this tool will first be further optimized in future directions experiment 1 to remove any potential behavioral confounds from the optimization experiments previously performed. Following final optimizations, GRAB\textsubscript{DA} will be used in behavioral trials to observe dopamine release during mating and subsequent partner interaction.

Future directions experiment 1 aims to perform a final optimization of GRAB\textsubscript{DA} before it is used in behavioral studies. This experiment will ensure proper optimization by removing
behavioral confounds. This will be done by eliciting dopamine release in the NAc of anesthetized subjects via channelrhodopsins in the VTA. Channelrhodopsins are membrane bound channel proteins that when exposed to light elicit an action potential in a neuron. In this experiment, specifically, channelrhodopsins will infect neurons in the VTA. Because there are many dopaminergic neurons projecting from the VTA to the NAc, dopamine release in the NAc is elicited by light.

Animals have been intracranially injected in the nucleus accumbens with GRABDA virus and fiber optic implant. In the same surgery, animals were injected with a channelrhodopsin virus and fiber optic implant in the VTA. Following recovery, and sufficient time to allow GRABDA and channelrhodopsin expression, voles will undergo anesthetized optimization trials. This experiment will utilize the Neurophotometrics fiber photometry system in the Donaldson Lab. Saline will be given as the vehicle for trials in which eticlopride is not given. Upon anesthetization and intraperitoneal injection of saline, a fiber photometry patch cable will be attached to the fiber optic implant in the nucleus accumbens of the subject. This patch cable will be connected to the Neurophotometrics fiber photometry system. Additionally, an optic fiber patch cable will be attached to the optic fiber implanted into the VTA. This fiber will be connected to a simple laser LED because only light is required to activate channelrhodopsins and nothing will be recorded via fiber photometry. Upon the start of the behavioral trial, blue light (473nm wavelength) will be used to optogenetically activate cell neurons with the channelrhodopsin in the VTA. This in turn should cause dopamine release in the NAc from the dopaminergic cell bodies in the VTA that project to the NAc. Quantity and timing of dopamine release following stimulation of VTA channelrhodopsins will be recorded with GRABDA and the fiber optic implant in the nucleus accumbens. Different intensities of light will be applied to the
VTA and the resulting dopamine signaling in the NAc will be observed. I hypothesize that an increase in light intensity in the VTA will result in an increase in fluorescence in the NAc.

After initial trials to observe dopamine release from VTA stimulation, trials will be repeated with the D2 receptor antagonist eticlopride hydrochloride. Just before anesthetization with isoflurane, voles will be given a 2mg/kg intraperitoneal injection of eticlopride hydrochloride. Following the injection, trials will be run as described above in the trials without antagonist. Information from these trials will be used to validate the specificity and efficacy of the GRABDA synthetic dopamine receptor in the absence of behavior.

I predict future directions experiment 1 will show significant differences between antagonist and vehicle trials. In this experiment, I hypothesize that I will see a robust fluorescence spike in animals receiving VTA optogenetic stimulation, and that this will scale with increased intensity of light applied in the VTA. Stimulation in the VTA will activate dopaminergic neurons in the NAc and dopamine release should be easily visible via fiber photometry detection of changes in fluorescence. Dopamine release should correlate directly with fluorescence spiking to indicate that GRABDA receptors are bound in this region. Because the animal will be anesthetized, this influx of dopamine will be due to VTA stimulation and nothing else. During the antagonist trials, fluorescence changes should be largely blocked. In the presence of antagonist, dopamine should be unable to bind to the synthetic receptors and therefore fluorescent signals should not change drastically from baseline. This will demonstrate that GRABDA synthetic receptor GFP conformation changes can be blocked by D2 antagonists. The absence of behavior in this experiment will ensure that the observed results are due to dopamine binding and that the lack of fluorescence increase is due to lack of dopamine binding – not lack of licking, motivation, or dopamine release.
Future directions experiment 2 will be instrumental in observing the dopamine dynamics in behaving animals during pre-bonding, mating, and post bonding behaviors. Behaviors including ability to recognize a partner, preference to interact with a partner and biparental care are recognized to be elements of a pair bond and can be visualized by a robust preference to interact with one vole (the partner) compared to any other vole \(^1\text{,}^9\text{-}^1\text{,}^1\). The presence of a pair bond can be tested in a partner preference test – a behavioral test that allows the subject vole to choose to spend time with a stranger or their partner. In bonded pairs, the subject will spend a majority (greater than 66.7% of the time) with their partner. In this experiment, the partner preference test will be utilized to examine behaviors relevant to social attachment. Training and testing during sucrose licking will be observed before and after mating and partner preference experiments to compare dopamine release in response to another, natural reward before and after the formation of a bond. This information will help identify whether changes in dopamine before and after bonding are due to social reward or other, natural rewards. Thus far, in preparation for these experiments, animals have been intracranially injected in the nucleus accumbens with GRAB\textsubscript{DA} virus followed by a fiber optic implant.

A study from the Aragona Lab examined dopamine release following pair bonding behavior utilizing FSCV and demonstrated significantly higher levels of dopamine release in the NAc of previously pair bonded voles as compared to non-bonded voles \(^1\text{,}^4\). Based on this experiment, I hypothesize that mating will produce more robust dopamine release than other behavior such as stranger interaction and sucrose licking. Increased dopamine release during mating would reinforce the knowledge that pair bond formation is catalyzed by mating. In addition, I predict partner interaction to be more rewarding than stranger interaction. Should results of this experiment follow my predictions, this will demonstrate the critical reward of
dopamine and reward learning involved in partner interaction. This may prove useful in understanding why a vole stays with its mate – helping to explain one of the underlying neuronal mechanisms of monogamy. This knowledge is essential in growing our understanding of social bonds and reward associated with monogamy and familiarity.

Moving forward, this tool may be used in many regions of the prairie vole brain to gain a more comprehensive understanding of dopamine temporal dynamics in a behaving prairie vole. GRAB_{DA} will hopefully aid in a greater understanding of the brain-wide neural mechanisms behind the robust social bonds witnessed in prairie voles. This understanding will one day be applicable to human behavior and may better our understanding of the complex social bonds we display and experience every day. If we begin to understand how and why social bonds form, we may begin to help those with abnormal or insufficient social bonding. GRAB_{DA} has the potential to make great strides in the field of molecular neurobiology now that it has been piloted for use in the prairie vole.
References