THE ROLE OF NUCLEUS ACCUMBENS DOPAMINE IN INCUBATION OF COCAINE
CRAVING

By

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LIST OF ABBREVIATIONS

AAV   Adeno-associated virus

AMPAR   α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

CREB   cyclic AMP response element-binding protein

D1R   Dopamine type 1 receptor

D2R   Dopamine type 2 receptor

DA   Dopamine

GABA   γ-aminobutyric acid

GFP   Green fluorescent protein

GPCR   G protein-coupled receptor

MSNs   Medium spiny neurons

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ABSTRACT

The nucleus accumbens plays a key role in guiding motivated behavior by integrating glutamate inputs, the major driver of neuronal activation, and dopamine (DA) inputs, a critical source of neuronal modulation. Therefore, it is important to understand how glutamate and DA transmission interact, particularly in reward-seeking behavior. In this dissertation, I use fiber photometry to extend our understanding of this interaction during two reward-seeking tasks.

Substance use disorder (SUD) is a brain disease in which reward-seeking becomes dysregulated. One hallmark of SUD is relapse despite desire or attempts to stop drug use, and this represents the most challenging aspect of treating this disorder. To better understand the persistent craving and relapse vulnerability seen in humans, in Chapter 1 I used the incubation of drug craving model, in which cue-induced drug seeking in rats progressively increases (incubates) during forced abstinence and then remains high for long periods of time. This phenomenon has also been demonstrated in humans. Previously, our lab and others demonstrated that expression of incubated cocaine seeking requires strengthening of glutamatergic synaptic transmission in the nucleus accumbens core. However, little was known about DA transmission in this model. To address this gap, I recorded DA transients in the nucleus accumbens core of male and female rats during cue-induced cocaine seeking. The magnitude of DA transients time-locked to responding did not differ when measured during early abstinence (prior to incubation) versus late abstinence (after incubation has plateaued). To test the functional relevance of these transients, I injected DA receptor antagonists into the nucleus accumbens core just before the seeking test. Blockade of either D1 or D2 DA receptors reduced cue-induced cocaine seeking after but not before incubation. These results suggest that DA contributes to

incubated cocaine seeking but the emergence of this role reflects changes in postsynaptic responsiveness to DA rather than presynaptic alterations.

Dopamine and glutamate signaling in the nucleus accumbens core are also implicated in seeking natural rewards such as food. Work using the classical extinction-reinstatement model has demonstrated that suppression of neuronal activity as well as blockade of DA signaling in the nucleus accumbens (core versus shell not specified) reduces reinstatement to food seeking. In Chapter 2, I further explored a role for both neuronal activity and DA using sensor multiplexing, a fiber photometry technique enabling simultaneous recording of changes in DA and calcium (a proxy for neuronal activation). DA transients time-locked to responding for food significantly increased with food self-administration training, while neuronal calcium transients showed a decrease from basal activity that became more pronounced with training. In the first session of extinction, I report a blunted DA increase from baseline and a blunted decrease in calcium. By the last extinction session, there were no detectable deflections from baseline in either trace. Rats then underwent a reinstatement test, a test of reward seeking, and showed increases from baseline time-locked to the lever-press in both DA and calcium recordings. These experiments support glutamate and DA acting cooperatively in the nucleus accumbens core during food reinstatement and validate sensor multiplexing in a model of motivated behavior.

INTRODUCTION

<u>Substance Use Disorder</u>

Substance Use Disorder (SUD) remains a persistent clinical issue. The World Health Organization reports that it claims ~500,000 lives globally every year, and in the United States (US), the National Institute on Drug Abuse (NIDA) reports deaths due to drug overdose have been increasing in recent years (NIDA 2023). According to the DSM V, SUD is defined as "chronic, relapsing disorder characterized by compulsive drug seeking and use despite adverse consequences" (DSM V 2014). There are many health consequences of excessive use of addictive substances, including increased risk of heart disease, stroke, and cancer. In addition, there is a tremendous societal toll of SUD, as individuals with SUD show reduced productivity which impacts their financial security, premature morbidity, and crime (Rand Corporation 2009).

Psychostimulants are a class of illicit drugs that includes methamphetamine, nicotine, and cocaine, and are considered to have high addiction liability. While opioid misuse and opioid use disorder have certainly skyrocketed in the past two decades, with the Department of Health & Human Services (HHS) declaring the opioid crisis a public health emergency in 2017, stimulant misuse remains a significant problem. In the US, 5.3 million people (1.9%) aged 12 and older used cocaine in the past year, with 1.4 million being diagnosed with cocaine use disorder (SAMHSA 2023). In fact, in 2021, cocaine-related overdose deaths were the third highest across all drugs assessed (NIDA 2023). Furthermore, according to the Department of Justice, more than 75% of drug offenders in the federal prison system had cocaine or methamphetamine as the primary drug type (S. Taxy 2015). In addition to active use and its consequences, relapse is a major problem for

individuals with SUD. For example, over 80% of persons with cocaine use disorder relapse within the first year of abstinence; this rate has remained constant over decades (Sinha 2011, Hunt, Barnett et al. 1971).

There is presently no FDA approved drug treatment for cocaine use disorder. The standard of care is behavioral therapy (motivational interviews, contingency management, community reinforcement, and cognitive behavioral therapy) (SAMHSA 2023). There is strong evidence for the efficacy of behavioral therapy in treatment of cocaine use disorder (Ronsley, Nolan et al. 2020), but many people do not receive or remain in treatment. This is attributed to issues on both the client and provider sides, including cost, inadequate social support, limited provider accessibility, ambivalence about abstinence, perceived difficulty of implementation, or lack of alignment with personal values (e.g., Kirby, Benishek et al. 2006, Palmer, Murphy et al. 2009). In regard to pharmacological agents, a clinical review concluded that these drugs were ineffective or there was not enough data to determine efficacy (Ronsley, Nolan et al. 2020). Overall, there is a need to develop better treatments for SUD. To address this, first there must be increased understanding of SUD as a brain disease, with a particular focus on relapse prevention.

Animal models of SUD

To better understand neural mechanisms underlying SUD, several pre-clinical models have been developed. The main two categories are non-contingent, in which the animal is given drug by an experimenter, and contingent, when an animal performs an action to receive drug. Behavioral sensitization is a major non-contingent model in which repeated intermittent injections of a drug produce an increase in its motor stimulant effect that persists for a period of time after discontinuing drug treatment (Kalivas and Stewart 1991 Wolf 1998, Wolf and Ferrario 2010, Kuhn, Kalivas et al. 2019). For psychostimulants

(cocaine and amphetamine), sensitization of incentive motivational effects also occurs (Robinson and Berridge 1993, Vezina 2004, Berridge and Robinson 2016). Much of the early research into drug-elicited changes in the brain began using this model to understand how drugs alter the functioning of the dopamine (DA) system and how this altered DA in turn leads to sensitization. Conditioned place preference (CPP) and conditioned place aversion (CPA) are models used to study the rewarding or aversive properties of drugs. These models, based on Pavlovian conditioning, focus on learned associations between the context of drug taking and effects of the drug. They typically involve a typically multi-chambered apparatus consisting of an intermediate section located in between two chambers that have defining features, like differing scents or visual stimuli (Napier, Herrold et al. 2013, McKendrick and Graziane 2020). During initial training there is a divider in place preventing the animal, typically a rat or mouse, from moving between the chambers. The animal receives an injection of the drug and is placed into a particular chamber and remains there for several minutes, and thus begins to experience the effects of that drug, either rewarding or aversive, in the context of that particular chamber. The subsequent day they are given a vehicle injection in the context of the opposite chamber. After repeated pairings the animal begins to associate the feelings elicited by that drug with the one chamber and form a preference or an aversion for that chamber. This can then be tested by removing the divider, allowing the animal to freely wander between the chambers and recording the time spent in each side (Prus, James et al. 2009). This model has been very important in discovering what underlies the formation of drug associated memories, as studies with both opiates and psychostimulants have shown DA is necessary for the formation of a conditioned place preference (Prus, James et al. 2009). Lastly, there are many methods for non-contingent drug delivery - such as oral gavage, drug-releasing surgical implants, or vapor chambers - which can be used to

study biological and behavioral effects of chronic drug exposure (Kuhn, Kalivas et al. 2019).

While non-contingent paradigms have utility when studying SUD, contingent models which allow for measurement of effort, motivation, and decision-making are considered the gold standard. Furthermore, it is well known that contingent voluntary models of intake can produce very different effects than the previously discussed non-contingent models (Green and Bardo 2020). The most prominent contingent model is drug self-administration (SA). SA is a behavior in which an animal learns to operantly respond, via lever press or nose poke, for drug delivery; this can occur intravenously, orally, or via vapor (Weeks 1962, Wise 1987, Meisch 2001, Vendruscolo and Roberts 2014). Drug delivery is typically paired with an environmental cue, such as a light or a tone. In the SA paradigm there are several distinct modalities that focus on the modeling of particular aspects of SUD in animals. For instance, the experimenter can adjust the length of time that the animal has access to the operant manipulanda/drug, thereby experimentally changing their drug intake. The three most common variants are short access, long access, and intermittent access. In short access regimens, animals typically self-administer drug for 1-3 hours per day. This is sometimes considered a model of recreational use. In long access regimens, animals typically have 6 hours per day to freely self-administer drugs. Long access SA is thought to better model SUD, with animals showing escalation of drug intake, stronger craving, and other behavioral and physiological differences from short access models (Ahmed 2012). The third is a more recent model, intermittent access, which involves short 5-10 minute periods of access to the drug followed by long forced pauses of 20-30 minutes for 6 hours per day. Intermittent access is thought to most closely mirror human drug use patterns, and, like long access,

replicates many of the behavioral and biological consequences of long-term drug use while keeping drug access restricted (Kawa, Bentzley et al. 2016).

In addition to differences in daily access to drug, SA regimens can differ by the length of the SA phase. Most typical SA studies will be 10-14 days, as previous work has shown this to be sufficient to produce behavioral changes that model aspects of addiction (Ahmed 2012). But studies that focus on escalation of drug intake, a criterion for SUD, will sometimes extend the SA phase to 3-4 weeks to see a significant increase in the number of daily infusions (Edwards and Koob 2013). Another variable that can be altered in SA is the reinforcement schedule. The more typical fixed ratio (FR) schedule requires a fixed number of operant responses to deliver the reinforcer (i.e. drug). One alternative is a variable ratio schedule, in which the number of responses has a fixed mean requirement but is unpredictable around that mean. Finally in a progressive ratio test the number of responses required increases as the session continues until the animal is no longer willing to respond (break-point). Regimens may also employ delay discounting, where the timing of reinforcement progressively extends, or probability discounting, where the likelihood of reinforcement changes. Changes in the amount of effort expended, time waited, or risk taken can indicate that the reward has become more valuable to the animal. Tuning these variables in SA studies can help address questions of how the value of a drug reward changes over time and under different conditions (Panlilio and Goldberg 2007).

However, as previously discussed, SUD is a relapsing disease, and the treatment and understanding of what causes relapse is a top priority. Therefore, many experimental models focus on replicating relapse-like behavior in animals following SA training. The classical model of relapse-like behavior is the extinction-reinstatement model. This model is a three phased experiment consisting of SA, extinction, and reinstatement phases. The

first SA phase is as described above, but following SA the animal enters extinction training. Extinction occurs when the animal learns that the previously rewarded operant response now has no consequence, leading to progressive reduction in operant responding. This is followed by a reinstatement test, in which the previously learned operant responding is reinvigorated usually following re-exposure to the reinforcer, an associated cue, or stress (Shaham, Shalev et al. 2003, Bossert, Marchant et al. 2013, Kuhn, Kalivas et al. 2019). A great deal of work has investigated the mechanisms by which these stimuli reinvigorate drug seeking after extinction (Mantsch, Baker et al. 2016, Scofield, Heinsbroek et al. 2016, Venniro, Caprioli et al. 2016). Furthermore, understanding mechanisms of extinction has the potential to lead to therapeutic approaches to reduce drug intake (Torregrossa and Taylor 2016, Hemstedt, Lattal et al. 2017).

Another model for relapse is the 'incubation of craving' model. This model is the leading model for studying vulnerability to cue-induced relapse after protracted abstinence from SA. A common version of the model entails a training period during which rats learn to self-administer, as described above, followed by a period of forced abstinence in the home cage. After different periods of abstinence, rats are reintroduced to the drug environment for a cue-induced seeking test, during which operant responses now deliver the cue previously paired with reward but no drug. The number of times the rats respond under these conditions is a measure of motivation to seek drug. It was discovered that cue-induced cocaine seeking progressively increased ("incubated") over the first month or so of abstinence and then remained high for additional months before falling off, providing a model for persistent vulnerability to relapse during abstinence (Neisewander, Baker et al. 2000, Grimm, Hope et al. 2001, Lu, Grimm et al. 2004). Incubation has subsequently been demonstrated for other addictive drugs, such as heroin, methamphetamine, alcohol,

and nicotine (Pickens et al 2011), and in humans for cocaine (Parvaz, Moeller et al. 2016), methamphetamine (Wang, Shi et al. 2013), alcohol (Li, Wu et al. 2015) and nicotine (Bedi, Preston et al. 2011). There are many different forms of this incubation model with forced abstinence being the classical model, but newer versions employ a volitional abstinence period during which the animal voluntarily suppresses their own drug use in favor of an alternative reward. This was designed to mirror the more modern contingency management treatment in humans and was developed through a reverse translational approach (Venniro, Caprioli et al. 2016, Venniro, Banks et al. 2020). Both forms of this model allow us to study relapse-like behavior in rodents, which enables us to ask direct questions about the biology underlying these events.

<u>The nucleus accumbens</u>

Among the many brain regions assessed in incubation of cocaine craving studies, a majority of the mechanistic studies have focused on the nucleus accumbens (Wolf 2016, Wright and Dong 2020). The nucleus accumbens, also sometimes referred to as ventral striatum, integrates inputs from cortical and limbic brain regions and energizes goal-directed behavior (Mogenson, Jones et al. 1980, Floresco 2015). The nucleus accumbens receives major axonal projections from excitatory (glutamate releasing) neurons in the prefrontal cortex, hippocampus, thalamus, and basolateral subregion of the amygdala (Sesack and Grace 2010). These glutamatergic projections synapse onto γ-aminobutyric acid (GABA)-releasing medium spiny neurons (MSNs), as well as interneurons. These MSNs are the principal/output neurons of the nucleus accumbens. They represent 95% of all the neurons in the nucleus accumbens and can be subdivided into two relatively equal populations of MSNs that express either the DA type 1 receptor (D1R) or the DA type 2 receptor (D2R) (Le Moine and Bloch 1995). The D1R is a G protein-coupled receptor

(GPCR) that, upon DA binding, triggers a second messenger cascade inside the cell via the G proteins $G\alpha_s$ or $G\alpha_{olf}$, stimulating the enzyme adenylate cyclase. The D2R acts in opposition to the D1R at this signaling level, being coupled to $G\alpha_{i/o}$, which inhibits adenylate cyclase (Neve, Seamans et al. 2004). Another significant input into the nucleus accumbens comes from ventral tegmental area neurons that release DA; effects of DA in the nucleus accumbens are critical for reward learning, as further detailed in the Section titled <u>Dopamine and incubation of cocaine craving</u> (Keiflin and Janak 2015, Bamford, Wightman et al. 2018, Berke 2018). Many addictive drugs exert their hedonic effects through the DA system; for example, cocaine blocks DA reuptake via binding to the DA transporter (DAT) thereby increasing the extracellular concentration of dopamine and extending its actions (Ritz, Lamb et al. 1987, Galloway 1988, Wolf 2011). DA's role in reward learning is largely enacted in the nucleus accumbens at the level of the MSNs; in some instances, DA has been reported to work with glutamatergic inputs to excite these typically hyperpolarized cells (Nicola, Surmeier et al. 2000, Moyer, Wolf et al. 2007, Sun, Milovanovic et al. 2008, Yagishita, Hayashi-Takagi et al. 2014). These MSNs in turn send inhibitory (GABA releasing) axons to various motor-related regions (Sesack and Grace 2010). Traditionally, these outputs are split into the direct and indirect pathways based on classic studies in dorsal striatum (Gerfen 1992).

According to the classical view, the direct pathway originates from D1R MSNs whose axons project to the internal globus pallidus subregion of the basal ganglia and substantia nigra, while the indirect pathway is composed of D2R MSNs that project to the external globus pallidus (Kreitzer and Malenka 2008). These pathways are quite segregated in the more dorsal regions of the striatum, but in the nucleus accumbens this separation is less distinct. In fact, both D1R and D2R expressing cells of the nucleus accumbens project to the ventral pallidum (VP) output region of the basal ganglia,

although these cell types do project to distinct subregions within the VP (Smith, Lobo et al. 2013, Scofield, Heinsbroek et al. 2016). Given their differing outputs and the opposing actions of DA on these cells, D1R MSNs have long been thought to enhance reward seeking while D2R MSNs oppose it (Hikida, Kimura et al. 2010, Lobo, Covington et al. 2010, Hikida, Yawata et al. 2013, Danjo, Yoshimi et al. 2014). However, there is growing evidence that D1 and D2-MSNs collaborate to shape motivated behavior, not only through their outputs but also through collateralization (Soares-Cunha, Coimbra et al. 2016, Burke, Rotstein et al. 2017, Bariselli, Fobbs et al. 2019, Lafferty, Yang et al. 2020, Allichon, Ortiz et al. 2021).

In addition to the MSNs, there are interneurons that form local inhibitory connections within the nucleus accumbens (Silberberg and Bolam 2015). These include parvalbumin expressing interneurons, somatostatin expressing interneurons, and cholinergic interneurons, which release acetylcholine. These cholinergic interneurons are interesting because, while they only represent 1-2% of neurons in the nucleus accumbens, they have large axonal arbors, meaning they can exert effects on a large population of cells. There is also some evidence that they can locally modulate DA release from the neurons that originate in the ventral tegmental area (Threlfell, Lalic et al. 2012, Nosaka and Wickens 2022). The parvalbumin expressing interneurons receive excitatory projections from many of the same regions that project to MSNs, and control MSN activity through feed-forward inhibition (Schall, Wright et al. 2021).

Within the nucleus accumbens there are two main subregions, the core and the shell, with a third potential subregion of the rostral pole (Zahm and Heimer 1993). The core and shell subregions have similar cellular characteristics and are both important for drug-related behaviors, albeit sometimes for different aspects of these behaviors (e.g., Ito,

Robbins et al. 2004). In addition, there are key differences in terms of inputs and outputs to these subregions (Zahm and Brog 1992). For instance, the medial prefrontal cortex input to the nucleus accumbens can be divided into two separate pathways, with the prelimbic (PL) medial prefrontal cortex projecting primarily to the nucleus accumbens core and infralimbic (IL) medial prefrontal cortex projecting mainly to the nucleus accumbens shell (Gorelova and Yang 1997). For my studies I have focused on the core subregion due to its well documented role in goal-directed behavior driven particularly by reward-associated cues (Kelley 2004, Burton, Nakamura et al. 2015) and cocaine self-administration (Ito, Robbins et al. 2004).

Glutamate and incubation of cocaine craving

Previous work in the Wolf lab has demonstrated that strengthening of glutamate transmission in the nucleus accumbens core during the period of forced abstinence following cocaine SA is necessary for the expression of cocaine incubation (Wolf 2016). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), an ionotropic glutamate receptor, provides the main source of excitatory drive to nucleus accumbens MSNs (Pennartz, Boeijinga et al. 1990, Hu and White 1996). In early forced abstinence, or when an animal is given saline as a control during the SA phase, synaptic transmission in nucleus accumbens MSNs is mediated primarily by calcium impermeable AMPARs (CI-AMPARs) (Kourrich, Rothwell et al. 2007, Conrad, Tseng et al. 2008, Reimers, Milovanovic et al. 2011). These receptors are defined by the presence of a GluA2 protein subunit in their structure, which renders the channel impermeable to calcium (Cull-Candy et al 2006; Isaac et al 2007). Thus, CI-AMPARs allow for passage of positively charged sodium ions into the cell when glutamate binds. During forced abstinence from extended-access cocaine SA, an atypical type of AMPAR that lacks the GluA2 subunit

accumulates in MSN synapses; these are calcium-permeable or CP-AMPARs (Conrad, Tseng et al. 2008, McCutcheon, Loweth et al. 2011, Wolf and Tseng 2012). In addition to calcium-permeability, CP-AMPARs also differ from CI-AMPARs in other properties including having higher single channel conductance (Cull-Candy et al 2006; Isaac et al 2007). Thus, an increase in CP-AMPAR levels leads to increased MSN responsiveness to glutamatergic inputs (Purgianto, Scheyer et al. 2013). Most importantly, blockade of these receptors prior to a cue-induced cocaine seeking test led to a significant reduction of seeking behavior (Conrad, Tseng et al. 2008); for reviews see (Wolf 2016, Wright and Dong 2020). Further research has delved more deeply into the mechanism of CP-AMPAR upregulation and showed a corresponding downregulation of mGlu1, a metabotropic glutamate receptor that normally serves to suppress CP-AMPAR levels in synapses (Loweth, Scheyer et al. 2014). Paralleling these synaptic changes, *in vivo* electrophysiology data demonstrate that nucleus accumbens core neurons show increased activity in response to drug-paired cues after protracted abstinence when compared to the last day of cocaine self-administration (SA) (Hollander and Carelli 2005, Hollander and Carelli 2007) and that activation of nucleus accumbens core MSNs is specifically related to incubation of cocaine craving (Guillem, Ahmed et al. 2014).

Dopamine and incubation of cocaine craving

Very little is known about the role of DA in the incubation of craving model. This is a significant knowledge gap given that DA in the nucleus accumbens core plays a major role in reward learning in other tasks, motivation for cocaine SA, and reinstatement to drug seeking (Phillips, Stuber et al. 2003, Saunders, Yager et al. 2013, Keiflin and Janak 2015, Bamford, Wightman et al. 2018, Berke 2018). Furthermore, cocaine exposure leads to complex adaptations in the DA system (Anderson and Pierce 2005, Volkow, Fowler et al.

2009) and that DA release in the nucleus accumbens core is implicated in cocaine related behaviors. Early work used fast-scan cyclic voltammetry (FSCV) to measure phasic DA transients in cocaine trained rats and found an elevation of DA in the nucleus accumbens core in response to cocaine cues that was shown to increase the likelihood of cocaine seeking (Phillips, Stuber et al. 2003). Additionally, cocaine associated cues lead to elevated DA in the nucleus accumbens (Ito, Dalley et al. 2000, Aragona, Day et al. 2009). Furthermore, cocaine alters DA signaling after both SA and non-contingent exposure (Willuhn, Burgeno et al. 2012, Ostlund, LeBlanc et al. 2014, Willuhn, Burgeno et al. 2014, Saddoris, Wang et al. 2016). Finally, functional inhibition of ventral tegmental area DA neurons reduces cocaine reinstatement (Mahler, Brodnik et al. 2019) and cocaine seeking after brief abstinence (Solecki, Wilczkowski et al. 2020). Given DA's broad role in reward learning, motivation and cocaine action, it is logical to assume it also plays a role in incubation of cocaine craving but there have been only three studies published. Our lab found modest changes in D1, D2 and D3 receptor surface expression in the nucleus accumbens core during incubation (Conrad, Ford et al. 2010). Further, intra-nucleus accumbens infusion of a D3R-preferring antagonist decreased cocaine seeking during abstinence (Xi, Li et al. 2013), but this decrease occurred independent of abstinence duration and the study measured context-induced seeking, not cue-induced seeking (as in our studies); these may involve distinct but partially overlapping pathways (Fuchs, Lasseter et al. 2008). Finally, a recent preprint used fast scan cyclic voltammetry to assess DA release in response to non-contingent presentation of cues previously paired with a cocaine regimen eliciting incubation (Burgeno, Farero et al. 2023), as discussed further in Chapter 1.

<u>Dopamine and glutamate interactions in motivated behavior</u>

DA alters how MSNs respond to glutamate signaling, although this relationship is complex and not fully understood (Nicola, Surmeier et al. 2000, Moyer, Wolf et al. 2007, Sun, Milovanovic et al. 2008, Yagishita, Hayashi-Takagi et al. 2014). One possibility is that DA may be acting through D1R to help facilitate MSN firing via transition to an 'up state' (Tseng, Snyder-Keller et al. 2007, Flores-Barrera, Vizcarra-Chacón et al. 2011), as MSNs *in vivo* have bi-stable resting potentials, existing in a hyperpolarized 'down state' and a depolarized 'up state' from which they can fire action potentials (O'Donnell 2003). In addition, presynaptic DA receptors modulate the ability of MSN to respond to glutamate inputs in the up state, potentially regulating signal-to-noise (O'Donnell 2003, Brady and O'Donnell 2004, Tseng and O'Donnell 2005). These mechanisms may be altered after repeated psychomotor stimulant exposure (Brady, Glick et al. 2005). Furthermore, the basolateral amygdala to nucleus accumbens glutamatergic input is modulated by DA when DA antagonism is paired with projection specific optogenetic stimulation in *ex vivo* slices (Stuber, Sparta et al. 2011). Therefore, it is likely that these two signaling molecules work in concert to lead to downstream changes in MSN firing.

<u>Biosensors to study incubation of cocaine craving</u>

The recent advent of genetically encoded biological sensors represents a massive step forward in the field of neuroscience. Biosensors are typically biological constructs made from a preexisting receptor or binding molecule that is linked to a fluorescent protein, such that when the molecule of interest binds it results in a conformational change in protein structure that leads to increased fluorescent emission (Patriarchi, Cho et al. 2019). This means that relative abundance of a compound of interest, say calcium, can be measured optically via changes in fluorescence (Nakai, Ohkura et al. 2001, Barnett,

Hughes et al. 2017). These sensors can be packaged into a viral vector, typically an adeno-associated virus (AAV), and expressed in neuronal populations of interest (Nassi, Cepko et al. 2015). Once the biosensor is expressed in the brain, changes in fluorescence can be measured using an optic fiber cannula implanted into the brain. Furthermore, comparing changes in fluorescence to fluctuations in the isosbestic wavelength (where emission is independent of sensor binding) allows for subtraction of any changes in light emission due to non-binding events (i.e. motion artifact due to cable twisting) (Zhang, Chao et al. 2022). This optical approach substantially increases temporal resolution and affords greater selectivity compared to traditional methods.

The first biosensors to gain prominence in neuroscience were genetically encoded calcium indicators. These sensors used the calcium binding protein calmodulin as the sensing domain bound to a green fluorescent protein (GFP) and have been termed GCaMPs (G-green fluorescent protein, CaM-calmodulin, P-peptide chain sequence from myosin light chain kinase) due to their protein composition. There have been many generations of these sensors, with each one improving the kinetics, signal to noise, and ability to read out neuronal activity more faithfully (Chen, Wardill et al. 2013, Zhang, Rózsa et al. 2023). However, it is still important to keep in mind that these sensors measure calcium, which is a proxy for neuronal activity, rather than directly measuring action potentials. All of this applies to studies of MSNs, where the use of such sensors has greatly enhanced our understanding of the role of MSNs in motivated behavior (Calipari, Bagot et al. 2016). However, of note, L-type Ca^{2+} channels are implicated in transitioning to the up state in nucleus accumbens and prefrontal cortex neurons (Vergara, Rick et al. 2003, Tseng and O'Donnell 2005, Tseng, Snyder-Keller et al. 2007), so an increase in MSN Ca^{2+} levels may be correlated with increased likelihood to respond to excitatory input. In fact,

there is good evidence these sensors, when expressed without cell compartment restriction, report more accurately dendritic calcium levels as opposed to somatic calcium levels in striatal neurons (Legaria, Matikainen-Ankney et al. 2022). Therefore, Ca^{2+} transients measured with GCaMPs provide a useful proxy for nucleus accumbens MSN activation state.

More recently, research on the functional significance of DA signaling has been rapidly evolving largely due to the introduction of new DA biosensors which allow for highly specific real-time measurement of changes in DA during behavioral tasks. Traditionally, DA has been measured using microdialysis and FSCV, methods with technological limitations of time scale and specificity, respectively (Bucher and Wightman 2015, Jaquins-Gerstl and Michael 2015). DA biosensors are genetically engineered DA receptors linked to a fluorophore, similar to the GCaMPs previously discussed (Patriarchi, Cho et al. 2018, Sun, Zeng et al. 2018). At present, there are two main DA biosensors available, GRAB_DA (Sun, Zeng et al. 2018, Sun, Zhou et al. 2020, Zhuo, Luo et al. 2023) and dLight (Patriarchi, Cho et al. 2018, Patriarchi, Mohebi et al. 2020). GRAB_DA's second generation, GRAB_DA2m, is built from a modified D2R and linked to circularly permutated (cp)-Enhanced Green Florescent Protein (EGFP) (Sun, Zeng et al. 2018, Sun, Zhou et al. 2020), while dLight is made from a modified D1R linked to cp-GFP (Patriarchi, Cho et al. 2018, Patriarchi, Cho et al. 2019, Patriarchi, Mohebi et al. 2020). Both biosensors offer several variants which differ in binding affinities for DA, maximal florescent response, and kinetics. Importantly, however, virus controls available for each sensor vary. For dLight, AAV-GFP is typically used. For GRAB_DA, however, a mutant variant is available that does not bind DA (Sun, Zeng et al. 2018). This is an excellent control as it achieves all the GFP control does (i.e., controls for effects of viral infection on cell health or possible

buffering of DA by the sensor), but also traffics the same as the sensor, thus controlling for any difference in surface expression of native receptors due to GRAB_DA expression.

The switch to optically based detection allows for sensor multiplexing, or the measurement of multiple molecules of interest. For example, one can simultaneously record both DA and Ca^{2+} transients within the same region as long as biosensors with distinct excitation and emission wavelengths are utilized. Typical wavelengths used are green (GFP) and red (cpm-Apple). Both genetically encoded calcium indicators and GRAB_DA sensors offer a red variant, namely jRGECO1a and GRAB_rDA, respectively (Dana, Mohar et al. 2016, Patriarchi, Mohebi et al. 2020, Sun, Zhou et al. 2020, Zhuo, Luo et al. 2023). However, the red variants present some technical challenges in that many red fluorophores show emission with blue light excitation; jRGECO1a is particularly notorious for this (Molina, Qian et al. 2019). Furthermore, even when sensors are engineered to not show blue light-based emission, such as the red GRAB_DA 1st generation (GRAB_rDA1) (Sun, Zhou et al. 2020), they are often very dim and difficult to work with. The second generation of red GRAB_DA has greatly improved its fluorescence, but at the cost of some blue light emission (Zhuo, Luo et al. 2023). This means that an experimenter must carefully select the type and generation of sensor when planning multiplexing experiments.

<u>Overarching hypothesis and specific aims</u>

The overarching goal was to test the hypothesis that DA is required for the expression of incubation of cocaine craving (Chapter 1) and develop an approach to integrate fiber photometry studies of DA with previously established glutamatergic mechanisms (Chapter 2).

The experiments in Chapter 1 used the DA biosensor GRAB_DA2m to measure DA transients in the nucleus accumbens core in association with cue-induced cocaine seeking in the incubation model. To date, only one study has used DA biosensors to study cocaine seeking after abstinence from cocaine SA (Pribiag, Shin et al. 2021). However, they used a short-access SA regimen and a relatively short withdrawal time (14 days), and tested context- but not cue-induced cocaine seeking. Context-induced seeking relies on associations between the drug SA chamber and drug delivery to elicit seeking, whereas cue-induced seeking entails the presentation of a discrete stimulus that was previously paired with drug. These types of seeking are distinct and show some mechanistic differences (Fuchs, Lasseter et al. 2008). Another study used voltammetry in an incubation paradigm and has showed increased DA release when measuring during non-contingent cue probe sessions with 30 days of forced abstinence between the recording days (Burgeno, Farero et al. 2023). In contrast, the experiments described here specifically investigate DA release during active cue-induced cocaine seeking after protracted abstinence. This is an advantage as we are looking at DA responding to the cue as it drives behavior. We found that there is an elevation of DA compared to baseline time-locked to the cocaine seeking action, and that the magnitude of this DA transient is not altered across incubation. Then, in order to assess the functional significance of this DA transient we performed pharmacological experiments. These experiments entailed the intracranial infusion of a D1R or D2R antagonist into the nucleus accumbens core. We found that either D1R or D2R antagonists, given prior to late withdrawal (incubated) cocaine seeking tests, lead to a significant reduction in cocaine seeking. This reduction was not seen when these antagonists were given prior to an early withdrawal (baseline) cocaine seeking test. Our conclusion from these studies is that there is no change in the magnitude of DA release associated with cue induced cocaine seeking across incubation,

but rather a postsynaptic adaptation, such that the same amount of DA release evokes a greater response and thus, invigorated cocaine seeking.

The experiments in Chapter 2 represent the first steps towards integrating these new DA data with our prior work on glutamate's role in cocaine incubation. These experiments used sensor multiplexing of a green genetically encoded calcium indicator, GCaMP8s, and a red GRAB_DA, GRAB_rDA3m, to assess feasibility and to learn about the relationship between DA release and MSN activity in a simpler paradigm (food self-administration) that nevertheless shares some features with our cocaine paradigm. We selected this food paradigm as it reduced the complexity of the surgery (no jugular catheter) and the length of the experiment. Our data show that DA tracks the reward predictive cue, lever entry, and that this response dissipates during extinction training. However, once responding is reinstated by the presentation of a priming food pellet along with the food-paired cue (pellet+cue), bootstrapping analysis indicates the resurgence of the DA transient. Within the same subjects, we found that calcium tightly tracked reward consumption, as we saw a persistent decrease from baseline during the SA phase. This decrease disappeared in extinction. Interestingly, during reinstatement, we found that calcium in MSNs increased with the presentation of the food cue (or pellet+cue), the inverse of what was seen during the SA phase. Our conclusions from these experiments are that these sensor multiplexing studies are indeed possible, and that DA and MSN activity can exhibit distinct relationships depending on behavioral state.

This work is represented in 3 Specific Aims:

- Aim 1 (from funded NRSA): Determine if DA transients change across incubation with fiber photometry.

- Aim 2 (from funded NRSA): Determine the functional significance of DA signaling during incubation using behavioral pharmacology.

- Aim 3: Assess DA release and MSN activity simultaneously during a food extinction-reinstatement task.

CHAPTER 1

Dopamine transmission at D1 and D2 receptors in the nucleus accumbens contributes to the expression of incubation of cocaine craving

Contributors to the manuscript version of this chapter (in revision for Neuropsychopharmacology):

Sophia J. Weber and Marina E. Wolf developed the experiments and wrote the manuscript. Sophia J. Weber, Alex B. Kawa, Alana L. Moutier, Madelyn M. Beutler, Lara M. Koyshman, Cloe D. Moreno, Jonathan G. Westlake conducted the experiments. Amanda M. Wunsch provided input on fiber photometry.

Abstract

Relapse represents a consistent clinical problem for individuals with substance use disorder. In the incubation of craving model of persistent craving and relapse, cue-induced drug seeking progressively intensifies or 'incubates' during forced abstinence. Previously, we and others have demonstrated that expression of incubated cocaine craving requires strengthening of excitatory synaptic transmission in the nucleus accumbens core. However, despite the importance of dopaminergic signaling in the nucleus accumbens core for motivated behavior, little is known about the role that dopamine (DA) plays in the incubation of cocaine craving. Here we used fiber photometry to measure DA transients in the nucleus accumbens core of male and female rats provoked by the drug-paired cue during cue-induced cocaine seeking tests in early abstinence, prior to incubation, and late abstinence, after incubation has plateaued. We observed DA transients time-locked to cue-induced responding, but their magnitude did not differ significantly when measured during early versus late abstinence seeking tests. Next, we tested for a functional role of these DA transients by injecting DA receptor antagonists into the nucleus accumbens core just before the drug seeking test. Blockade of either D1 or D2 DA receptors reduced cue-induced cocaine seeking after but not before incubation. We found no main effect of sex or interaction with any other variables in our experiments. These results suggest that DA contributes to incubated cocaine seeking but the emergence of this role reflects changes in postsynaptic responsiveness to DA rather than presynaptic alterations.

Introduction

Substance Use Disorder (SUD) is a serious global health problem. A major difficulty in treating SUD is the propensity for relapse in abstinent individuals. Preclinical models have been developed to better understand neural mechanisms underlying relapse. The 'incubation of craving' model is a leading model for studying vulnerability to cue-induced relapse after protracted abstinence from drug self-administration. It is based on the discovery that cue-induced cocaine seeking in rats progressively increased ("incubated") over the first month or so of abstinence and then remained high for additional months before declining, providing a model for persistent vulnerability to relapse (Neisewander, Baker et al. 2000, Grimm, Hope et al. 2001, Lu, Grimm et al. 2004). Incubation has subsequently been demonstrated for many addictive substances across drug classes, including nicotine, heroin and alcohol, among others (Lu, Grimm et al. 2004, Pickens, Airavaara et al. 2011, Reiner, Fredriksson et al. 2019, Altshuler, Lin et al. 2020), and in humans for nicotine, methamphetamine, alcohol, and cocaine (Bedi, Preston et al. 2011, Wang, Shi et al. 2013, Li, Wu et al. 2015, Parvaz, Moeller et al. 2016).

Although many brain regions contribute to incubation of cocaine craving (Pickens, Airavaara et al. 2011), our lab and others have shown that synaptic plasticity leading to strengthening of excitatory synapses on medium spiny neurons (MSNs) in the nucleus accumbens core and shell subregions is necessary for the expression of incubated cocaine craving (Wolf 2016, Wright and Dong 2020). In contrast, very little is known about the role of nucleus accumbens dopamine (DA) in the incubation of cocaine craving. This is a significant knowledge gap since DA transmission in the nucleus accumbens core is essential for stimulus-reward learning and motivated behavior (Keiflin and Janak 2015, Bamford, Wightman et al. 2018, Berke 2018, Sippy and Tritsch 2023). Most relevant to the

incubation model, DA release is associated with presentations of reward-related cues and the ability of such cues to promote reward seeking behavior [e.g., (Nicola, Taha et al. 2005, Roitman, Wheeler et al. 2005, Day, Roitman et al. 2007, Stuber, Klanker et al. 2008)]. For example, one early study showed that non-contingent presentation of cues paired with cocaine self-administration elicited DA release in the nucleus accumbens core, measured using microdialysis (Ito, Dalley et al. 2000). Another used fast-scan cyclic voltammetry (FSCV) to measure phasic DA transients during cocaine self-administration and found an elevation of DA in the nucleus accumbens core in response to cocaine cues that promoted cocaine seeking (Phillips, Stuber et al. 2003). Since then, many voltammetry studies have demonstrated that cocaine-associated cues lead to elevated DA in the nucleus accumbens (e.g., Aragona, Day et al. 2009), and more recent fiber photometry studies have detected DA transients in nucleus accumbens core during cue-induced reinstatement of cocaine seeking (Luján, Oliver et al. 2023) and in lateral shell in association with context-induced cocaine seeking (Pribiag, Shin et al. 2021).

Given DA's broad role in reward learning, motivation, and cocaine action, it is logical to assume it also plays a role in incubation of cocaine craving. However, no studies have measured DA release during expression of cocaine incubation. Here we used fiber photometry paired with the DA biosensor GRAB_DA2m (Sun, Zeng et al. 2018, Sun, Zhou et al. 2020) to assess DA release in the nucleus accumbens core during cue-induced seeking tests following long access cocaine self-administration. The cue elicited a DA transient of similar magnitude on forced abstinence day (FAD) 1-2, prior to incubation, and FAD40-50, after incubation. Then we tested the functional significance of this DA release, focusing on DA D1-class and D2-class receptors (D1R and D2R). Infusing either a D1R or D2R receptor antagonist into the nucleus accumbens core prior to a seeking test

reduced cocaine seeking on FAD40-50 but not FAD1-2. We also examined the effects of these antagonists on locomotor activity during the seeking tests, using photobeams in the operant boxes, and in the open field as a control for their effects on general activity. These results are the first to demonstrate DA release during expression of incubation and a requirement for DA receptor signaling in this incubation.

Methods

Subjects

We used male and female Long-Evans rats, either obtained from Charles River (Wilmington, MA) or bred in house. Rats received from Charles River were ~9 weeks upon receipt and were group housed for ~1 week after arrival to acclimate prior to surgery. Rats bred in house were group housed with same sex litter mates from weaning until surgery at 10-17 weeks of age. After surgery, all rats were single housed. Throughout the experiment, rats had free access to standard laboratory chow and water in their home cages and were maintained on a reverse 12:12h light/dark cycle (lights off at 10 AM prior to surgery and 9-9:30 AM post-surgery due to a switch in housing rooms after surgery). All procedures were approved by the OHSU Animal Care and Use Committee and followed NIH guidelines outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). From a total of 79 male and 76 female rats assigned to experimental groups, 10 were excluded due to failure to acquire self-administration, equipment failure during testing (n = 7), significant outlier values during behavioral testing (n = 2), or unexpected deaths during forced abstinence (n = 2). After histology, 32 were excluded due to misplaced intracranial cannula and 1 due to signs of infection surrounding the cannula shaft. See Table 1 for specifics.

Table 1. Number of subjects that successfully reached different phases of each experiment

Expt #	Group	Self-Administration	Pre-screening	Seeking test	Animals included after histology
1	GRAB_Damut	7 (3M, 4F)	7 (3M, 4F)	7 (3M, 4F)	6 (2M, 4F)
	GRAB_DA2m	28 (13M, 15F)	25 (12M, 13F)	23 (11M,12F)	17 (8M, 9F)
Expt #	**Group**	**Self-Administration**	**Seeking test**		**Animals included after histology**
2	FAD1-2	16 (8M, 8F)	14 (8M, 6F)		13 (7M, 6F)
	FAD40-50	40 (18M,22F)	39 (17M,22F)		28 (12M, 16F)
3	FAD1-2	16 (7M, 9F)	15 (6M, 9F)		13 (5M, 8F)
	FAD40-50	34 (19M, 15F)	38 (18M, 15F)		23 (12M, 11F) *
Expt #	**Group**	**Open Field testing**			**Animals included after histology**
4	Open field D1	8 M			7 M
	Open field D2	6 (3M, 3F)			5 (3M, 2F)
Total		155 (79M, 76F) from surgery			112 (56M, 56F)

*7 rats were excluded due to pump failure during microinjection
F, female; M, male

Drugs

We received cocaine HCl from the NIDA Drug Supply Program. Cocaine was dissolved in sterile 0.9% saline and buffered with NaOH to reach pH 7. The final concentration of the stock solution was 5 mg/mL. Cocaine was stored at 4°C once in solution. We dissolved the dopamine (DA) D1 receptor selective antagonist SCH39166 hydrobromide (Tocris Bioscience, Cat. #2299) in DMSO (Sigma Aldrich, #276855) at 500 mM. We then diluted 1:10 in sterile saline to make 50 mM stock vials that were stored at -20°C. On the day of the experiment, this stock was diluted 1:10 in sterile saline to make a 5 mM working stock solution; 0.5 µl was injected into each hemisphere, delivering 1 µg SCH 39166/hemisphere. This dose was based on efficacy in prior intra-nucleus accumbens core infusion studies (Rossi, Reverte et al. 2020). As the SCH39166 working

stock was 1% DMSO in sterile saline, we used 1% DMSO in sterile saline in the vehicle group for SCH39166 experiments. We dissolved the DA D2 receptor selective antagonist L-741,626 (Tocris Bioscience, Cat. #1003/10) in 200 proof ethanol (Decon Laboratories, Inc; #2701) at 100 mM, diluted 1:2 with Tween-80 (Sigma, P4780), and finally diluted 1:10 with sterile water for a final working solution of 5 mM L-741,626. Accordingly, we used a solution of 5% ethanol, 5% Tween-80, and 90% sterile water for vehicle groups in L-741,626 experiments. This vehicle and dose were based on other intra-nucleus accumbens core studies (Papp, Gruca et al. 2017). Working solutions were stored at -20ºC and slow thawed/vortexed the day of testing. SCH39166 was selected based on its high affinity for the D1R relative to other DA and 5-HT receptors (McQuade, Duffy et al. 1991, Alburges, Hunt et al. 1992). While there is a potential for actions of SCH39166 at the D5R, the overall expression of D5R in the nucleus accumbens core is very low (Khan, Gutiérrez et al. 2000, Beaulieu and Gainetdinov 2011). L-741,626 was selected as a D2R antagonist due to its 50-fold higher affinity for the D2R than the D3R (Kulagowski, Broughton et al. 1996, Grundt, Husband et al. 2007).

Surgery

Surgical procedures depended on whether rats were destined for: 1) cocaine self-administration (SA) followed by fiber photometry measurements of DA levels in the nucleus accumbens core during cue-induced seeking tests, 2) cocaine SA followed by intracranial infusion of DA antagonists prior to cue-induced seeking tests, or 3) intracranial DA receptor antagonist infusion into the nucleus accumbens core prior to open field experiments.

Intravenous catheter implantation: Rats destined for cocaine SA experiments were implanted with silastic catheters as previously described (Conrad, Tseng et al. 2008,

Loweth, Scheyer et al. 2014). Briefly, rats were anesthetized with inhalable isoflurane (5% for induction, 1-3% maintenance) and a catheter (Plastics One part #313000BM-15 with Liveo Laboratory Tubing #508-001) was passed subcutaneously from the mid-scapular region and inserted into the right jugular vein. The external portion of the catheter is attached to a mesh back mount platform to which an infusion line can be connected during intravenous drug SA.

Intracranial cannula implantation: For rats receiving intracranial drug infusions after drug SA, intravenous catheterization was immediately followed by intracranial surgery to implant bilateral 23-gauge guide cannula (Plastics One C317G) above the nucleus accumbens core. For intra-nucleus accumbens core drug infusion prior to open field tests, no catheter surgery was performed prior to intracranial surgery. Briefly, rats were mounted in a stereotaxic device. After making an incision in the skin on the skull, the nose bar was adjusted such that the change in dorsal-ventral coordinate from Lambda to Bregma was >0.1mm. We implanted the cannula at the following stereotaxic coordinates relative to Bregma: antero-posterior (AP) +1.3 mm; medio-lateral (ML) ±2.4 mm; dorsal-ventral (DV) -6.3 mm, 6° angle (Paxinos and Watson 2014). We anchored the cannula to the skull with 1/8" pan head sheet metal screws (Fastenere #842176107226) and dental cement (Stoelting #51458) and inserted blockers (Plastics One #C317DC) into the cannula barrel for protection.

Intracranial virus infusion and fiber optic cannula implantation: For rats destined for fiber photometry experiments, immediately following intravenous catheterization we performed bilateral intracranial infusions of virus expressing either the GRAB_DA2m biosensor (AAV9-hSyn-GRAB_DA2m, Addgene #140553) or the GRAB_DAmut control virus (AAV9-hSyn-GRAB_DAmut, Addgene #140555) into the nucleus accumbens core followed by

implantation of fiber optic cannula (Thor Labs, CFM15L10) bilaterally above each infusion site. Viruses were used after diluting 1:3 in sterile saline. Briefly, rats were mounted in a stereotaxic device and, after making an incision, the nose bar was adjusted such that the change in dorsal-ventral coordinates from Lambda to Bregma was <0.1 mm. In each hemisphere, we performed two infusions (250 nL each) of virus, to achieve better dorsal/ventral spread, using the following stereotaxic coordinates (relative to Bregma): AP +1.3; ML ±2.4 mm; DV -7.2 mm for first infusion and DV -7.0 mm for the second infusion; 6° angle. We infused virus at 100nL/min and left the injector in place for 5 min following each infusion. Once the two infusions were completed, we raised the needle by 0.1 mm and waited 2 additional min prior to removal. Following the virus infusion, we implanted fiber optic cannula (AP +1.3 mm; ML ±2.4 mm; DV -6.8 mm; 6° angle). We anchored the cannula to the skull with 1/8" pan head sheet metal screws (Fastenere #842176107226) and dental cement (Stoelting #51458) and covered the fiber optic cannula with dust caps (Thor Labs, CAPF) for protection.

Post-operative Procedures: Rats received 5 mg/kg subcutaneous meloxicam (Covetrus, 6451602845, SKU #49755) as a post-operative analgesic. Rats for photometry experiments recovered for 4 days prior to beginning habituation/7 days prior to beginning SA (see Fig. 1A for timeline), and rats for pharmacology experiments recovered for 7 days prior to beginning SA (timelines in Figs. 7 and 10). For rats used for drug SA, catheters were flushed daily with cefazolin (0.1-0.3 mL of 0.1 g/mL in sterile 0.9% saline; Covetrus #54847) to prevent infection and maintain patency.

<u>Behavioral Apparatus</u>

Self-administration chambers: We trained and tested rats in Med-Associates (Fairfax, VT) self-administration (SA) chambers (#ENV-008-VPX) enclosed in sound attenuating

chambers. Each chamber was equipped with two nose-poke holes (#ENV-114BM) on opposite sides of the apparatus which served as the operant manipulanda. The holes on the right side and left sides of the chamber represented the active and inactive ports, respectively. Photobeams (#ENV-253SD) located at 1/3 and 2/3 of the length of the chamber were used to track activity. We used a single speed syringe pump (#PHM-100 or #PHM-108) that was mounted to the wall of the sound attenuating chamber or placed on top of the SA chamber to deliver infusions of intravenous cocaine. The pump was used to drive a 10 mL syringe that was connected (via a liquid swivel) to the rat's catheter using polyethylene-50 tubing protected by a metal spring.

Open field: Open field locomotion experiments took place in a 100 cm x 100 cm X 17 cm plexiglass open field (Stoelting 60200). We kept the apparatus 2 ft minimum from any other surface and elevated ~3 ft above the ground.

<u>Behavioral Procedures</u>

Open field studies following intra-nucleus accumbens core injection of DA receptor antagonists: Open field studies were performed after recovery from surgery to implant bilateral intracranial guide cannulas. They began with 3 days of habituation to control for the effect of novelty on locomotion and accustom rats to the microinjection procedure. On the first day of habituation, rats were moved to the room where the microinjections would occur, placed on a desk for 30 sec of gentle head restraint, and then placed in their home cage for 15 min prior to being placed into the open field for 1 h to match the length of the cocaine seeking test. On the second habituation day, rats were returned to the room, their cannula blockers were removed, and then blockers were reinserted after rinsing sequentially in saline, 100% ethanol, and gentamicin (5 mg/mL diluted from 100mg/mL Covetrus, #6913). Rats again waited 15 min before being placed into the open field for 1

h. On the last day of habituation, the cannula blockers were removed and the injectors (Plastics One C317I) were lowered into the guide cannula and kept in place for 30 sec. Caps and injectors were rinsed as described above before reinsertion. Rats waited 15 min before being placed into the open field for 1 h. Rats received a day off prior to the first microinjection day and a day off in between microinjections (washout day) if two counterbalanced microinjections were given. On microinjection days, cannula blockers were removed. Injectors filled with drug or vehicle solution (an air bubble marked the start of the injected solution) were connected via Polyethylene Cannula Tubing PE50 10' (Plastics One, C313CT) to 10 µL Hamilton syringes (Hamilton, 80065) mounted in a syringe pump (World Instruments, UMP3 and Micro4). Injectors were inserted, extending 1 mm past the guide cannula into the nucleus accumbens core. Bilateral injection of vehicle or DA antagonist (1 µg/0.5 µL per hemisphere for both SCH39166 and L-741,626) was performed over 1 min. Injection was confirmed via air bubble movement in the tubing. Injectors were left in place for an additional min to allow for diffusion. Injectors were then removed and cannula blockers were reinserted after rinsing as described for habituation days. We waited 15 min prior to beginning behavioral testing as done in previous studies (Rossi, Reverte et al. 2020). Then, rats were placed in the open field apparatus and allowed to explore for 1 h. We recorded their behavior via a ceiling mounted webcam (Logitech C615) and OBS recording software (Version 27.2.3). Values for total distance traveled were generated with ANY-maze (version 7.16) video tracking software with a custom protocol. These values were then inputted into GraphPad Prism (version 10.2.2) and analyzed with an unpaired (SCH39166) or paired (L-741,626) t-test.

Cocaine self-administration: We trained rats to self-administer cocaine using an extended access regimen (6 h/day for 10 sessions; 5 days on, 2 days off, and 5 days on). A 10-

session regimen has been used in our lab for many years (e.g., Conrad, Tseng et al. 2008), while the 2 days off have been introduced more recently (Kawa, Hwang et al. 2022). The start of the session was indicated by the onset of white noise and a single cocaine infusion paired with port illumination. Following this, rats nose-poked in the active port to receive an infusion of cocaine (0.5 mg/kg/infusion), as done in previous studies (Conrad, Tseng et al. 2008, Loweth, Scheyer et al. 2014, Kawa, Hwang et al. 2022), paired with 4 s port illumination which also represented the time-out period during which any additional nose-pokes were recorded but did not result in an infusion. We kept the concentration of cocaine in the syringe constant and varied the timing of infusion to correct for body weight (1.2-3.5 s) to deliver the same dose of cocaine to each rat. Responses in the inactive port were recorded but had no consequence. During SA training, food and water were provided ad libitum in the operant chamber. We tested rats that either failed to acquire SA (average infusions on SA days 8-10 < 25) or did not show greater responses in the active versus inactive port for catheter patency with 0.1 mL sodium brevital (Covetrus, #72465). Animals that failed the patency test were removed from the study (n=2).

For virus group comparisons in the photometry experiments, we analyzed all data in GraphPad Prism (version 10.2.2) with mixed effects analysis with an assumption of sphericity (equal variability of differences). For assessment of nose poke behavior and discrimination between ports, we set fixed effects of virus (GRAB_DA2m or GRAB_DAmut), session (SA1-SA10), and port (active or inactive), with a random effect of subject. The factor of virus was between-subject while session/port were within-subject. To assess cocaine infusions, we performed another mixed effect analysis as previously described except with fixed effects of virus and session.

For analysis of potential sex effects on cocaine self-administration in rats used in photometry experiments, we collapsed across virus groups (GRAB_DA2m or GRAB_DAmut), to increase our sample size and thereby our ability to detect potential sex effects, and performed another mixed effects analysis, using same parameters as above, with fixed effects of sex (male or female), session (SA1-SA10) and port (active or inactive). The factor of sex was between-subject while session/port were within-subject. We assessed cocaine infusions via mixed effect analysis as previously described except with fixed effects of sex and session.

Fixed effects input into the model and output are specified in Tables 1-10. When applicable we removed terms to fit a simpler model to our data. If multiple comparisons were run, we performed a Holm-Šídák correction on post-hoc analyses. Family-wise alpha threshold for confidence was set to 0.05.

Cue-induced seeking tests during forced abstinence: All rats received a cue-induced cocaine seeking test on forced abstinence day (FAD) 1 or 2, i.e., 24-48 h following the last day of SA (session 10). In the food experiments in Chapter 2 we evaluated reinstatement in response to both a food paired cue and a food pellet prime (paired with a cue), but here, due to our focus on incubation, we did not do any cocaine-primed seeking tests as cocaine-primed seeking does not show incubation (Lu, Grimm et al. 2004). The experimental conditions during the seeking test and SA training were kept as similar as possible, except that active port responses now delivered the cue but no cocaine infusion. In addition, during the test rats did not have access to food or water and in photometry experiments rats were not tethered via their back port as they had been during SA training. The number of responses on the active and inactive ports were recorded and active responses served as our measure of cocaine seeking. Locomotion during the test was

monitored as photobeam breaks (see *Behavioral Apparatus*). Following the FAD1-2 seeking test, rats underwent forced abstinence for 40-50 days in home cages (singly housed, as during SA). This time period of forced abstinence was selected as this is when incubation and associated glutamatergic plasticity in the nucleus accumbens core are stably expressed (Wolf and Tseng 2012). During forced abstinence, rats were handled and weighed at least once a week, and estrous cycle monitoring was performed during certain periods as described below. Rats then underwent a second cocaine seeking test identical to the test performed on FAD1-2. For rats implanted with fiber optic cannulas, photometry measures were made throughout a 30 min test as detailed below under *Fiber photometry recordings*. Rats that received intra-cranial injections prior to testing had 1-h seeking tests. Data on the early and late FAD tests were compared via mixed effects analysis performed in GraphPad Prism (version 10.2.2) with an assumption of sphericity (equal variability of differences). For testing for an effect of virus expression on cocaine seeking, we set fixed effects of virus (GRAB_DA2m or GRAB_DAmut), test day (FAD1-2 or FAD40-50), and port (active or inactive), with a random effect of subject. The factor of virus was between-subject and test day/port were within-subject.

For analysis of potential sex effects on cue-induced cocaine seeking in rats used in photometry experiments, we collapsed across virus groups (GRAB_DA2m or GRAB_DAmut) performed another mixed effects analysis, same parameters as above, with fixed effects of sex (male or female), test day (FAD1-2 or FAD40-50), and port (active or inactive). The factor of sex was between-subject and test day/port were within-subject.

Fixed effects input into the model and output are specified in supplemental tables. When applicable we removed terms to fit a simpler model to our data. If multiple

comparisons were run, we performed a Holm-Šídák correction on post-hoc analyses. Family-wise alpha threshold for confidence was set to 0.05.

Cue-induced seeking tests preceded by intra-nucleus accumbens core microinjections: Rats for pharmacology studies received intra-nucleus accumbens core infusions of DA receptor antagonists 15 min prior to either the FAD1-2 or FAD40-50 seeking test. These rats were habituated to the microinjection procedure as described above (*Open field studies following intra-nucleus accumbens core injection of DA receptor antagonists*) except that rats were injected in a room separate to testing and then moved after the 15 min wait. On the day of the seeking test, bilateral infusion of vehicle or DA receptor antagonists into the nucleus accumbens core was performed 15 min prior to the start of a 1-h cue-induced seeking test. Rats were sorted into vehicle or treatment groups based on average infusions for the last 3 days of SA and, if being tested on FAD40-50, FAD1-2 active nose pokes. These animals were given a longer 1-h test to mirror similar studies from our lab (Loweth, Scheyer et al. 2014) and pokes on FAD40-50 were analyzed with a mixed effects model with fixed effects of treatment (DA antagonist vs. vehicle) and port (active vs. inactive). Post hoc analysis of active port responding (DA antagonist vs. vehicle) was performed and corrected using Holm-Šídák tests. We also analyzed data disaggregated for sex.

Fiber photometry recordings

Habituation: For fiber photometry experiments, we began by habituating rats to the fiber optic cable 3 days prior to the start of SA training. We weighed each rat, cleaned their cannula with electronic grade 99.9% anhydrous isopropyl alcohol (MG Chemicals, #824-1L), and then placed the rat into the operant chamber. They were connected to a fiber optic patch cable (Thor labs Custom: FP-400URT, 0.5NA, 0.4m length, FT023SS tubing

with a 2.5mm stainless steel ferrule) via a ceramic connector (Thor Labs, ADAF1-5) and the cable was passed through the top of the box and attached to a steel arm with a counterweight (Med Associates, PHM-110-SAI). The rats were then allowed to explore the operant box while tethered for 20 min after which they were returned to their home cage. Rats underwent 2 more days of habituation prior to SA. On the last day of habituation rats received highly palatable food pellets (Lab Diet 5TUL) in the home cage to reduce neophobia when the same pellets were presented later in the experiment.

Recording parameters: Prior to all recordings, fiber optic cables were bleached at 200 mA for 8 h overnight. On the morning of the recording day, the LED power level and DC were adjusted so that both the 488 nm LED and 405 nm LED had a power output of 40 µW at the end of the fiber optic patch cable as measured using a Thor labs power meter (PM100D+). If the recording day lasted greater than 5 h, power output was checked for stability and changes were made to maintain 40 µW output. For recordings, we passed excitation wavelengths (488 nm and 405 nm) from the TDT RZ10x system via TDT patch cables (200 µm core, 2 m length, 0.5 NA). These TDT cables then connected to a Doric Minicube (FMC6) which in turn was coupled to two Thor fiber optic patch cables (FP400URT-Custom: FP400URT, FT0.5SS tubing, 4 m length coupled to a smaller 0.4 m patch cable via a TDT cable coupler) and then connected to the rat as described in *Habituation.* Emissions (500-550 nm and 460-490 nm) were received via the same patch cable and then decoupled at the Minicube and returned to the RZ10x via TDT response cables (600 µm, 2 m length, 0.5 NA). We acquired these emissions via the RZ10x photodetectors, digitized at 6kHz, and recorded in the TDT Synapse Software (version 95-44132P) on WS4 at frequency 330 Hz for 465 nm and 210 Hz for 405 nm. All recordings had a 6 Hz lowpass filter and a 9.5 V clip threshold.

Prescreening: Prescreening was conducted to verify that a DA signal was detected and identify the hemisphere with the best signal prior to advancing a rat to cue-induced seeking tests. We prescreened rats for DA signal on one of the two days off after SA session 5 and before SA session 6. For the prescreen, we cleaned the fiber optic cannula and then connected the rat to the recording set-up as described in *Recording parameters.* After 3 s of recording, the rat was placed in a novel context (Rubbermaid 71 qt container with same bedding as home cage) and allowed to explore for 1-2 min. Then, the rat was presented with highly palatable food pellets (LabDiet 5TUL) and allowed to investigate and consume for 1 min. The rat was then removed from the novel context and the recording was repeated for the second hemisphere.

Recording during cocaine seeking: For recordings during cue-induced seeking tests, the rat was removed from the home cage, weighed, and had their fiber optic cannula cleaned. We connected the fiber optic patch cable via a ceramic connector as described in *Recording parameters*. The patch cable was passed through the SA chamber on a counterweight system as described in *Habituation*. Rats were placed into the operant chamber and the photometry recording began. The Med Associates program (to record nose-pokes and infusions) was started 5 s later to avoid light artifact obscuring any start of session signals. The photometry recording was continuous for the entire 30-min seeking test. During the test, rats were also recorded via a Logitech webcam (#C615) and checked every 10 min for possible disconnection or tangling of the fiber optic cable. If an intervention was required, it was done as quickly and unobtrusively as possible and the time of the intervention during the test session was noted. After the seeking test ended, the photometry recording was halted and the rat was returned to the home cage.

Data analysis: Fiber photometry data were analyzed using the analysis suite GuPPy created by the Lerner Lab and can be downloaded on their GitHub page; the details of the data manipulations used to generate the GRAB_DA2m signal traces are described in (Sherathiya, Schaid et al. 2021). In brief, for each recording session, we applied least-squares linear fit to align isosbestic and signal channels to one another and use fluctuations in the isosbestic channel to account for florescence changes in the signal channel that are not a result of DA binding. Any disconnects during the recording, defined by a rapid substantial shift (>10 mV) in average mV, were snipped prior to fitting the traces and any nose pokes within those snips were excluded from further analysis. Then we calculated a change in fluorescence measure (ΔF/F=Signal-Fitted Control/Fitted Control) and applied z-score normalization to control for between-session and between-rat differences in virus expression or recording. Finally, we averaged normalized traces across behavioral event replicates for each rat, then averaged by day (early or late seeking test).

For analysis of the entire session trace (Fig. 5E), we examined the average spike amplitude (spikes are defined as transients that are >7 median absolute deviations above the median of the 15-s moving window, regardless of whether they coincide with a nose-poke) as well as the frequency of these spikes during the seeking tests. This threshold was determined by analyzing our GRAB_DAmut recordings and selecting parameters that lead to zero spike detection in these recordings. We analyzed these whole session values with a mixed effects model to assess whether test day had a significant effect on frequency or average spike amplitude.

For DA transients associated with nose-pokes, first we extracted z-scored ΔF/F traces within a 6-sec window around each behavioral response (active or inactive nose-

poke) and averaged all traces to generate an average response for the rat during each test. We identified significant DA transients using continuous threshold bootstrapping methods. Bootstrapping is a statistical method in which multiple stimulated data sets are generated from an original data set to enable hypothesis testing and calculation of confidence intervals. When applied to fiber photometry data we consider each timepoint in the trace to be a data set, with each rat contributing 1 data point from their individual averaged trace. We then generate 1000 replications of this data set by resampling with replacement from our original 17 data points (one for each rat). This generates a normal distribution of values for each timepoint, which we can then use to generate confidence intervals. This enables us to say with 95% confidence what the z-score would be at that timepoint. This is used as a method to detect likely transients (i.e., z-score $\neq 0$) in the entire 6-sec time window analyzed. However, rapid non-physiological changes in fluorescence can occur, for example due to a cable hitting a wall; to account for these non-physiological events, we set a consecutive threshold where the 95% confidence interval cannot not contain 0 (baseline) for a consecutive number of samples set in the code parameters. We selected a consecutive threshold of 125 for our 6 kHz acquisition, which equates to 0.02083 s, based on running the mutant recordings through the analysis and selecting a threshold which resulted in no significant transients. Bootstrapping is thought to provide an unbiased way to identify relevant transients in photometry without experimenter selection of a time window and can be considered a data driven approach. Using this analysis, we identified instances in our recorded traces where the z-score is 95% likely to not be at baseline and marked these epochs with horizontal bars above the traces. In addition to this analysis, we performed permutation tests on the bootstrapped distributions. This is a statistical method in which we compare the mean of the simulated data for each timepoint and assess if it is significantly different from the mean of another bootstrapped

sample distribution (i.e., timepoint 0-sec for FAD1-2 vs timepoint 0-sec for FAD40-50). In tandem with this analysis, we also performed the classical hypothesis driven approach in which we selected time windows before and after our behavioral event of interest and calculated the Area Under the Curve (AUC) for the z-scored ΔF/F trace within this time window. Using these AUC values, we performed a linear mixed effects model with factors of test day (FAD1, FAD40+) and sex (M, F). We analyzed DA responses separately for active pokes that triggered the cue, active pokes during the 4-s cue/time-out period, and inactive pokes. We also analyzed the data after separating the 30-min test into three 10-min bins to assess potential within-test changes in DA responding.

Estrous cycle monitoring

Estrous cycle monitoring was conducted for 4-8 days around both the FAD1-2 and FAD40-50 seeking tests. The monitoring consisted of weighing the animal and, if female, performing a vaginal lavage; if male, we gently prodded the testes as a control manipulation. To habituate rats to these procedures, they were also performed (but estrous cycle stage was not scored) once a week during the forced abstinence phase. Vaginal lavages were performed as described (Becker, Arnold et al. 2005). Briefly, female rats were weighed and then held with their tail lifted to expose their genitalia. A glass blunt-tipped eye dropper (TecUnite, 1mL) containing ~0.3-0.5 mL sterile 0.9% saline was placed at the entrance of the vagina; the saline was quickly inserted and withdrawn. This saline was then placed in a labeled 24-well plate and kept at 4°C for a maximum of 4 days prior to image analysis. Lavages were imaged in the 24-well plate. Images were acquired at 20 X magnification using a Leica DMi8 inverted microscope equipped with an ORCA-Flash4.0 LT+ Digital CMOS camera (Hamamatsu). Leica Application Suite X (LASX) Premium Software (version 3.7.5.24914) was used for image acquisition and ImageJ was used for

analysis. After all images were acquired, we examined the lavages for each rats across days and assigned the lavage as estrus (identified by large numbers of cornified cells that lack nuclei), proestrus (identified by large numbers of nucleated cells), diestrus (identified by large numbers of lymphocytes), or metestrus (identified by a mix of lymphocytes and cornified cells) (Becker, Arnold et al. 2005). Our ability to test for effects of estrus on cocaine seeking was limited because estrus is of short duration and because our females are freely cycling, although adequately powered studies have detected increased seeking in female rats in the estrus phase (Kerstetter, Aguilar et al. 2008, Nicolas, Russell et al. 2019, Corbett, Dunn et al. 2021). Nevertheless, we highlight individuals in estrus on supplemental graphs to demonstrate consideration for these effects, as has been done in one of our previous publications (Funke, Hwang et al. 2023).

Histology

Upon completion of experiments, rats were euthanized via lethal injection of Fatal Plus (Covetrus, #35946) diluted to 80 mg/kg with sterile saline. Once animals no longer displayed reflexive motor responses, they were perfused transcardially first with 1x phosphate buffer saline (PBS) followed by 4% formaldehyde/1% methanol in 1x PBS, pH ~7, at a rate of ~100 mL/s over 5 min. Brains were extracted and allowed to rest in the formaldehyde solution for up to 24 h. Brains were transferred to 1x PBS with 0.01% Sodium azide and then sliced on a vibratome (Leica VT1000s; frequency 8, speed 70, blade DORCO plat. 5T300) at 60 µm. Slices were kept in a 24 well plate in 1x PBS with 0.01% sodium azide at 4°C until the start of immunohistochemistry or Cresyl violet staining.

Cresyl violet staining: Brains from intracranial DA receptor antagonist microinfusion experiments were mounted on Superfrost Plus slides (Fisher Scientific, Cat# 1255015)

after slicing and allowed to dry completely. Once dry the tissue was rinsed on the slides in decreasing concentrations of EtOH (100%, 95%, 70%, 0%) for 3 min in each concentration. The slides were then placed in a Cresyl violet acetate solution (1 g Cresyl Violet Acetate/2.5 mL 100% Glacial Acetic Acid/1 L H_2O) and remained there for 2-4 min or until stain was sufficiently dark. After staining, slides were placed into increasing concentrations of EtOH (0%, 70%, 95%, 100%) for 15 sec each. Next the tissue was rinsed with CitriSolv Hybrid (Decon Labs Inc., Cat #1601H) twice for 5 min each. Tissue was coverslipped (Fisher Scientific, 12541026) directly after the last CitriSolv rinse with Permount (Fisher Scientific, Cat #SP15-100) and allowed to dry for 2 days. After slides dried we examined the tissue to ensure that our guide cannulas/injectors were located in the nucleus accumbens core.

Immunohistochemistry: We performed immunohistochemistry (IHC) on tissue from photometry experiments to amplify the GFP signal prior to imaging to confirm virus expression and placement. We began IHC with three 30-min washes, first in 1x PBS (diluted from 10x PBS, Quality Biological, 119-069-151) then twice in 1x PBS with 0.5% (v/v) Triton-X100 (Electron Microscopy Sciences, #22140). After this we permeabilized the tissue for 2 h in 1x PBS with 0.5% (v/v) Triton-X100, 20% (v/v) DMSO (Sigma Aldrich, #276855), and 2% (w/v) Glycine (Sigma Aldrich, #G8898) at room temperature (RT) on a rocking shaker. Next, we blocked tissue in 1x PBS with 0.5% (v/v) Triton-X100, 10% (v/v) DMSO, and 6% (v/v) Normal Donkey Serum (NDS, Jackson Immuno Research, 017-200-121) for 2 h at RT on a rocking shaker. After blocking we incubated the tissue overnight at RT in 1:1000 Anti-GFP (Aves, GFP-1010) in 1x PBS with 0.5% (v/v) Tween-20 and 0.01% (w/v) Heparin (Sigma Aldrich, #H3393-100KU) with 3% NDS and 10% DMSO on a rotator. The following day, we washed the tissue 3 times for 30 min each in 1x PBS with 0.5% (v/v)

Tween-20 (Thermo Scientific, #J20605-AP) and 0.01% (w/v) Heparin before incubating in

1:250 Anti-chicken-488 (Jackson Immuno Research, 775-546-155) in 1x PBS with 0.5%

(v/v) Tween-20 and 0.01% (w/v) Heparin with 3% NDS overnight at RT on a rotator. The

third day, we performed two 30-min washes in 1x PBS with 0.5% (v/v) Tween-20 and

0.01% (w/v) Heparin before beginning a final wash in 1x PBS (30 min). Slices were kept

in 1x PBS with 0.01% sodium azide at 4°C until being mounted onto Superfrost Plus slides

and coverslipped using Vectashield Vibrance with DAPI (Vector Labs, H-1800-10).

Imaging Images were acquired using a Leica DMi8 inverted microscope equipped with an

ORCA-Flash4.0 LT+ Digital CMOS camera (Hamamatsu). LASX Premium Software was

used for image acquisition and ImageJ was used for analysis. Exposure was determined

using the software to avoid saturation while maximizing the pixel distribution in the

histogram. For DAPI and FITC at 2.5X, we used a 3-s exposure.

Results

Expression of GRAB_DA2m does not alter cocaine self-administration or incubation of craving

As our experiments are long in duration, we began by determining the stability of

virus expression. Drug-naïve rats received nucleus accumbens core infusion of an AAV

expressing GRAB_DA2m and were killed after 3 weeks or 10 weeks to parallel timing of

cue-induced seeking tests in subsequent experiments. Immunohistochemical analysis of

the nucleus accumbens core revealed no difference in maximal fluorescence between

groups, demonstrating stable expression over the period of interest (Fig. 1B; t_{11}=0.110,

p=0.915; see Table 2 for details of this and subsequent statistical analyses for Fig. 1).

Next, we tested whether expression of the sensor, which potentially could lead to buffering of DA levels, might be affecting our measured behaviors. Rats underwent infusion of GRAB_DA2m or a mutant version that does not bind DA, GRAB_DAmut, into the nucleus accumbens core, implantation of a fiber optic cannula above the area of virus expression, and implantation of a jugular catheter to enable intravenous drug self-administration, followed by cocaine self-administration training (Fig. 1A). Cannula placements are shown in Fig. 1C. During cocaine self-administration training, nose-pokes in the active port triggered cocaine infusion, a 4-sec light cue, and a 4-sec time-out period. Both virus groups acquired cocaine self-administration, discriminating between active and inactive ports (Fig. 1D left; Mixed effects model, session: $F_{9,189}$=3.93, p=0.0001; port: $F_{1,21}$=37.4; p<0.0001; virus: $F_{1,189}$=0.154, p=0.696) and earning an equivalent number of cocaine infusions (Fig. 1D middle; virus: $F_{1,21}$=0.248, p=0.624). For one rat, inactive port data on self-administration training days 6-7 were excluded as outliers; full data can be found in Fig. 2. After completing 10 days of self-administration training, rats received cue-induced seeking tests on FAD1 or 2 and again on FAD40-50 (within-subject design), during which pokes in the active port triggered the 4-sec cue light but no cocaine infusion. Mixed effects analysis revealed no group difference in drug seeking (Fig. 1B right; virus: $F_{1,21}$=0.391, p=0.538) and an interaction between fixed effects of port and test day, indicative of incubation of cocaine craving (test day x port: $F_{1,21}$=9.071, p=0.00664). There was no significant three-way interaction (virus x test day x port: $F_{1,21}$=0.0445, p=0.835). Post-hoc analysis demonstrated that GRAB_DA2m rats showed significantly increased responding on the active port (t_{42}=0.868, p=0.00146), while GRAB_DAmut rats did not, possibly reflecting smaller sample sizes. These same comparisons showed no significant difference in FAD40-50 responding between virus groups. Additionally, we found no significant main effect of sex for active/inactive port responding (Fig. 3A left: $F_{1,21}$=0.640,
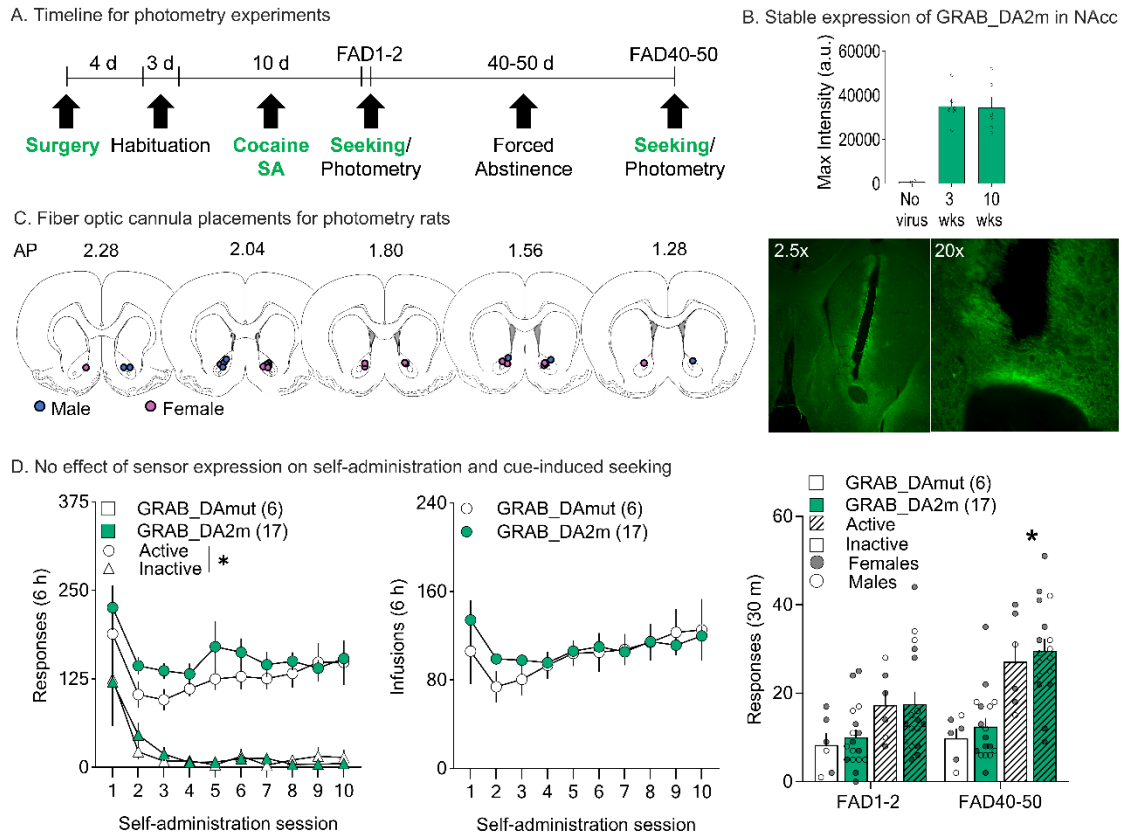
Figure 1. Behavior and virus expression in rats destined for fiber photometry. **A**. Timeline for photometry experiment (green denotes stages shown in this figure). **B**. Top: AAV9-hSyn-GRAB_DA2m expression in NAcc quantified (mean ± SEM) over time in three groups: no virus control group (n = 2 hemispheres), 500nL of virus assessed after 3 weeks of expression (n = 3 rats/6 hemispheres), and 500nL of virus after 10 weeks of expression (n = 3 rats/6 hemispheres). Dots show values for individual hemispheres. Bottom: Representative image after 3 weeks of expression at two different magnifications. **C**. Fiber optic cannula placement for photometry rats. The dot signifies the end of the cannula (females, pink; males, blue). Placement was determined after immunohistochemistry for GFP to confirm virus expression at the end of the cannula. Sections adapted from Paxinos & Watson 7th edition. **D**. Cocaine self-administration and cue-induced seeking data. Left: Rats expressing either GRAB_DA2m (n = 17) or GRAB_DAmut (n = 6) learned to nose poke into the active port for an infusion of cocaine and discriminated between the drug-associated (active) port and the inactive port (*p<0.05; Table 2). Middle: Cocaine infusions for sensor and mutant expressing rats during 10 days of self-administration training. Mean (±SEM) for each 6-h session is shown. Right: Cue-induced seeking tests on FAD1-2 and FAD40-50 for sensor and mutant expressing rats. Bars show mean (±SEM) for each 30-min seeking test while dots indicate individual rats (open circles, males; closed circles, females). * indicates significant main effect of test day but not virus for active pokes on FAD40-50 versus FAD1-2 (Table 2). AP, anterior posterior; FAD, forced abstinence day.

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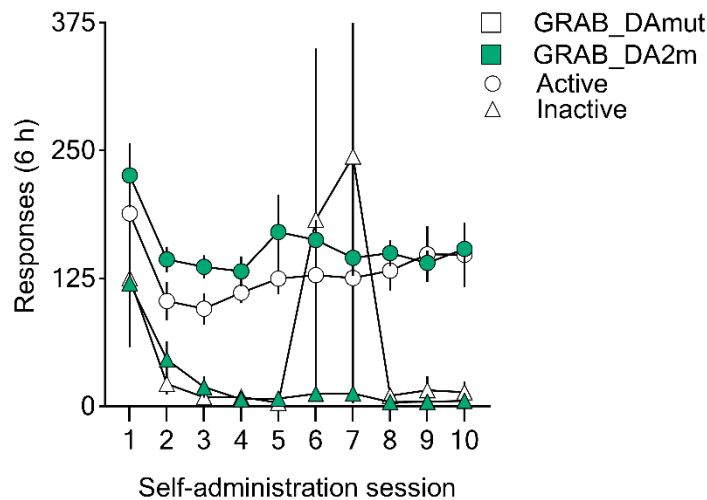
Figure 2. Cocaine self-administration and incubation data with no exclusions. Inactive responses were excluded for a single female rat that from Fig. 1D are included here. Rats expressing both GRAB_DA2m and GRAB_DAmut learned to nose-poke into the active port for an infusion of cocaine and discriminated between the drug-associated port and the inactive port (*p<0.05). Mean (±SEM) for each 6-h session is shown.

p=0.433, Table 3), cocaine infusions (Fig. 3A middle; sex: $F_{1,21}$=0.360, p=0.555) or cue-induced cocaine seeking (Fig. 3A right: $F_{1,21}$=0.296, p=0.5924) which mirrors our previous findings using the same regimen (Kawa, Hwang et al. 2022). We also tested for interactions between sex and other factors and found no significant interactions (Table 3). Of note, it was shown previously that female rats in estrus express more robust cocaine incubation (Kerstetter, Aguilar et al. 2008, Nicolas, Russell et al. 2019, Corbett, Dunn et al. 2021). Our female rats were freely cycling, with only 3 in estrus at the time of the seeking test (2 on FAD1-2, 1 on FAD40-50; purple data-points in Fig. 3A right), so we cannot draw conclusions about estrous cycle effects from our study. Overall, these results suggest that expression of the sensor in the nucleus accumbens core does not affect cocaine self-administration or incubation of craving and confirm prior results demonstrating very similar incubation for male and female rats.

Table 2. Statistical output for immunohistochemical and behavioral data in Figs. 1 and 6

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Fig.
N/A	Fluorescence Intensity	Group	$F_{2,11}=10.97$	0.0024	**	1B
		Holm-Šídák[††]				
		No virus vs. 3 weeks	$t_{11}=4.419$	0.0031	**	
		No virus vs. 10 weeks	$t_{11}=4.341$	0.0031	**	
		3 weeks vs. 10 weeks	$t_{11}=0.1095$	0.915	n.s.	
SA	Nose pokes over 6-h (n=17 GRAB_DA2m, 6 GRAB_DAmut)	Virus x Session x Port				1D, left
		Virus	$F_{1,189}=0.1536$	0.6955	n.s.	
		Session	$F_{9,189}=3.93$	0.0001	***	
		Port	$F_{1,21}=37.4$	<0.0001	****	
		Virus x Session	$F_{9,189}=1.726$	0.0856	n.s.	
		Virus x Port	$F_{1,189}=3.991$	0.0472	*	
		Session x Port	$F_{9,189}=2.106$	0.0309	*	
		Virus x Session x Port	$F_{9,189}=2.040$	0.0371	*	
SA	Infusions over 6-h (n=17 GRAB_DA2m, 6 GRAB_DAmut)	Virus x Session				1D, middle
		Virus	$F_{1,21}=0.2480$	0.6237	n.s.	
		Session	$F_{9,189}=4.172$	<0.0001	****	
		Virus x Session	$F_{9,189}=1.101$	0.3641	n.s.	
Cocaine Seeking	Nose pokes over 30-m (n=17 GRAB_DA2m, 6 GRAB_DAmut)	Virus x Test Day x Port				1D, right
		Virus	$F_{1,21}=0.3913$	0.5384	n.s.	
		Test Day	$F_{1,21}=7.526$	0.0122	*	
		Port	$F_{1,21}=50.05$	<0.0001	****	
		Virus x Test Day	$F_{1,21}=0.1149$	0.7380	n.s.	
		Virus x Port	$F_{1,21}=0.04717$	0.8320	n.s.	
		Test Day x Port	$F_{1,21}=9.071$	0.00664	**	
		Virus x Session x Port	$F_{1,21}=0.04448$	0.8350	n.s.	
Cocaine Seeking	Nose pokes over 30-m (n=10M, 13F)	Test Day x Bin[†]				6B
		Test Day	$F_{1,17}=10.7$	0.0001	***	
		Bin	$F_{2,34}=12.02$	0.0045	**	
		Test Day x Bin	$F_{2,34}=0.6467$	0.5301	n.s.	

[†]Random effects with SD = 0 excluded from model
[††]adjusted P-values reported for post hoc comparisons
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
F, female; M, male

Cues previously paired with cocaine infusion elicit similar DA responses in early and late forced abstinence

Fiber photometry recordings were performed in both GRAB_DA2m and GRAB_DAmut rats during FAD1-2 and FAD40-50 seeking tests (Fig. 5A). As expected, in the GRAB_DAmut-expressing rats there was no apparent signal (Fig. 4). We therefore used the mutant photometry data to define analysis thresholds, as detailed below.

Photometry data for GRAB_DA2m-expressing rats on FAD1-2 and FAD40-50 are presented in Fig. 5B. DA levels, plotted relative to each nose-poke (time 0), are shown for pokes in the active port that triggered 4-s light cue presentation (Fig. 5B left), pokes in the active hole that did not trigger cue presentation because they occurred during the 4-s time-out period when the cue light is already on (Fig. 5B center), and pokes in the inactive port that had no consequence (Fig. 5B right). Horizontal lines above the traces indicate the duration of DA responses assessed using continuous threshold bootstrapping methods (Table 6 for details), where the 95% confidence interval does not cross baseline (i.e., z-score = 0) for the consecutive threshold period (Jean-Richard-Dit-Bressel, Clifford et al. 2020, Liu, Jean-Richard-Dit-Bressel et al. 2020, Yau and McNally 2022). Time periods during which bootstrapping revealed differences between groups are indicated by a dark blue line. GRAB_DAmut data were used to identify a consecutive threshold for bootstrapping analysis (see Methods for details).

Focusing on active pokes triggering the cue, analysis of both FAD1-2 and FAD40-50 revealed an increase in DA levels at cue onset (0-1 s) as well as a smaller amplitude but longer-lasting increase during the remainder of cue light presentation (1-4 s) (Fig. 5B). Although there were small differences in the exact timing of DA increases (see colored bars above FAD1-2 and FAD40-50 traces), we found no significant differences between
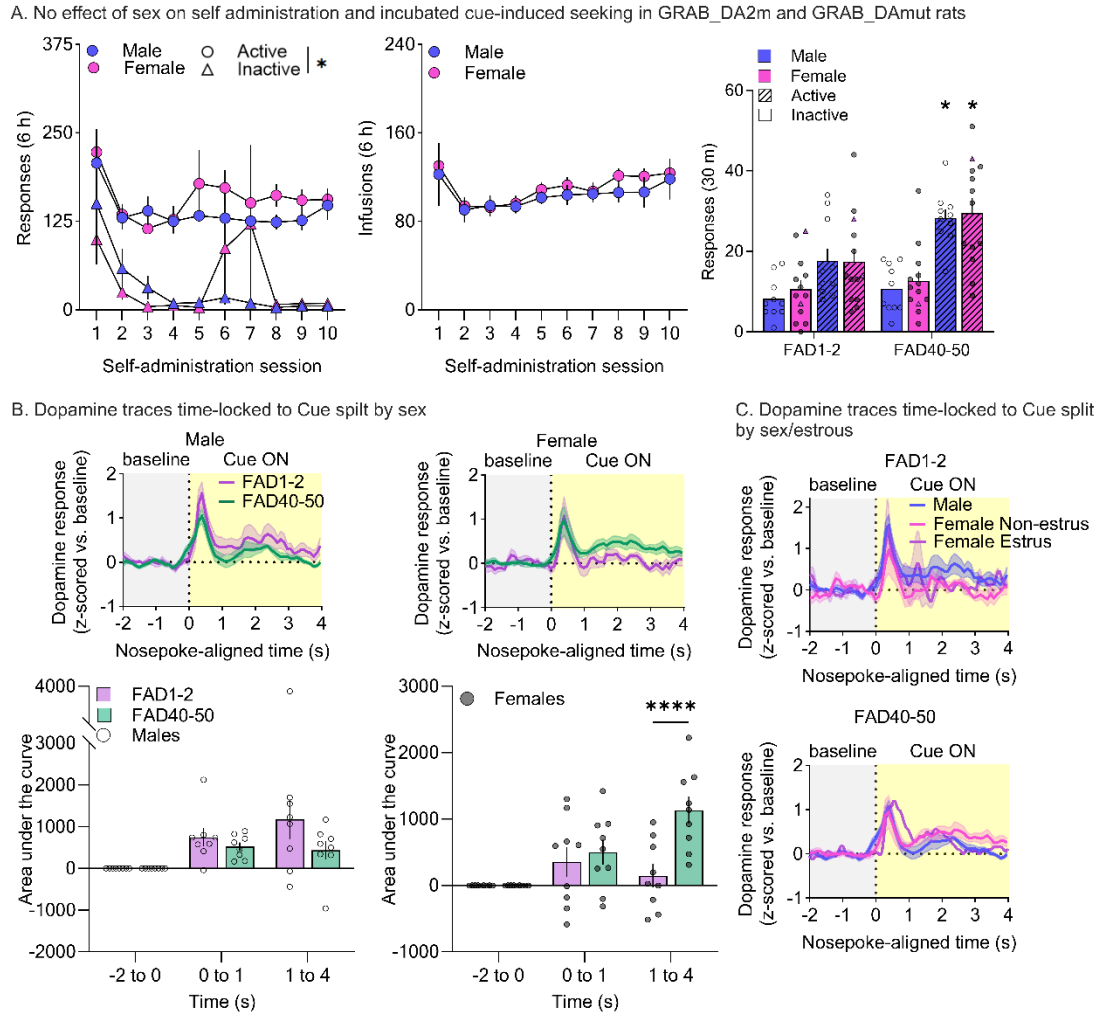
Figure 3. Behavioral and fiber photometry data split by sex. **A**. Self-administration and incubation data from Fig. 1 are shown for males and females separately. *Left:* Both male and female rats expressing GRAB_DA2m or GRAB_DAmut learned to nose-poke into the active port for an infusion of cocaine and discriminated between the drug-associated port and the inactive port (*p<0.05). Mean (±SEM) for each 6-h session is shown. *Middle:* Cocaine infusion data collapsed by virus group and spilt by sex. Mean (±SEM) for each 6-h session is shown. *Right:* Cocaine seeking data for FAD1-2 and FAD40-50 tests collapsed by virus and spilt by sex GRAB_DA2m n= 8 males/9 females, GRAB_DAmut n = 2 males/4 females) (*p<0.05 vs respective FAD1-2 active port data). Bars show mean (±SEM) for each 30-min seeking test while dots indicate individual rats. Purple triangles indicate female rats that were in estrus at the time of the seeking test. **B**. DA traces time-locked to active pokes that triggered the cue (0-4 sec), from Fig. 5, are shown for males and females separately. *Left:* z-scored mean DA response for male rats normalized to a baseline period (-2 to 0 s) on FAD1-2 and FAD40-50 (n = 8, within subject design). SEM is shown in shaded area around the mean. Bar graphs below DA trace show AUC spilt into time epochs of baseline (-2 to 0 s), initial peak (0 to 1 s) and secondary peak (1 to 4 s). Bars show mean (±SEM) for each time period around the behavioral event while dots indicate

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individual rats. *Right:* The same z-scored mean DA response and area under the curve results are shown for female rats (n = 9). **C**. DA traces time-locked to active pokes that triggered the cue (0-4s) spilt by sex and by estrous cycle stage. *Top:* z-scored mean DA response normalized to a baseline period (-2 to 0 s) on FAD1-2 (n = 8 males, n = 7 females non-estrus, n = 2 females estrus). SEM is shown in shaded area around the mean. *Bottom:* z-scored mean DA response normalized to a baseline period (-2 to 0 s) on FAD40-50 (n = 8 males, n = 8 females/non-estrus, n = 1 female/estrus). FAD, forced abstinence day.

Table 3. Statistical output for behavioral data in Fig. 3

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Figure
SA	Nose pokes over 6-h (n=10M, 13F)	Sex x Session x Port				3A, left
		Sex	$F_{1,21}=0.6399$	0.4327	n.s.	
		Session	$F_{9,189}=3.845$	0.0002	***	
		Port	$F_{1,21}=53.85$	<0.0001	****	
		Sex x Session	$F_{9,189}=1.002$	0.4397	n.s.	
		Sex x Port	$F_{1,21}=0.1336$	0.7184	n.s.	
		Session x Port	$F_{9,189}=0.8727$	0.5507	n.s.	
		Sex x Session x Port	$F_{9,189}=0.6181$	0.7808	n.s.	
SA	Infusions over 6-h (n=10M, 13F)	Sex x Session				3A, middle
		Sex	$F_{1,21}=0.3597$	0.5551	n.s.	
		Session	$F_{9,189}=4.188$	<0.0001	*****	
		Sex x Session	$F_{9,189}=0.2139$	0.9922	n.s.	
Cocaine Seeking	Nose pokes over 30-m (n=10M, 13F)	Sex x Test Day x Port				3A, right
		Sex	$F_{1,21}=0.2955$	0.5924	n.s.	
		Test Day	$F_{1,21}=10.62$	0.0038	**	
		Port	$F_{1,21}=63.60$	0.0001	****	
		Sex x Test Day	$F_{1,21}=0.01097$	0.9176	n.s.	
		Sex x Port	$F_{1,21}=0.2576$	0.6171	n.s.	
		Test Day x Port	$F_{1,21}=12.07$	0.0023	**	
		Sex x Test Day x Port	$F_{1,21}=0.1273$	0.7248	n.s.	

F, female; M, male
**p<0.01, ***p<0.001, ****p<0.0001

Table 4. Statistical output for fiber photometry data related to Figs. 3 and 6E

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Figure
Cocaine seeking (30-min)	DA response to active nose pokes that trigger cue, AUC (n=17)	Time x Sex x Test Day				Data from 5C left, re-analyzed with sex as a factor
		Time	$F_{2,30}=11.46$	0.0002	***	
		Sex	$F_{1,30}=1.10$	0.304	n.s.	
		Test Day	$F_{1,15}=1.740$	0.2069	n.s.	
		Time x Sex	$F_{2,30}=0.6153$	0.5472	n.s.	
		Time x Test Day	$F_{2,30}=1.417$	0.2582	n.s.	
		Sex x Test Day	$F_{1,30}=2.110$	0.1567	n.s.	
		Time x Sex x Test Day	$F_{2,30}=2.789$	0.0775	n.s.	
Cocaine seeking (30-min)	DA response to active nose pokes that trigger cue, AUC (n=8M)	Time x Test Day				3B, bottom left
		Time	$F_{2,14}=6.862$	0.0084	**	
		Test Day	$F_{1,7}=2.738$	0.1420	n.s.	
		Time x Test Day	$F_{2,14}=0.4512$	0.0705	n.s.	
Cocaine seeking (30-min)	DA response to active nose pokes that trigger cue, AUC (n=9F)	Time x Test Day				3B, bottom right
		Time	$F_{2,16}=6.588$	0.0083	**	
		Test Day	$F_{1,8}=11.96$	0.0086	**	
		Time x Test Day	$F_{2,16}=21.37$	<0.0001	****	
		Holm-Šídák: FAD1-2 vs. FAD40-50[††]				
		-2 to 0	$t_{24}=0.001099$	0.9991	n.s.	
		0 to 1	$t_{24}=1.011$	0.3223	n.s.	
		1 to 4	$t_{24}=6.848$	<0.0001	****	
		Holm-Šídák: FAD1-2[††]				
		-2 to 0 vs. 0 to 1	$t_{32}=1.802$	0.2238	*	
		-2 to 0 vs. 1 to 4	$t_{32}=0.7506$	0.5114	***	
		0 to 1 vs. 1 to 4	$t_{32}=1.051$	0.5114	n.s.	
		Holm-Šídák: FAD40-50[††]				
		-2 to 0 vs. 0 to 1	$t_{32}=2.538$	0.0162	*	
		-2 to 0 vs. 1 to 4	$t_{32}=5.733$	<0.0001	****	
		0 to 1 vs. 1 to 4	$t_{32}=3.195$	0.0063	**	
Cocaine seeking	DA response to 1st active nose poke that triggered cue, AUC (n=17)	Time x Test Day[†]				AUC data from traces in Fig. 6E
		Time	$F_{2,32}=3.31$	0.0496	*	
		Test Day	$F_{1,16}=0.5601$	0.4651	n.s.	
		Time x Test Day	$F_{2,32}=0.2274$	0.7979	n.s.	

F, female; M, male
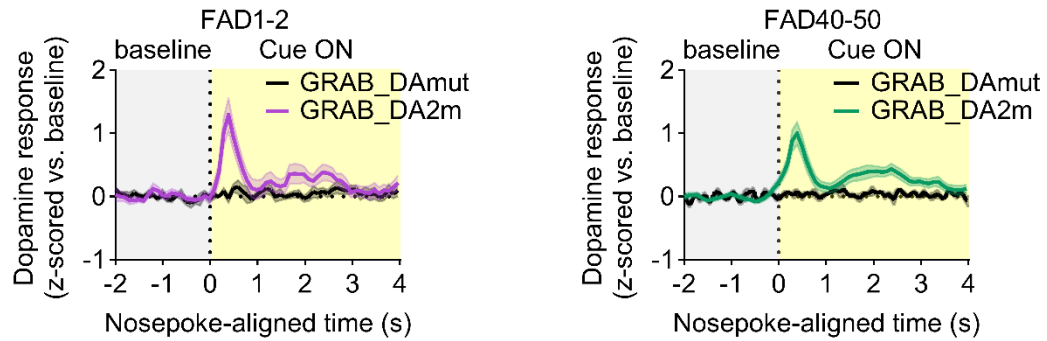*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 4. DA signals are evident in the nucleus accumbens core of rats expressing GRAB-DA2m but not rats expressing GRAB-DAmut. **A.** DA traces time-locked to active pokes that triggered the cue (0-4s) during the cue-induced seeking tests for GRAB_DA2m and GRAB_DAmut expressing rats. *Left:* z-scored mean DA response normalized to a baseline period (-2 to 0 s) on FAD1-2 (n = 17 GRAB_DA2m, n = 6 GRAB_DAmut). SEM is shown in shaded area around the mean. *Right:* The same analysis is shown for these rats on FAD40-50 (within-subject design).

test days when comparing bootstrapped confidence intervals. We also quantified both periods relative to baseline (-2 to 0 s) via area under the curve (AUC) (Fig. 5C left). Mixed effects analysis revealed a significant effect of time (s) ($F_{2,32}$=14.0*, p*=<0.0001), suggesting that DA levels are changing significantly across the analysis window, but not test day (FAD1-2 vs FAD40-50; $F_{1,16}$=0.128, *p*=0.725) and no significant time x test day interaction ($F_{2,32}$=0.451, *p*=0.641). These data indicate that active pokes triggering the cue elicit DA release of similar magnitude on FAD1-2 and FAD40-50 when averaged across trials and subjects. However, there was considerable individual variability in DA responses, as depicted in heat maps showing the average DA response for each subject (Fig. 5D). Next, we performed the same analyses for active port nose-pokes made within the time-out period (Cue light on) (Fig. 5B middle). The pattern of DA responses was similar to that shown in Fig. 5B left, but less robust, with only the initial response detected by bootstrapping analysis. Again, no difference between test days was indicated. Mixed

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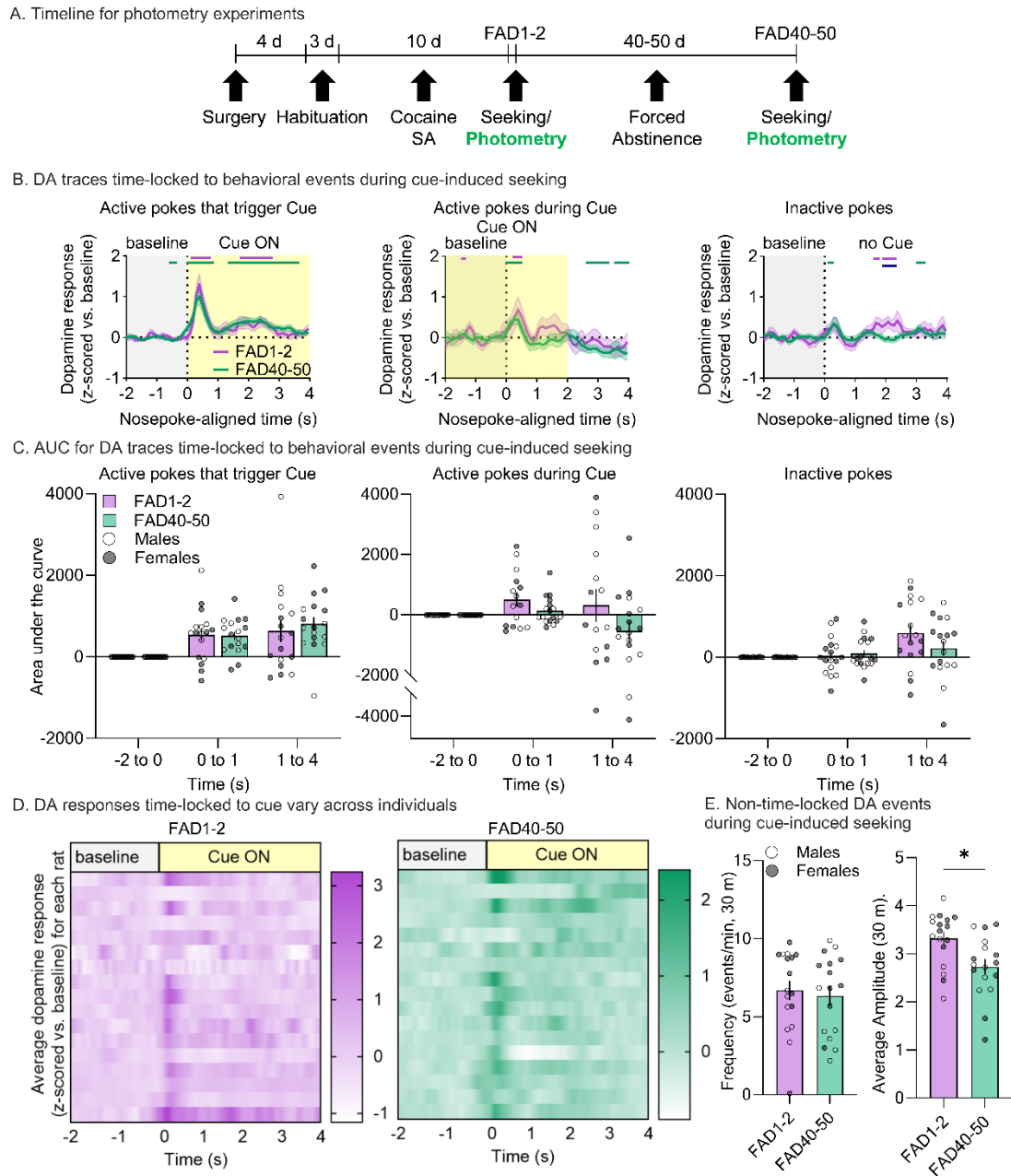
Figure 5. Fiber photometry recordings during cue-induced seeking tests. **A**. Timeline for photometry experiment (green denotes stages shown in this figure). **B**. DA traces time-locked to behavioral events (n = 17 rats). *Left:* z-scored mean DA response normalized to a baseline period (-2 to 0 s) for active pokes that triggered the cue on FAD1-2 and FAD40-50. SEM is shown in shaded area around the mean. The matching-colored lines above the traces show periods in the 6-sec window during which bootstrapping indicates 95% confidence that the mean is not equal to zero. *Middle:* z-scored DA response for active pokes during cue light on/time-out period on FAD1-2 and FAD40-50. *Right:* z-scored DA response for inactive pokes on FAD1-2 and FAD40-50. **C**. Area under the curve for traces shown in (B). Traces are split into time epochs of baseline (-2

to 0 s), initial peak (0 to 1 s) and secondary peak (1 to 4 s). Bars show mean (±SEM) for each time period around the behavioral event while dots indicate individual rats. **D**. Heat maps showing the average DA trace for each rat that contributed to (B). *Left:* DA responses to active pokes that trigger the cue for individual rats on FAD1-2. *Right:* Individual responses on FAD40-50. Darker colors represent higher z-score. **E**. Analysis of DA events not time-locked to a behavioral event. Data obtained using the mutant sensor GRAB_DA2mut were used to define a threshold for DA transient identification such that 0 peaks were detected in the mutant trace; this threshold was applied to the entire 30 min recording for each seeking test for all rats (n = 17). *Left:* Frequency (average number of events per minute) on FAD1-2 and FAD40-50. *Right:* Average amplitude of all events above threshold in the 30 min recording for FAD1-2 and FAD40-50. Bars show mean (±SEM) for each 30-min seeking test while dots indicate individual rats (open circles, males; closed circles, females). *p<0.05 FAD1-2 versus FAD40-50 (Table 5). AUC, area under the curve; FAD, forced abstinence day.

Table 5. Statistical output for fiber photometry data in Fig. 5

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Figure
Cocaine seeking (30-min)	DA response to active nose pokes that trigger cue, AUC (n=17)	Time x Test Day				5C, left
		Time	$F_{2,32}=14.0$	<0.0001	****	
		Test Day	$F_{1,16}=0.1281$	0.7251	n.s.	
		Time x Test Day	$F_{2,32}=0.4512$	0.6409	n.s.	
Cocaine seeking (30-min)	DA response to active nose pokes during cue, AUC (n=15 FAD1-2, n=17 FAD40-50)	Time x Test Day[†]				5C, middle
		Time	$F_{2,32}=1.361$	0.2709	n.s.	
		Test Day	$F_{1,16}=3.312$	0.0875	n.s.	
		Time x Test Day	$F_{2,32}=1.266$	0.2987	n.s.	
Cocaine seeking (30-min)	DA response to inactive nose pokes, AUC (n=17)	Time x Test Day				5C, right
		Time	$F_{2,32}=6.97$	0.00307	**	
		Test Day	$F_{1,16}=1.020$	0.3275	n.s.	
		Time x Test Day	$F_{2,32}=2.802$	0.0756	n.s.	
Cocaine seeking (30-min)	Frequency of events	Paired t-test				5E, left
		Test Day	$t_{16}=0.7640$	0.4388	n.s.	
Cocaine seeking (30-min)	Amplitude of events	Paired t-test				5E, right
		Test Day	$t_{16}=2.84$	0.0117	*	

[†]Random effects with SD = 0 excluded from model
*p<0.05, **p<0.01, ****p<0.0001
F, female; M, male

Table 6. Statistical output for bootstrapping analyses in Fig. 5

Expt phase	Measure	Factors in analysis	Time 95% CI ≠ 0	FAD1-2 vs FAD40-50	Figure
Cocaine seeking (30-min)	DA response to active nose pokes that trigger cue, z-scored trace (n=17)	Bootstrapping FAD1-2	0.14 – 0.76 s 1.61 – 2.85 s	n.s.	5B, left
		FAD40-50	-0.59 – -0.46 s -0.12 – 0.92 s 1.2 – 3.6 s		
Cocaine seeking (30-min)	DA response to active nose pokes during cue, z-scored trace (n=15 FAD1-2, n=17 FAD40-50)	Bootstrapping FAD1-2	-1.26 – -1.13 s 0.29 – 0.54 s	n.s.	5B, middle
		FAD40-50	0.05 – 0.44 s 2.62 – 3.44 s 3.47 – 4.0 s		
Cocaine seeking (30-min)	DA response to inactive nose pokes, z-scored trace (n=17)	Bootstrapping FAD1-2	1.62 – 1.80 s 1.93 – 2.22 s	Yes, 1.93 – 2.22 s	5B, right
		FAD40-50	0.17 – 0.36 s 2.97 – 3.23 s		

effects analysis of AUC for 0-1 s and 1-4 s time periods (relative to -2 to 0 s baseline) found no effect of time ($F_{2,32}$=1.36, p=0.271) or test day ($F_{1,16}$=3.31, p=0.0875) (Fig. 5B middle).

We analyzed frequency and amplitude of all DA transients over the 30-min test, regardless of whether they were time-locked to a nose-poke (Fig. 5E). For this analysis, GRAB_DA2m data were used to define a threshold for DA transient identification such that 0 peaks were detected in the mutant trace after applying this threshold (see Methods). The frequency of DA transients did not differ between test days (Fig. 5E left; t_{16}=0.764, p=0.439), but the average amplitude of all above-noise DA events was significantly reduced on FAD40-50 compared to FAD1-2 (Fig. 5E right; t_{16}=2.84, p=0.0117). This could
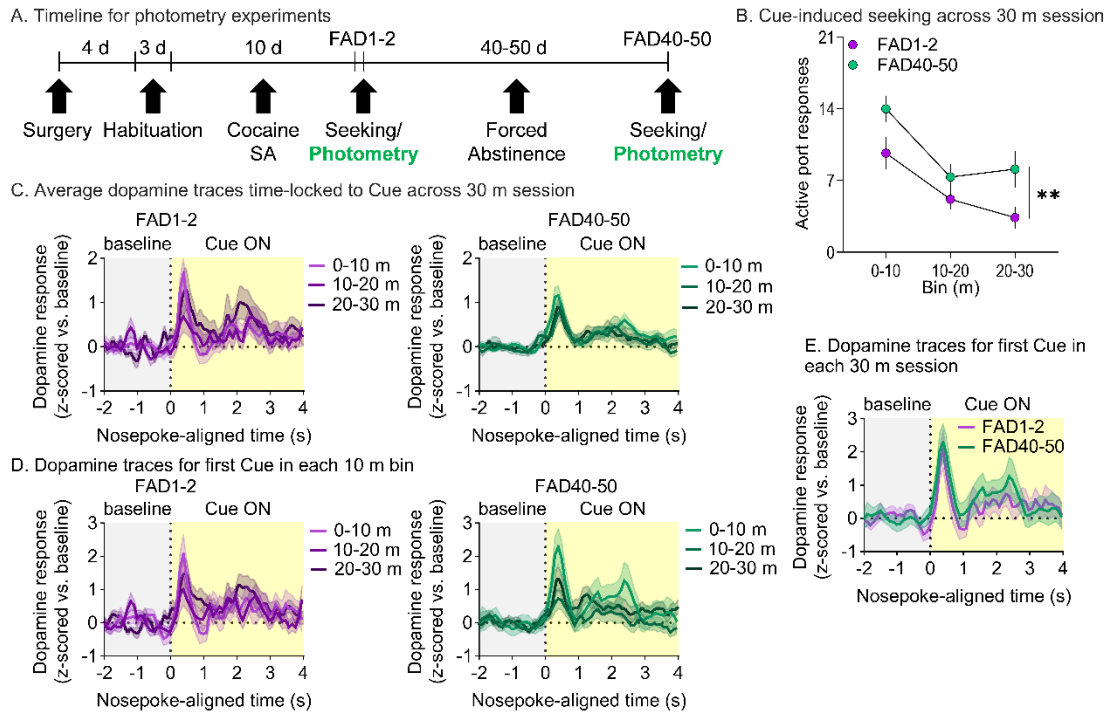
Figure 6. Analysis of behavior and DA responses during specific periods of cue-induced seeking tests. **A**. Timeline for photometry experiment (green denotes stages shown in this figure). **B**. Mean (±SEM) active port responses for seeking test data separated into 10-min bins (n = 17 rats in this and all subsequent panels). **C**. Mean DA trace (±SEM) time-locked to active pokes that triggered the cue, split into 10-min bins, on FAD1-2 (*Left*) and FAD40-50 (*Right*). Separate bins are shown as different colored lines. **D**. Mean DA trace (±SEM) time-locked to the first poke that triggered a cue in each 10-min bin shown in (C). Separate bins are shown as different colored lines. **E**. Mean DA trace (±SEM) time-locked to the first poke that triggered a cue in each 30-min session for FAD1-2 and FAD40-50. FAD, forced abstinence day.

reflect alterations in tonic DA release as a function of abstinence time and/or a response to the context previously paired with cocaine self-administration.

We also investigated within-session effects by binning data from the 30-min seeking tests into 3 10-min bins. Behaviorally, we observed greater active port responses across bins for the FAD40-50 test compared to the FAD1-2 test, reflecting incubation of craving (Fig. 6B; main effect of test day: $F_{1,17}$=10.7, p=0.00446). For photometry data,

considerable variability was observed across bins on FAD1-2, with the highest magnitude DA response in the first 10 min, whereas responses in each bin were similar on FAD40-50 (Fig. 6C). A concern when comparing the two test days is that we averaged different numbers of responses. Thus, because of the incubation phenomenon, rats made more active port responses on FAD40-50, and therefore the number of measured DA responses contributing to that average is higher than for FAD1-2. This would be expected to reduce variability for the FAD40-50 test. In order to control for this, we analyzed the first nose-poke in each 10-min bin, so that each rat has only one poke contributing to each bin across both test days. When analyzed in this manner, the pattern of responding across bins was similar on FAD1-2 and FAD40-50 (Fig. 6D). We also compared the DA response to the first nose-poke of each session and observed no difference between test days (Fig. 6E). This was mirrored by analysis of AUC (data not shown) that found a significant effect of time ($F_{2,32}$=3.31, p=0.0496) but not test day ($F_{1,16}$=0.560, p=0.465) and no significant interaction ($F_{2,32}$=0.227, p=0.798) (Table 5). Overall, these data indicate that within-session DA responses do not differ between test days when accounting for the number of responses in the data-sets.

DA release is similar in male and female rats during cue-induced seeking tests

For nose-pokes that trigger cue presentation (Fig. 5B left), individual data-points for corresponding AUC data (Fig. 5C left) suggest similar DA elevation in males and females, and re-analysis of these data (Mixed effects analysis with factors of time, test day and sex) found no significant effect of sex ($F_{1,30}$=1.10, p=0.304) or test day ($F_{1,15}$=1.74, p=0.207) but a significant effect of time ($F_{2,30}$=11.5, p=0.000200). Furthermore, there were no significant interactions between sex and other variables (sex x time: $F_{2,30}$=0.615, p=0.547; sex x test day: $F_{1,30}$=2.11, p=0.157; sex x time x test day: $F_{2,30}$=2.79, p=0.0775).

Nevertheless, to thoroughly address sex as a biological variable, DA traces for active pokes triggering the cue are shown disaggregated for sex (Fig. 3B top) and AUC was analyzed for each sex (Fig. 3B bottom). For males, there was a significant effect of time ($F_{2,14}$=6.86, p=0.00838), but no effect of test day ($F_{1,7}$=2.74, p=0.142) or interaction ($F_{2,14}$=0.451, p=0.0705). However, females showed a significant effect of time ($F_{2,16}$=6.59, p=0.00827), test day ($F_{1,8}$=12.0, p=0.00859), and interaction ($F_{2,16}$=21.4, p<0.0001). We therefore performed post hoc analysis of the female data and found a significant increase on FAD40-50 vs FAD1-2 in DA levels 1-4 s after the cue onset (t_{24}=6.85, p<0.0001). Overall, these results reveal a similar pattern between the sexes in DA release time-locked to cue-induced cocaine seeking, although small time-dependent differences are suggested. Finally, as there is some evidence that DA transmission is modulated by the estrous cycle (Yoest, Cummings et al. 2014, Zachry, Nolan et al. 2021), we replotted DA responses triggering cues to compare males to females in non-estrus phases or estrus at the time of the test (Fig. 3C). As there were only 2 females on FAD1-2 and 1 female on FAD40-50 in estrus, conclusions cannot be drawn.

Intra-nucleus accumbens core administration of a D1 receptor antagonist decreases expression of cocaine incubation

To determine whether DA responses during cue-induced seeking tests have behavioral significance, we infused D1 or D2 receptor antagonists into the nucleus accumbens core 15 min prior to cue-induced seeking tests on FAD1-2 or FAD40-50. Results obtained with the D1 antagonist SCH39166 are shown in Fig. 7. All rats self-administered cocaine using the same 10-day regimen employed in Figs. 1-6 and then were divided into equivalent groups, destined for vehicle or SCH39166 infusion, based on cocaine infusions on the last three days of self-administration (sessions 8-10) (Fig. 7B

A. Timeline for FAD1 D1-R antagonism

7 d — 10 d — FAD1

Surgery | Cocaine SA | **Seeking/ Pharmacology**

B. D1-R blockade during baseline (FAD1) cue-induced seeking

Group Balancing

□ Vehicle (7) ● Females
■ SCH39166 (7) ○ Males

Infusions (6 h) — Self administration session (8, 9, 10)

FAD1 cue-induced seeking test

Nosepokes (1 h) — Inactive, Active

Active nosepokes — Time (m): 20, 40, 60

C. Timeline for FAD40-50 D1-R antagonism

7 d — 10 d — FAD1 — 40-50 d — FAD40-50

Surgery | Cocaine SA | Seeking | Forced Abstinence | **Seeking/ Pharmacology**

D. D1-R blockade during incubated (FAD40-50) cue-induced seeking

Group Balancing

|| Vehicle (15) ● Females
■ SCH39166 (13) ○ Males

Infusions (6 h) — Self administration session (8, 9, 10)

FAD40-50 cue-induced seeking test

Nosepokes / 1 h — Inactive, Active *

Active nosepokes — Time (m): 20, 40, 60 ***

E. D1-R blockade and locomotion

FAD1

□ Vehicle (7) ● Females
■ SCH39166 (7) ○ Males

Beam breaks (1 h) — FAD1-2 *

FAD40-50

□ Vehicle (14)
■ SCH39166 (12)

Beam breaks (1 h) — FAD40-50

Drug naïve rats - open field

○ Males (6)

Distance Traveled (m) — Vehicle, SCH39166

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Figure 7. Intra-NAcc infusion of the D1R antagonist SCH39166 reduces cue-induced seeking on FAD40-50 but not FAD1. **A**. Timeline for FAD1 D1R antagonist experiment (yellow denotes stages that are the focus panel B). **B**. Behavioral data for FAD1 D1R antagonist experiment. *Left:* Groups destined for vehicle or SCH39166 infusion on FAD1 were balanced based on mean (±SEM) cocaine infusions for the last three 6-h sessions of self-administration training. *Middle:* Bars show mean (±SEM) active and inactive pokes during the 1-h FAD1 cue-induced seeking test for rats receiving intra-NAcc vehicle (n = 7) or SCH39166 (n = 7). Dots indicate individual rats (open circles, males; closed circles, females)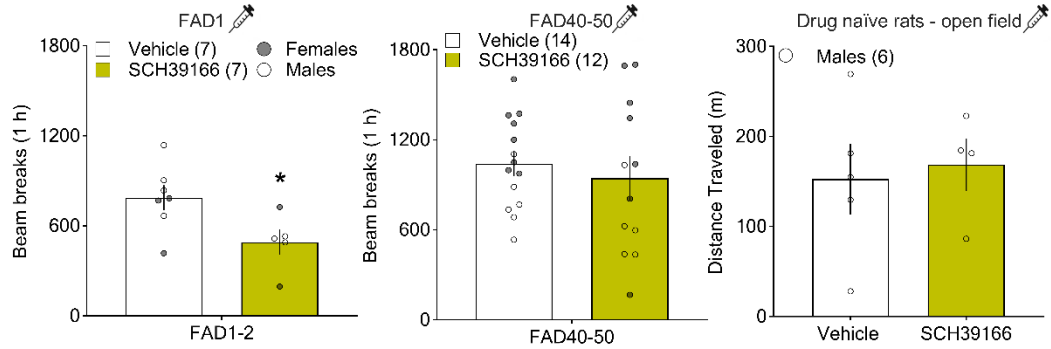. *Right:* Mean (±SEM) active pokes for FAD1 seeking test data split into three 20-min bins. **C**. Timeline for FAD40-50 D1R antagonist experiment (yellow denotes stages that are the focus of panel D). **D**. Behavioral data for FAD40-50 D1R antagonist experiment. *Left:* Groups destined for vehicle or SCH39166 infusion on FAD40-50 were balanced based on mean (±SEM) cocaine infusions for the last three 6-h sessions of self-administration training (shown here) as well as FAD1 seeking data (Fig. 9A). *Middle:* Mean (±SEM) pokes during the 1-h FAD40-50 cue-induced seeking test for rats receiving intra-NAcc vehicle (n = 15) or SCH39166 (n = 13) (*p<0.05 vs vehicle; Table 7). *Right:* Mean (±SEM) active pokes for FAD40-50 seeking test data split into three 20-min bins. Incubated cue-induced seeking was suppressed in the SCH39166 group (***p<0.001 main effect of treatment, SCH39166 vs vehicle; Table 7). **E**. Operant box photobeam breaks during seeking tests and a separate open field experiment conducted in drug-naïve rats. *Left:* Mean (±SEM) beam breaks during the FAD1 seeking test shown in (B) (n = 7 rats/group). *Middle:* Mean (±SEM) beam breaks during the FAD40-50 seeking test shown in (D) (n = 14 for vehicle, n = 12 for SCH39166; two operant boxes lacked functional photobeams). *Right:* Mean (±SEM) total distance traveled in meters (m) during a 1-h open field test conducted after intra-NAcc injection of vehicle or SCH39166 in drug-naïve rats (n = 6 total; 3 rats received both vehicle and SCH39166 in a counter-balanced design with one day between tests, 2 rats received only vehicle, and 1 rat received only SCH39166). SCH39166 significantly reduced beam breaks given prior to the FAD1 test (*p<0.05 vs vehicle), but not when given before the FAD40-50 test and had no effect on open field locomotion (Table 7). Dots show data for individual rats. FAD, forced abstinence day.
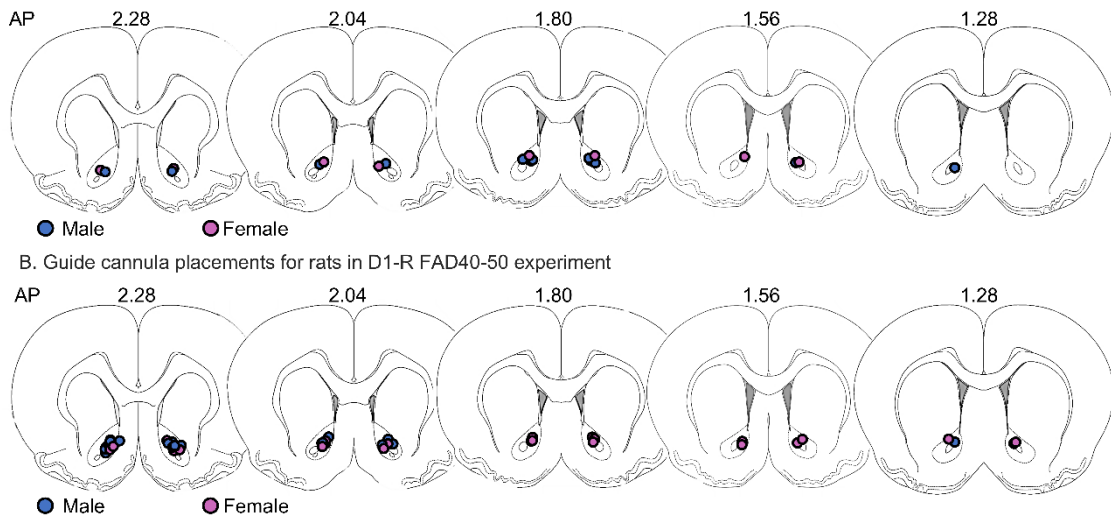

B. Guide cannula placements for rats in D1-R FAD40-50 experiment

Figure 8. Cannula placements for experiments shown in Fig. 7. **A**. Cannula placements for FAD1 D1R antagonist experiment shown in Fig. 7B. Placement of injector tips was determined using Cresyl violet counterstaining of formalin-fixed tissue. Dots show injector tip for each cannula (bilateral) for each rat, and color indicates sex of the rat. Sections adapted from Paxinos & Watson 7th edition. **B**. Cannula placements for FAD40-50 D1R antagonist experiment shown in Fig. 7D. Placement of injector tips was determined as described for (A). AP, anterior posterior; FAD, forced abstinence day.

Table 7. Statistical output for D1R antagonist experiments in Fig. 7

Expt phase	Measure	Fixed effects	F-value	P-value	Significant?	Figure
Cocaine Seeking FAD1	Nose pokes over 1-h (n=6 SCH39166, n=7 vehicle)	Treatment x Port				7B, middle
		Treatment	$F_{1,11}=0.1495$	0.7064	n.s.	
		Port	$F_{1,11}=24.54$	0.0004	***	
		Port x Treatment	$F_{1,11}=0.001385$	0.9710	n.s.	
Cocaine Seeking FAD1	Nose pokes in 20-m bins (n=6 SCH39166, 7 vehicle)	Treatment x Bin				7B, right
		Treatment	$F_{1,12}=0.1543$	0.7014	n.s.	
		Bin	$F_{2,24}=5.21$	0.0132	*	
		Bin x Treatment	$F_{2,24}=0.03536$	0.9653	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=13 SCH39166, 15 vehicle)	Treatment x Port				7D, middle
		Treatment	$F_{1,26}=14.13$	0.0009	***	
		Port	$F_{1,26}=115.6$	<0.0001	****	
		Port x Treatment	$F_{1,26}=6.03$	0.0210	*	
		Holm-Šídák††				
		Inactive	$t_{52}=1.182$	0.2425	n.s.	
		Active	$t_{52}=4.44$	<0.0001	***	
Cocaine Seeking FAD40-50	Nose pokes in 20-m bins (n=13 SCH39166, 15 vehicle)	Treatment x Bin				7D, right
		Treatment	$F_{1,26}=16.7$	0.0124	**	
		Bin	$F_{2,52}=4.79$	0.000377	**	
		Bin x Treatment	$F_{2,52}=1.090$	0.3438	n.s.	
Cocaine Seeking FAD1	Beambreaks over 1-h (n=6 SCH39166, 7 vehicle)	Unpaired t test				7E, left
		Treatment	$t_{10}=2.43$	0.0352	*	
Cocaine Seeking FAD40-50	Beambreaks over 1-h (n=13 SCH39166, 15 vehicle)	Unpaired t test				7E, middle
		Treatment	$t_{24}=0.6012$	0.5533	n.s.	
Open Field	Distance traveled (n=7M)	Unpaired t test				7E, right
		Treatment	$t_7=0.3153$	0.7617	n.s.	

††adjusted P-values reported for post hoc comparisons
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
Analysis shown for combined female (F) and male (M) data.

left). For rats assigned to the FAD40-50 test group, vehicle and SCH39166 groups were also balanced based on results of the FAD1 seeking test (Fig. 9A).

Infusion of SCH39166 (1 µg/0.5 µL) in the nucleus accumbens core (placements shown in Fig. 8) did not affect cocaine seeking on FAD1-2 compared to vehicle-infused rats (Fig. 7B right; mixed effects analysis showed a significant effect of port, but no effect of treatment or treatment x port interaction; Table 7). When we binned the 1-h test into three 20-min bins, a mixed effects analysis showed a significant effect of bin ($F_{2,24}$=5.210, p=0.0132) but not treatment and no significant treatment x bin interaction (Table 7). In contrast, when the D1R antagonist was infused prior to a FAD40-50 test, it significantly suppressed incubated cocaine seeking (Fig. 7D middle; treatment x port: $F_{1,26}$=6.03, p=0.0210; see Table 7 for full model) specifically on the active port (t_{52}=4.44, p<0.0001). Analysis of binned data from FAD40-50 (Fig. 7D right) revealed a significant effect of treatment ($F_{1,26}$=16.7, p=0.000377) and bin ($F_{2,52}$=4.79, p=0.0124), but no interaction ($F_{2,54}$=1.09, p=0.344). These data suggest that, even though DA release is unchanged from FAD1-2 to FAD40-50, the D1R-mediated effects triggered by DA release have more impact on cocaine seeking during the late abstinence test.

We analyzed the FAD40-50 data in Fig. 7D to test for an effect of sex via a mixed effects model with fixed effects of treatment, sex, and port. We did not perform this analysis on FAD1-2 data due to low group sizes and lack of effect in the full data-set. Our analysis of FAD40-50 revealed significant main effects of treatment ($F_{1,22}$=14.5, p=0.000975) and port ($F_{1,22}$=105, p<0.0001), as expected from analysis described above, but did not reveal a significant main effect of sex ($F_{1,22}$=0.0214, p=0.885). We also analyzed these data split by sex, using a mixed effects analysis with fixed effects of treatment and port for each sex (Fig. 9B, Table 8). For FAD40-50, both males and females showed a significant main effect
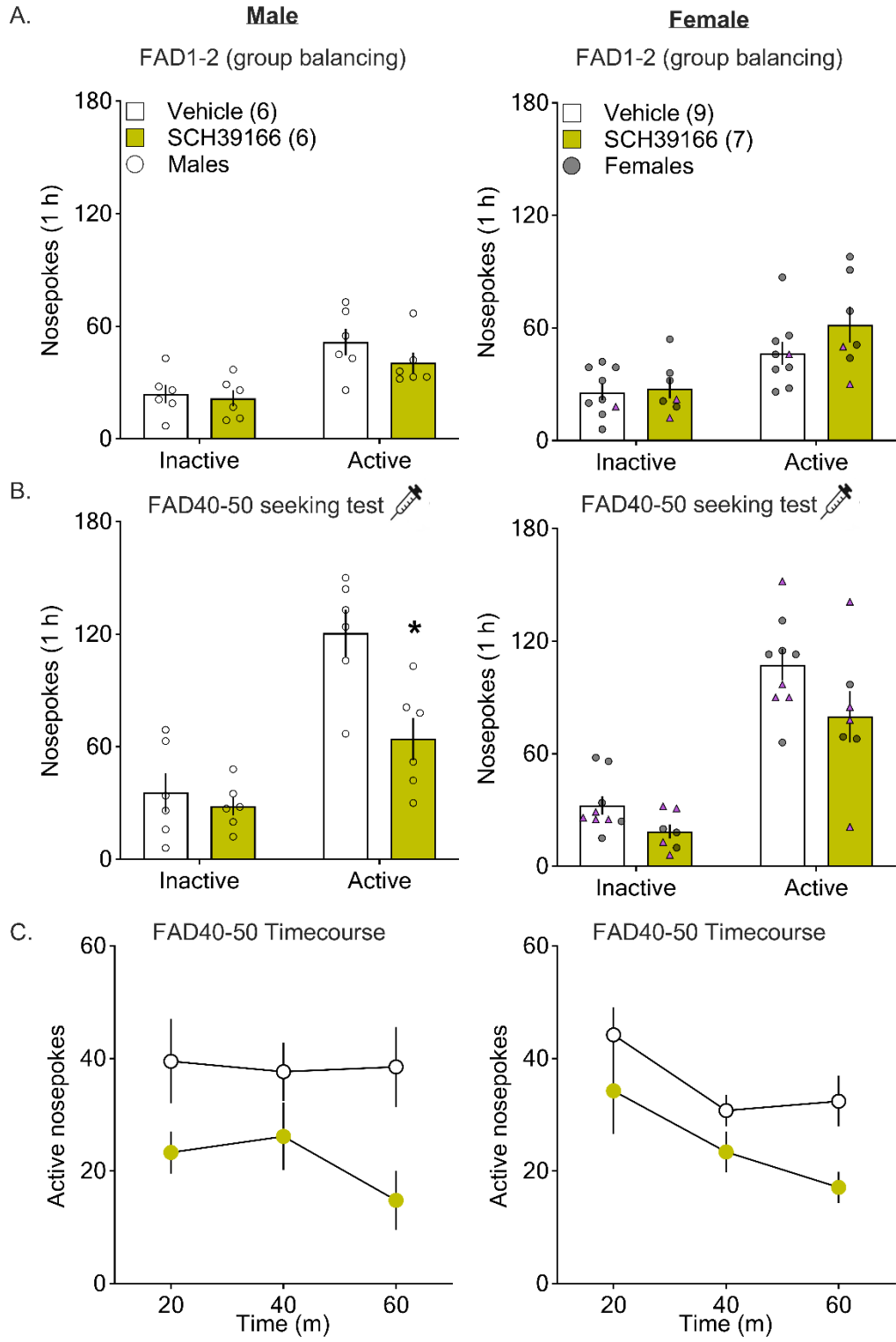
A.

Male

FAD1-2 (group balancing)

Female

FAD1-2 (group balancing)

B.

FAD40-50 seeking test

FAD40-50 seeking test

C.

FAD40-50 Timecourse

FAD40-50 Timecourse

Figure 9. Behavioral data for the D1R antagonist experiment shown in Fig. 7 split by sex. Male data are shown in the left column and female data in the right column. **A**. Mean (±SEM) for active port responses on FAD1-2 (no microinjection) used to balance groups (in combination with infusion data shown in Fig. 7D *Left*) prior to the intracranial injection and seeking test. **B.** Mean (±SEM) pokes for the 1-h FAD40-50 cocaine seeking for rats receiving intra-nucleus accumbens core vehicle or SCH39166 prior to the test split by sex (n = 6 male/9 female for vehicle, n = 6 male/7 female for SCH39166). **C.** Mean (±SEM) pokes for FAD40-50 seeking test data split into 3 20-minute bins. Dots show data for individual rats. Purple triangles indicate female rats that were in estrus at the time of the seeking test (FAD1-2: n = 2 SCH39166, n = 1 vehicle; FAD40-50: n = 4 SCH39166, n = 4 vehicle). FAD, forced abstinence day.

Table 8. Statistical output for D1R antagonist experiments related to Fig. 9

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Figure
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=13 SCH39166, 15 vehicle)	Sex x Treatment x Port				Data from 7D middle, re-analyzed with sex as a factor
		Sex	$F_{1,22}=0.02136$	0.8851	n.s.	
		Treatment	$F_{1,22}=14.46$	0.0010	***	
		Port	$F_{1,22}=104.8$	<0.0001	****	
		Sex x Treatment	$F_{1,22}=0.2906$	0.5952	n.s.	
		Sex x Port	$F_{1,22}=0.5476$	0.4671	n.s.	
		Treatment x Port	$F_{1,22}=6.766$	0.0163	*	
		Sex x Treatment x Port	$F_{1,22}=1.549$	0.2264	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=6M SCH39166, 6M vehicle)	Treatment x Port				9B, left
		Treatment	$F_{1,10}=8.515$	0.0154	*	
		Port	$F_{1,10}=41.41$	<0.0001	****	
		Port x Treatment	$F_{1,10}=6.791$	0.0262	*	
		Holm-Šídák††				
		Inactive	$t_{20}=0.5092$	0.6162	n.s.	
		Active	$t_{20}=3.912$	0.0017	**	
Cocaine Seeking FAD40-50	Nose pokes in 20-m bins (n=6M SCH39166, 6M vehicle)	Treatment x Bin				9C, left
		Treatment	$F_{1,10}=10.16$	0.0097	**	
		Bin	$F_{2,20}=0.5330$	0.5949	n.s.	
		Bin x Treatment	$F_{2,20}=0.5980$	0.5595	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=7F SCH39166, 9F vehicle)	Treatment x Port				9A, right
		Treatment	$F_{1,14}=5.391$	0.0358	*	
		Port	$F_{1,14}=79.50$	<0.0001	****	
		Port x Treatment	$F_{1,14}=0.8050$	0.3848	n.s.	
Cocaine Seeking FAD40-50	Nose pokes in 20-m bins (n=7F SCH39166, 9F vehicle)	Treatment x Bin				9C, right
		Treatment	$F_{1,14}=6.067$	0.0273	*	
		Bin	$F_{2,28}=6.850$	0.0038	**	
		Bin x Treatment	$F_{2,28}=0.4669$	0.6317	n.s.	

††adjusted P-values reported for post hoc comparisons
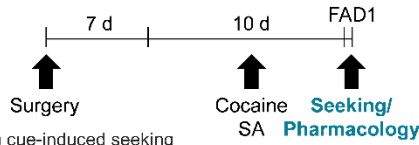*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. F, female; M, male

of treatment (male: $F_{1,10}=8.52$, $p=0.0154$; female: $F_{1,14}=5.39$, $p=0.0358$), but only males showed a significant port x treatment interaction (male: $F_{1,10}=6.79$, $p=0.0262$; female: $F_{1,14}=0.805$, $p=0.385$). Post hoc tests revealed that SCH39166 significantly reduced active port responding on FAD40-50 in males ($t_{20}=3.91$, $p=0.00173$), whereas this effect trended in females but did not achieve significance ($t_{28}=2.34$, $p=0.0517$). Overall, there is no effect of sex in the aggregated data-set. However, when disaggregated by sex these results may suggest the possibilities of sex differences in the effect of D1R blockade on incubated cocaine seeking that could be pursued in future studies.

Because DA antagonists could reduce drug seeking via a general reduction in motor behavior, we monitored locomotor activity during seeking tests by recording infrared beam-breaks in operant chambers. SCH39166 significantly reduced beam-breaks on FAD1-2 (Fig. 7E left; $t_{10}=2.43$, p=0.0352) but did not affect beam-breaks in the FAD40-50 test (Fig. 7E middle; $t_{24}=0.601$, p=0.553). We also tested the effect of intra-nucleus accumbens core vehicle or SCH39166 (same dose) on open field behavior in drug-naïve rats and found no effect of treatment (Fig. 7E right; $t_7=0.315$, p=0.761). These results, combined with no significant effect of SCH39166 on inactive nose-pokes (Fig. 7D middle), suggest that the observed effect of D1R antagonism on FAD40-50 drug seeking is not due to a general reduction in motor behavior.

Intra-nucleus accumbens core administration of a D2 antagonist decreases expression of cocaine incubation

We performed a parallel experiment using the highly specific D2R antagonist L-741,626 and its vehicle (Fig. 10). Intra-nucleus accumbens core infusion of L-741,626 (1 µg/0.5 µL; placements shown in Fig. 11) did not affect cocaine seeking when given prior to the FAD1-2 test (Fig. 10B middle; significant effect of port, but no significant effect of

A. Timeline for FAD1 D2-R antagonism
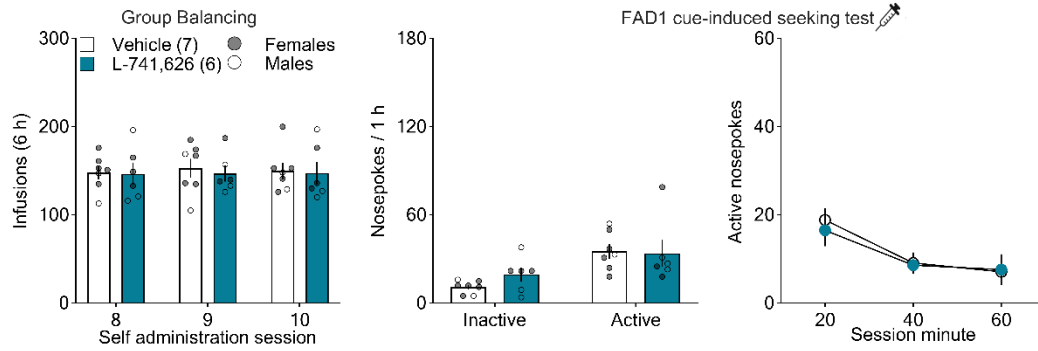
7 d | 10 d | FAD1

Surgery | Cocaine SA | Seeking/ Pharmacology

B. D2-R blockade during baseline (FAD1) cue-induced seeking

Group Balancing

Vehicle (7) — Females
L-741,626 (6) — Males

FAD1 cue-induced seeking test

C. Timeline for FAD40-50 D2-R antagonism

7 d | 10 d | FAD1 | 40-50 d | FAD40-50

Surgery | Cocaine SA | Seeking | Forced Abstinence | Seeking/ Pharmacology

D. D2-R blockade during incubated (FAD40-50) cue-induced seeking

Group Balancing

Vehicle (11) — Females
L-741,626 (12) — Males

FAD40-50 cue-induced seeking test

E. D2-R blockade and locomotion

FAD1

Vehicle (7) — Females
L-741,626 (6) — Males

FAD40-50

Vehicle (10)
L-741,626 (12)

Drug naïve rats - open field

Females (2)
Males (3)

65
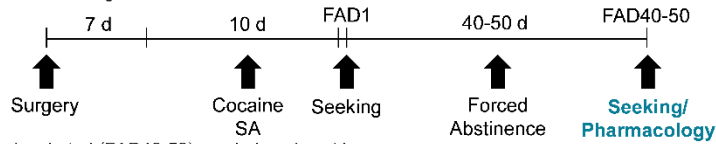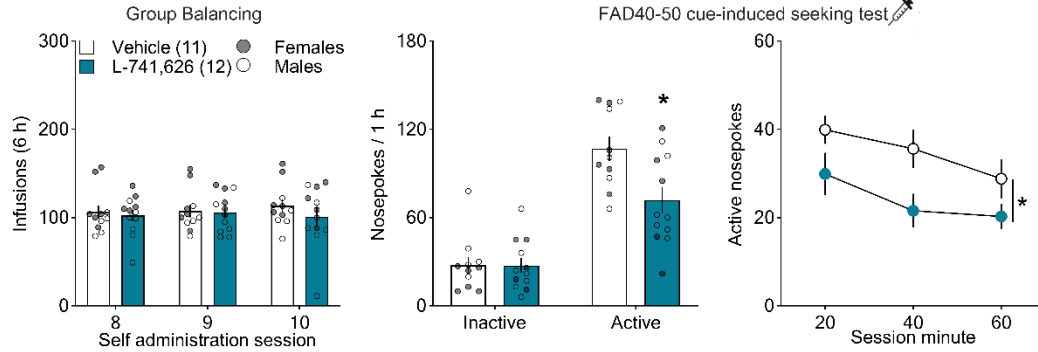
Figure 10. Intra-NAcc infusion of the D2R antagonist L-741,626 reduces cue-induced seeking on FAD40-50 but not FAD1. **A**. Timeline for FAD1 D2R antagonist experiment (teal denotes stages that are the focus of panel B). **B**. Behavioral data for FAD1 D2R antagonist experiment. *Left:* Groups destined for vehicle or L-741,626 infusion on FAD1 were balanced based on mean (±SEM) cocaine infusions for the last three 6-h sessions of self-admi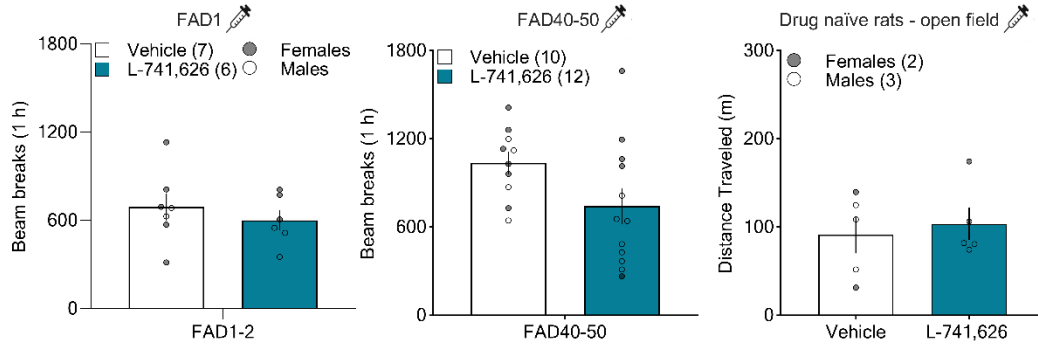nistration training. *Middle:* Mean (±SEM) pokes for the 1-h FAD1 cue-induced seeking test for rats receiving intra-NAcc vehicle (n = 7) or L-741,626 (n = 6). Dots indicate individual rats (open circles, males; closed circles, females). *Right:* Mean (±SEM) active pokes for FAD1 seeking test data split into three 20-min bins. **C**. Timeline for FAD40-50 D2R antagonist experiment (teal denotes stages that are the focus of panel D). **D**. Behavioral data for FAD40-50 D2R antagonist experiment. *Left:* Groups destined for vehicle or L-741,626 infusion on FAD40-50 were balanced based on mean (±SEM) cocaine infusions for the last three 6-h sessions of self-administration training (shown here) as well as FAD1 seeking data (Fig. 12A). *Middle:* Mean (±SEM) pokes for the 1-h FAD40-50 cue-induced seeking test for rats receiving intra-NAcc vehicle (n = 11) or L-741,626 (n = 12) (*p<0.05 vs vehicle; Table 9). *Right:* Mean (±SEM) active pokes for FAD40-50 seeking test data split into three 20-min bins (*p<0.05 main effect of treatment, L-741,626 versus vehicle; Table 9). **E**. Operant box photobeam breaks during seeking tests and a separate open field experiment conducted in drug-naïve rats. *Left:* Mean (±SEM) beam breaks during the FAD1 seeking test shown in (B) (n = 7 for vehicle, n = 6 for L-741,626). *Middle:* Mean (±SEM) beam breaks during the FAD40-50 seeking test shown in (D) (n = 10 vehicle, n = 12 L-741,626; two operant boxes lacked functional photobeams). *Right:* Mean (±SEM) total distance traveled in meters (m) during a 1-h open field test conducted after intra-NAcc injection of vehicle or L-741,626 in drug- naïve rats (n = 5 total, all rats received both vehicle and L-741,626 in a counter-balanced design with one day between tests). FAD, forced abstinence day.

A. Guide cannula placements for rats in D2-R FAD1 experiment

AP   2.28   2.04   1.80   1.56   1.28

○ Male   ○ Female

B. Guide cannula placements for rats in D2-R FAD40-50 experiment

AP   2.28   2.04   1.80   1.56   1.28

○ Male   ○ Female

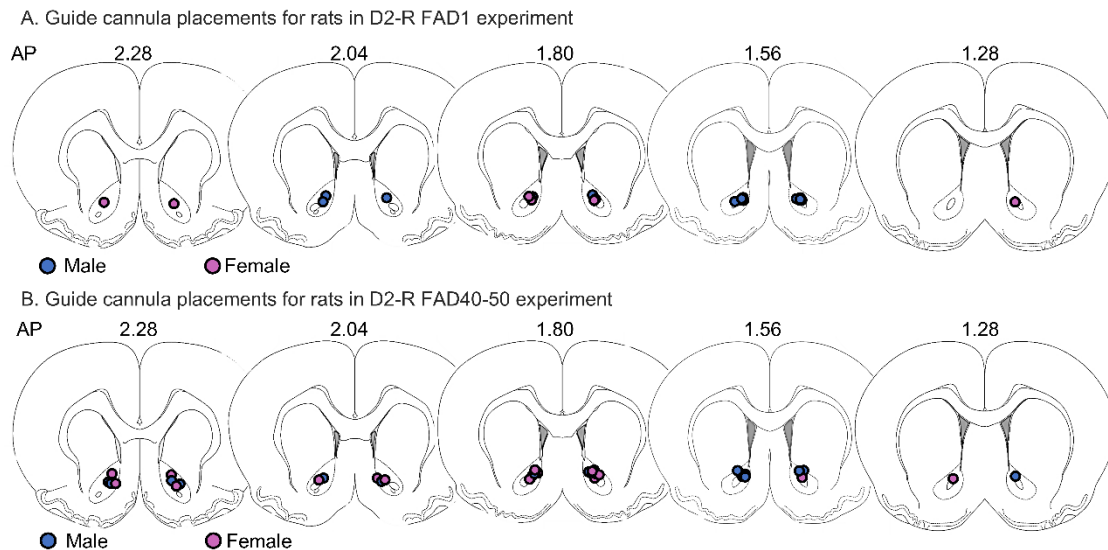**Figure 11**. Cannula placements for experiments shown in Fig. 10. **A**. Cannula placements for FAD1 D2R antagonist experiments. Placement of injector tips was determined using Cresyl violet counterstaining of formalin-fixed tissue. Dots show injector tip for each cannula (bilateral) for each rat, and color indicates sex of the rat. Sections adapted from Paxinos & Watson 7th edition. **B**. Cannula placements for FAD40-50 D2R

antagonist experiments. Placement of injector tips was determined as described for (A). AP, anterior posterior; FAD, forced abstinence day.

Table 9. Statistical output for D2R antagonist experiments in Fig. 10

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Figure
Cocaine Seeking FAD1	Nose pokes over 1-h (n=6 L-741,626, 7 vehicle)	Treatment x Port[†] Treatment Port Port x Treatment	 $F_{1,22}=0.4189$ $F_{1,22}=12.17$ $F_{1,22}=0.8257$	 0.5242 0.0021 0.3734	 n.s. ** n.s.	10B, middle
Cocaine Seeking FAD1	Nose pokes in 20-m bins (n=6 L-741,626, 7 vehicle)	Treatment x Bin Treatment Bin Bin x Treatment	 $F_{1,13}=0.07718$ $F_{2,26}=13.31$ $F_{2,26}=0.2231$	 0.7855 0.0001 0.8015	 n.s. *** n.s.	10B, right
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=12 L-741,626, 11 vehicle)	Treatment x Port Treatment Port Port x Treatment Holm-Šídák[††] Inactive Active	 $F_{1,21}=5.217$ $F_{1,21}=89.72$ $F_{1,21}=7.06$ $t_{42}=0.03070$ $t_{42}=3.46$	 0.0329 <0.0001 0.0148 0.9757 0.00251	 * **** * n.s. **	10D, middle
Cocaine Seeking FAD40-50	Nose pokes in 20-m bins (n=12 L-741,626, 11 vehicle)	Treatment x Bin Treatment Bin Bin x Treatment	 $F_{1,21}=6.32$ $F_{2,42}=5.41$ $F_{2,42}=0.4008$	 0.0202 0.00811 0.6723	 * ** n.s.	10D, right
Cocaine Seeking FAD1	Beambreaks over 1-h (n=6 L-741,626, 7 vehicle)	Unpaired t test Treatment	 $t_{11}=0.7417$	 0.4738	 n.s.	10E, left
Cocaine Seeking FAD40-50	Beambreaks over 1-h (n=12 L-741,626, 11 vehicle)	Unpaired t test Treatment	 $t_{20}=1.950$	 0.0654	 n.s.	10E, middle
Open Field	Distance traveled (n= 2F, 3M)	Unpaired t test Treatment	 $t_{4}=0.5496$	 0.6118	 n.s.	10E, right

Analysis shown for combined female (F) and male (M) data.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

treatment or treatment x port interaction; Table 9). There was also no effect of treatment when we split the 1-h FAD1-2 seeking test into 20-min bins (Fig. 10B right; $F_{1,13}=0.0772$, $p=0.786$; Table 9). However, similar to our D1R antagonism experiment, we observed a significant suppression of incubated cocaine seeking when L-741,626 was given prior to the FAD40-50 test (Fig. 10D right; treatment x port interaction: $F_{1,21}=7.06$, $p=0.0148$; Table 9). Post hoc analysis confirmed that L-741,626 specifically reduced active port responding ($t_{42}=3.46$, p=0.00251). We performed the same analysis after binning the data and found a main effect of treatment ($F_{1,21}=6.32$, $p=0.0202$) and bin ($F_{2,42}=5.41$, $p=0.00811$), but no interaction ($F_{2,42}=0.401$, $p=0.672$). Overall, these data indicate that D2R blockade significantly reduced incubated cocaine seeking but did not influence FAD1-2 seeking.

We analyzed the FAD40-50 data set shown in Fig. 10D via a mixed effect model with factors of test day, port, and sex to investigate possible sex differences. FAD1-2 data was not tested for sex effects due to low group sizes and lack of collective effect. We did not observe a main effect of sex ($F_{1,19}=0.101$, $p=0.754$). However, when spilt by sex (Fig. 12B), the male rats showed a significant effect of treatment ($F_{1,10}=5.36$, $p=0.0432$) while the females did not ($F_{1,9}=0.839$, $p=0.384$). Males failed to show a significant interaction between port and treatment ($F_{1,10}=1.26$, $p=0.289$). Full statistical output is shown in Table 10. Overall, as discussed for D1R antagonism, this suggests that the same level of DA release is having a more pronounced effect via D2Rs on FAD40-50 compared to FAD1-2 with no significant effect of sex. Disaggregated data hint at a potential sex differences in D2R antagonism effects, but we lack the sample size to make firm conclusions.

To test for effects of the D2R antagonist on locomotion, we analyzed beam breaks in the operant chamber during seeking tests and found no significant difference between the L-741,626 and vehicle groups on FAD1-2 ($t_{11}=0.742$, $p=0.474$) or FAD40-50 ($t_{20}=1.95$,

p=0.0654) (Fig. 10E left and middle). To further establish that this dose of L-741,626 does not affect motor behavior, we tested a separate cohort of drug-naïve rats for open field locomotion and found no effect of treatment (t_4=0.550, *p*=0.612) (Fig. 10E right). These data, along with no effect of L-741,626 on inactive nose-pokes (Fig. 10D middle), indicate that the observed effect of D2R antagonism on incubated cocaine seeking is not due to motor impairment.

Discussion

DA has long been recognized as critical for the ability of cues to elicit motivated behavior (see Introduction). This work adds to that body of literature by showing that DA in the nucleus accumbens core contributes to expression of incubated cocaine craving. Specifically, during cue-induced seeking tests performed during forced abstinence from cocaine self-administration, we used fiber photometry to demonstrate DA transients time-locked to nose-pokes that triggered presentation of the cue previously paired with cocaine infusions. The magnitude of this DA response did not differ significantly on FAD1-2 and FAD40-50, suggesting that cue-elicited DA release is unchanged over the course of cocaine incubation. To test the functional significance of this DA release, we performed intra-nucleus accumbens core injections of the D1R antagonist SCH39166 prior to FAD1-2 or FAD40-50 seeking tests. When given on FAD1-2, we found no effect of D1R antagonism on active port responses. However, when SCH39166 was given on FAD40-50, when cocaine seeking has incubated, we found a significant suppression of active port responses. We performed a parallel study with L-741,626, a specific D2R antagonist, and similarly found suppression of FAD40-50 cocaine seeking with no effect on FAD1-2. Given that the magnitude of DA release is similar during both tests, these results suggest a
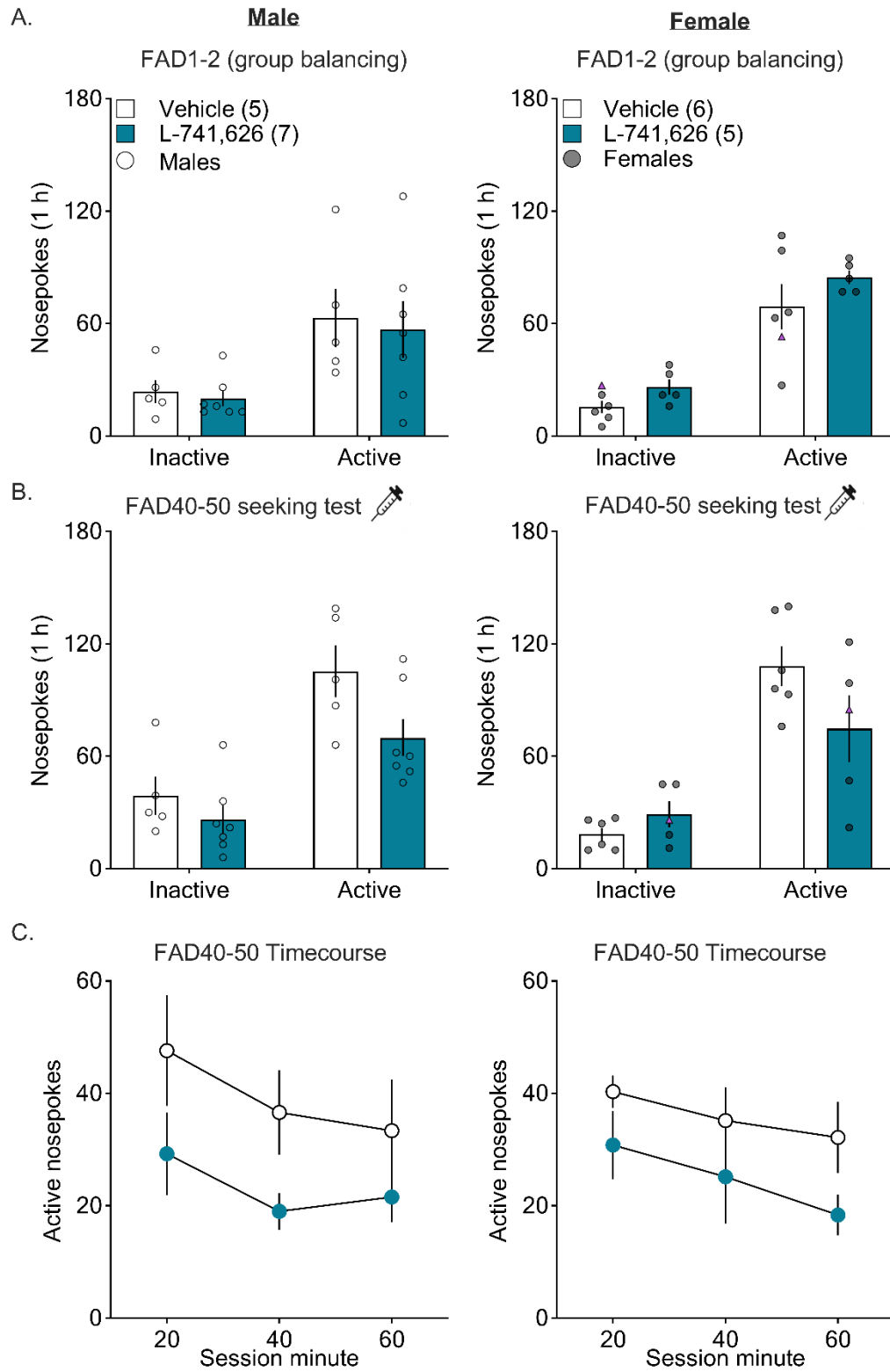
A.

Male

FAD1-2 (group balancing)

Female

FAD1-2 (group balancing)

B.

FAD40-50 seeking test

FAD40-50 seeking test

C.

FAD40-50 Timecourse

FAD40-50 Timecourse

Figure 12. Behavioral data for D2R antagonist experiment shown in Fig. 10 split by sex. Male data are shown in the left column and female data in the right column. **A.** Mean (±SEM) for active port responses on FAD1-2 (no microinjection) used to balance groups (in combination with infusion data from Fig. 10D *Left*) prior to the intracranial injection and seeking test. **B.** Mean (±SEM) pokes for the 1-h FAD40-50 seeking test for rats receiving intra-nucleus accumbens core vehicle or L-741,626 prior to the seeking test spilt by sex (n = 5 males/6 females for vehicle, n = 7 males/5 females for L-741,626). **C.** Mean (±SEM) pokes for FAD40-50 seeking test data spilt into 3 20-min bins. Dots show data for individual rats. Purple triangles indicate female rats that were in estrus at the time of the seeking test (FAD1-2: n = 0 L-741,626, n = 1 vehicle; FAD40-50: n = 1 L-741,626, n = 0 vehicle). FAD, forced abstinence day.

Table 10. Statistical output for D2-R antagonist experiments related to Fig. 12

Expt phase	Measure	Fixed effects in model	F-value	P-value	Significant?	Figure
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=12 L-741,626, 11 vehicle)	Sex x Treatment x Port				Data from 10D middle, re-analyzed with sex as a factor
		Sex	$F_{1,19}$=0.1012	0.7538	n.s.	
		Treatment	$F_{1,19}$=4.859	0.0400	*	
		Port	$F_{1,19}$=84.85	<0.0001	****	
		Sex x Treatment	$F_{1,19}$=0.6303	0.4370	n.s.	
		Sex x Port	$F_{1,19}$=0.9264	0.3479	n.s.	
		Treatment x Port	$F_{1,19}$=6.289	0.0214	*	
		Sex x Treatment x Port	$F_{1,19}$=0.6325	0.4363	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n=7M L-741,626, 5M vehicle)	Treatment x Port				12B, left
		Treatment	$F_{1,10}$=5.358	0.0432	*	
		Port	$F_{1,10}$=29.15	0.0003	***	
		Port x Treatment	$F_{1,10}$=1.256	0.2885	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 20-m (n=7M L-741,626, 5M vehicle)	Treatment x Bin				12C, left
		Treatment	$F_{1,10}$=4.048	0.0719	n.s.	
		Bin	$F_{2,20}$=3.154	0.0645	n.s.	
		Bin x Treatment	$F_{2,20}$=0.2563	0.7764	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 1-h (n= 5F L-741,626, 6F vehicle)	Treatment x Port				12B, right
		Treatment	$F_{1,9}$=0.8392	0.3835	n.s.	
		Port	$F_{1,9}$=65.09	<0.0001	****	
		Port x Treatment	$F_{1,9}$=6.860	0.0278	*	
		Holm-Šídák††				
		Inactive	t_{18}=0.7124	0.4853	n.s.	
		Active	t_{18}=2.229	0.0762	n.s.	
Cocaine Seeking FAD40-50	Nose pokes over 20-m (n= 5F L-741,626, 6F vehicle)	Treatment x Bin				12C, right
		Treatment	$F_{1,9}$=2.804	0.1284	n.s.	
		Bin	$F_{2,18}$=3095	0.0699	n.s.	
		Bin x Treatment	$F_{2,18}$=0.1587	0.8545	n.s.	

†Random effects with SD = 0 excluded from model. ††adjusted P-values reported for post hoc comparisons. *p<0.05, ***p<0.001, ****p<0.0001. F, female; M, male

postsynaptic change in nucleus accumbens core MSNs that underlies this changing role of DA with incubation.

Methodological Considerations

There are several methodological issues that should be acknowledged. Regarding the photometry studies, we cannot rule out a difference in DA release when comparing FAD1-2 to FAD40-50 that it is too small for our sensor to detect. We selected GRAB_DA2m for these studies over the higher affinity GRAB_DA2h due to its faster off kinetics (Sun, Zhou et al. 2020) and its use in similar studies (Pribiag, Shin et al. 2021, Luján, Oliver et al. 2023). Another potential concern is buffering of DA by the sensor. We found no difference in cocaine self-administration and incubation between rats expressing GRAB_DA2m and a mutant sensor that does not bind DA. This argues against substantial buffering, although it is possible that DA buffering is still occurring but that some form of compensation has occurred. Finally, we observed significant individual variability in DA responses among the 17 rats evaluated during cocaine seeking. This variability could have may origins, including individual differences [e.g., (Deroche-Gamonet, Belin et al. 2004)], the area of nucleus accumbens core targeted, or experimental parameters that influence signal detection, but we lack the robust sample size necessary to explore this.

In regard to pharmacology studies, the major concern is specificity of the chosen drugs for their receptors, as lack of selectivity could lead to off-target effects. We selected SCH39166 based on its high affinity for the D1R relative to other DA and 5-HT receptors and L-741,626 due to its 50-fold higher affinity for the D2R than the D3R (see Methods for details). Results of a prior study argue against a contribution of D3-Rs to our observed effects with L-741,626 (see section below titled *D1 and D2 receptors contribute to incubated cocaine craving).* Overall, our study was designed to maximize selectivity, but

we cannot completely rule out the possibility of interactions with other receptors. Another concern is that DA receptor antagonism can lead to suppression of motor activity, which could reduce responding during our seeking tests. To avoid this, we selected a SCH39166 dose that reduced methamphetamine seeking without eliciting motor deficits as measured by operant responding for sucrose/maltodextrin (Rossi, Reverte et al. 2020). Consistent with these prior results, we found no significant reduction of locomotion during seeking tests or open field activity. For L-741,626, we likewise found no evidence of non-specific reduction of motor activity, consistent with another study that administered various doses of this drug into the nucleus accumbens core and reported no effects on locomotion (Papp, Gruca et al. 2017). An additional potential concern is a 'floor effect' in FAD1-2 pharmacology studies, although this concern is lessened by our use of a 1-h seeking test during which significant responding occurs even on FAD1-2. Our lab has successfully conducted such studies in the past (Werner, Stefanik et al. 2018, Christian, Stefanik et al. 2021).

The magnitude of cue-induced DA release is not altered over incubation

When measuring DA release during cue-induced cocaine seeking tests, and time-locking to the seeking response (active pokes that trigger the cue), we found that the average magnitude of DA release was the same on FAD1-2 and FAD40-50. This suggests that incubated seeking is not related to an increase in DA release in the nucleus accumbens core on FAD40-50. While it is possible that DA plays an equivalent role in pre- and post-incubation responding, the lack of effect of DA-R antagonism on FAD1-2 seeking paired with the suppression of incubated cocaine seeking when DA-R antagonists are administered on FAD40-50 suggests there may be postsynaptic adaptations such that the same amount of DA release evokes a greater response. It has long been known that

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adaptations in DA receptor expression and post-receptor signaling occur after cocaine exposure including cocaine self-administration (White and Wolf 1991, Anderson and Pierce 2005, Volkow, Fowler et al. 2009). We previously examined surface expression of DA-Rs in the nucleus accumbens core after the same cocaine self-administration regimen used here and found no change in D1Rs and small decreases in D2Rs during cocaine incubation (Conrad, Ford et al. 2010), so a change in signaling downstream of these receptors seems more likely to account for our results.

However, the role of changes in DA release in cue responding may depend on the design of the study. For example, a recent study measured DA release in response to non-contingent presentation of a cue previously paired with cocaine SA and found greater DA release after 30 days of forced abstinence (Burgeno, Farero et al. 2023). The difference likely reflects the fact that this study measured the DA response to the cue in isolation whereas we measured the DA response to the seeking action paired with the cue. Furthermore, this study did not demonstrate a causal relationship between greater DA release during the probe test and expression of incubated cocaine seeking, but rather confirmed the relationship between DA release and approach behavior. Another recent study measured DA release in the lateral nucleus accumbens shell during cocaine seeking in mice and found an increase in the frequency of DA transients measured on FAD24 compared to pre-SA baseline (Pribiag, Shin et al. 2021). We did not observe a change in frequency, perhaps because our recordings were performed in the core subregion or because we compared FAD40-50 to FAD1. Finally, in slice voltammetry studies performed in the nucleus accumbens core after 4 weeks of abstinence from cocaine SA regimens leading to incubation of craving, electrically stimulated DA release did not differ

significantly from DA release in cocaine naïve rats although cocaine was more potent at DA uptake inhibition (Alonso et al 2022).

Recent theories emphasize the significance of DA release not only for learning about cues and rewards but also for encoding motivational vigor and saliency (Hamid, Pettibone et al. 2016, Mohebi, Pettibone et al. 2019, Kutlu, Zachry et al. 2021, Jeong, Taylor et al. 2022). Our data are generally consistent with these findings although our behavioral task is not designed to isolate these separate facets of DA-related behavior.

D1 and D2 receptors contribute to incubated cocaine craving

Our DA antagonist studies revealed that blocking either the D1R or D2R similarly attenuated incubated cocaine seeking. This may seem inconsistent with the canonical view of oppositional effects of D1R expressing MSN (D1-MSN) and D2R expressing MSN (D2-MSN) [e.g., (Hikida, Kimura et al. 2010, Lobo, Covington et al. 2010)]. Two things are important to keep in mind. First, our pharmacology experiments were focused at the level of D1R and D2R, not D1-MSN and D2-MSN. Second, even at the level of MSN, recent models support cooperative interactions between D1-MSN and D2-MSNs in regulating motivated behavior (Burke, Rotstein et al. 2017, Bariselli, Fobbs et al. 2019, Allichon, Ortiz et al. 2021). We also acknowledge that DA receptors are present not only on MSN (where they act as neuromodulators to influence excitability, glutamate transmission, and intracellular signaling) but also on presynaptic glutamate and GABA terminals, and on interneurons, indicating a multiplicity of potential interactive regulatory mechanisms (Tritsch and Sabatini 2012).

Focusing on cocaine incubation, our study is the first to examine roles for D1R or D2R in the nucleus accumbens. However, there is evidence that activation of both

receptors in the nucleus accumbens core but not shell is required for the expression of incubation of methamphetamine craving (Rossi, Reverte et al. 2020). Another study found that activation of both D1R and D2R in the nucleus accumbens core shell is necessary and sufficient for drug-primed reinstatement of cocaine seeking (Schmidt, Anderson et al. 2006, Schmidt and Pierce 2006). Overall, these results support cooperative actions of DA signaling at D1R and D2R in cocaine seeking. Consistent with these findings at the level of the nucleus accumbens, functional inhibition of ventral tegmental area DA neurons reduces cocaine reinstatement (Mahler, Brodnik et al. 2019) and cocaine seeking after brief abstinence (Solecki, Wilczkowski et al. 2020).

While we focused on D1R and D2R as these are the most widely expressed DA receptors in the nucleus accumbens core, some nucleus accumbens MSN also express the D3R (Le Moine and Bloch 1996). Indeed, one study found a role for nucleus accumbens D3 receptors (D3R) in context-induced cocaine seeking during forced abstinence but effects of D3R antagonists were independent of abstinence duration (Xi, Li et al. 2013). Based on this study, it is unlikely that D3Rs contribute to the effects of the D2R antagonist L-741,626 on cocaine seeking in our study, as our effects were specific to incubated cue-induced cocaine seeking. However, although we acknowledge that there can be differences in mechanisms of cue- and context-induced seeking (Fuchs, Lasseter et al. 2008).

Consideration of sex differences

We found no main effect of sex or any significant interactions across any of our experiments, similar to studies of AMPAR plasticity from our lab using this regimen (Kawa, Hwang et al. 2022). However, sex differences have been found in many aspects of human addiction (Kosten, Gawin et al. 1993, Carroll and Smethells 2015, McHugh, Votaw et al.

2018). Some sex differences have been recapitulated in rodent models, with female rats acquiring cocaine SA faster than males (Hu, Crombag et al. 2004) and showing greater reinstatement (Fuchs, Evans et al. 2005). For cocaine incubation there is no difference between males and females in non-estrus phases of the estrous cycle but females in the estrus phase show significantly increased seeking (Kerstetter, Aguilar et al. 2008, Nicolas, Russell et al. 2019, Corbett, Dunn et al. 2021). This potentiated seeking could be due to the sex hormone estradiol modulating DA-R signaling (Yoest, Cummings et al. 2014). Additionally, female mice in estrus show increased phasic activity of ventral tegmental area DA neurons measured using single-unit recordings (Calipari, Juarez et al. 2017). We can't rule out small differences in DA release, not detectable with our methods, related to the estrous cycle. It should be noted that incubation studies showing estrus effects used between-subject designs. A study using a within-subject design found no differences in incubation of cocaine craving between males and females or between females in estrus and non-estrus phases of the cycle (Alonso, O'Connor et al. 2022). We point out that our sample sizes were likely too low to adequately test for sex differences and this is certainly the case for estrous cycle effects. In addition, we cannot rule out the possibility that differences in pharmacokinetics between males and females contributed to our results (Soldin and Mattison 2009, de Vries and Forger 2015). Finally, there is evidence for sex differences in D2R regulation of striatal dopamine dynamics (Walker, Ray et al. 2006).

Integration with other incubation mechanisms in the nucleus accumbens

This study demonstrates a role for DA signaling at D1R and D2R in the nucleus accumbens core in incubated cocaine seeking, while our previous work has demonstrated that incubation depends on strengthening of glutamate transmission in the nucleus accumbens core via synaptic insertion of high conductance Ca^{2+}-permeable AMPA

receptors (CP-AMPARs) (Conrad, Tseng et al. 2008, Loweth, Scheyer et al. 2014, Wolf 2016, Kawa, Hwang et al. 2022). CP-AMPAR upregulation in the nucleus accumbens shell is also required for the incubation of cocaine craving (Wright and Dong 2020). This dual necessity of DA and glutamate suggests these neurotransmitters may be interacting to support incubated cocaine seeking. There is precedent for such an interaction. For example, the reinforcing properties of the basolateral amygdala to nucleus accumbens glutamate pathway are gated by D1Rs in the nucleus accumbens (Stuber, Sparta et al. 2011). More generally, DA has been shown to alter how MSNs respond to glutamate signaling, although this relationship is complex and not fully understood (Nicola, Surmeier et al. 2000, Tritsch and Sabatini 2012, Sippy and Tritsch 2023). Studies in cultured nucleus accumbens neurons suggest interactions between DA and glutamatergic plasticity; for example, D1R activation primes AMPAR for synaptic insertion (Sun, Milovanovic et al. 2008). Another possibility is related to the fact that MSNs *in vivo* have bi-stable resting potentials, existing in a hyperpolarized 'down state' and a depolarized 'up state' from which they can fire action potentials (O'Donnell 2003). DA may be acting through D1R to facilitate transition to the 'up state' (Tseng, Snyder-Keller et al. 2007, Flores-Barrera, Vizcarra-Chacón et al. 2011). Alternatively, DA may indirectly increase MSN activity by reducing inhibitory tone. Activation of D2R on the D2-MSN, which collateralize on D1-MSNs, reduces GABA release from the D2-MSN resulting in a disinhibition of D1-MSN. This mechanism contributes to cocaine-induced locomotor activity (Dobbs, Kaplan et al. 2016). Furthermore, we must consider DA actions on D2R present on cholinergic interneurons, which have been shown to modulate MSN firing when inhibited via optogenetics (Witten, Lin et al. 2010).

Conclusions

Cue-induced cocaine seeking is accompanied by a similar magnitude of nucleus accumbens core DA release in early and late abstinence, suggesting that 'incubated' seeking does not reflect enhancement of DA release (i.e., a presynaptic effect). Instead, the suppression of incubated seeking (FAD40-50) but not FAD1 seeking) by D1R or D2R antagonists suggests that postsynaptic changes occur that enable the same amount of DA release to drive stronger behavioral responding. To investigate this possibility, future studies will need to measure the response in the postsynaptic MSN. One possible approach is to record MSN activation using a calcium sensor while simultaneously recording from a DA biosensor. This would provide insight into how DA and glutamate interact postsynaptically to set the gain on cue reactivity during cocaine abstinence. In Chapter 2, we validate this dual sensor approach in a model of food seeking.

CHAPTER 2

Dopamine dynamics and calcium signaling in the nucleus accumbens core during food seeking

Contributors to the version of this chapter that is being prepared for submission: Sophia J. Weber and Marina E. Wolf developed the experiments and wrote the manuscript. Sophia J. Weber, Gillian S. Driscoll, Madelyn M. Beutler, Haley M. Kuhn, and Jonathan G. Westlake conducted the experiments.

Abstract

Extinction-reinstatement paradigms have been used to study reward seeking for both food and drug rewards. The nucleus accumbens is of particular interest in reinstatement due to its ability to energize motivated behavior. Indeed, previous work has demonstrated that suppression of neuronal activity or dopaminergic signaling in the nucleus accumbens reduces reinstatement to food seeking. In this study, we sought to further establish a connection between glutamatergic input, measured by proxy via a genetically encoded calcium indicator, and dopamine tone, measured simultaneously with a red-shifted DA biosensor. We performed this sensor multiplexing in the nucleus accumbens core in the classic extinction-reinstatement paradigm with food reward. We detected DA transients that changed in magnitude or temporally shifted over the course of self-administration training. In our calcium traces we observed a decrease from baseline time-locked to the lever press for food reward, which became more prominent with training. Both patterns were reduced in the first session of extinction with no deflections from baseline detected in either the DA or calcium traces in the last extinction session. When we recorded during reinstatement tests, bootstrapping analysis detected a calcium response when reinstatement was primed by cue or pellet+cue presentation, while a DA response was detected for pellet+cue reinstatement. These data further establish a role for nucleus accumbens core activity and DA in reinstatement of food seeking and represent the first attempt to simultaneously record the two.

Introduction

Reward-associated stimuli help guide motivated behavior, and these learned associations between an environmental cue and rewarded outcome are generally adaptive. However, the ability of reward-associated cues to elicit behavior can become problematic when these rewards also result in negative consequences, as with addictive drugs. Reinstatement of reward seeking has been studied for many decades to interrogate drivers of maladaptive reward seeking behavior, particularly as an analog for relapse in substance use disorder (Shaham, Shalev et al. 2003, Bossert, Marchant et al. 2013, Knackstedt and Kalivas 2009). However, this model has also been used with palatable food reward instead of drug reward and similar behavioral and mechanistic results have been reported (Kelley, Schiltz et al. 2005, Calu, Chen et al. 2014). This model is unique in that it allows for the examination of neurobiological circuits of reward learning across multiple phases: acquisition, extinction, and reinstatement. By examining brain regions across multiple states, we can advance our understanding of the circuitry that underlies reward seeking.

The nucleus accumbens is an important locus for goal-directed behavior, integrating information from cortical, limbic, and midbrain inputs and sending output to motor regions (Floresco 2015). Furthermore, the nucleus accumbens has emerged as a particularly important region in food seeking and consummatory behavior (Kelley 2004). It has long been known that neurons in the nucleus accumbens exhibit a pause in firing during consummatory behavior (Nicola, Yun et al. 2004, Roitman, Wheeler et al. 2005), with more recent single unit recording studies replicating this pause and going further to show that electrical stimulation of the nucleus accumbens inhibits licking behavior (Krause, German et al. 2010). There is speculation that this pause may be primarily in

dopamine receptor 2 (D2R) expressing medium spiny neurons (MSNs) (Frazier and Mrejeru 2010). However, consumption is different from instrumental action; in fact, previous studies recording nucleus accumbens neurons found an increase in a subset of MSN firing prior to lever pressing for food or water reward (Carelli, Ijames et al. 2000). This activity is causally relevant, as another study found that pharmacological inactivation of the nucleus accumbens core, but not shell, impaired responding on a food reinstatement task (Floresco, McLaughlin et al. 2008). In addition to the activity of the nucleus accumbens MSNs, dopamine (DA) transmission in the nucleus accumbens is necessary for reward seeking in food tasks, despite not being necessary for the hedonic value of reward (Berridge and Robinson 1998). A more recent study expanded upon this, providing evidence for DA actions at D1 dopamine receptors (D1Rs) in the nucleus accumbens core in food reinstatement (Guy, Choi et al. 2011). These findings suggest that DA and glutamatergic input into the nucleus accumbens core may converge to facilitate food reinstatement, which is supported by evidence for these systems working together in other circumstances (Nicola, Surmeier et al. 2000, Moyer, Wolf et al. 2007, Sun, Milovanovic et al. 2008, Yagishita, Hayashi-Takagi et al. 2014).

We sought to more directly interrogate both glutamate and DA signaling during a food extinction-reinstatement paradigm. To do so, we employed fiber photometry with sensor multiplexing to simultaneously record nucleus accumbens core DA release and MSN calcium levels, a proxy for glutamatergic activation, during all phases of a food extinction-reinstatement task. We found that DA release increased during the learning or self-administration phase, and that eventually this peak shifted earlier in time to precede the lever press. This DA signal then declined with extinction and re-emerged with reinstatement (based on bootstrapping analysis). In contrast, MSN calcium levels

decreased from baseline after the lever press during self-administration. This decrease disappeared after the first day of extinction. Reinstatement was associated with an increase in MSN calcium. These results are the first to examine both MSN activity via calcium proxy and DA release during an extinction-reinstatement task. Furthermore, this study paves the way for future multiplexing studies of addictive drugs, such as those conducted in Chapter 1, perhaps helping us better understand how DA acts on the postsynaptic cell during incubation of craving.

Methods

Subjects

We used male and female Long-Evans rats obtained from Charles River (Wilmington, MA). They were ~9 weeks old upon arrival and were group housed for ~1 week after arrival to acclimate prior to surgery. After surgery, all rats were single housed. During recovery, rats had free access to standard laboratory chow and water in their home cages and were maintained on a reverse 12:12h light/dark cycle (lights off at 10 AM prior to surgery and 9-9:30 AM post-surgery; variance reflects a switch in housing rooms after surgery). One week prior to starting behavioral training, rats began food restriction and were maintained at 85% of their free-feeding weight, although water was freely accessible in the home cage throughout the experiment. Weights were recorded throughout food restriction. All procedures were approved by the OHSU Animal Care and Use Committee and followed NIH guidelines outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). From a total of 8 male and 7 female rats assigned to experimental groups, 4 were excluded after histology due to misplaced intracranial cannulas.

Intracranial virus infusion and fiber optic cannula implantation: We performed bilateral (n=8, 4 male/4 female; for these rats, one hemisphere was selected for recording) or unilateral (n=3, 2 male/1 female) intracranial infusions of viral cocktail of GCaMP8s, a genetically encoded calcium indicator (AAV9-syn-jGCaMP8s-WPRE, Addgene #162374), and a red-shifted dopamine biosensor, rGRAB_DA3m (AAV2/9-hsyn-rDA3m, Biohippo #BHV12400545), into the nucleus accumbens core followed by implantation of fiber optic cannula (Thor Labs, CFM15L10) above each infusion site. We performed unilateral surgeries on a subset of rats due to lack of supplies after shipping was delayed due to an ice storm. We infused 500nL of a working stock consisting of 3 μL GCaMP8s, 3 μL rGRAB_DA3m, and 3 μL sterile saline. Briefly, rats were mounted in a stereotaxic device and, after making an incision, the nose bar was adjusted such that the change in dorsal-ventral coordinates from Lambda to Bregma was <0.1 mm. In each hemisphere (or in one hemisphere for unilateral animals), we performed two infusions (250 nL each) of virus, to obtain better dorsal/ventral spread), using the following stereotaxic coordinates (relative to Bregma): AP +1.3; ML ±2.4 mm; DV -7.2 mm for first infusion and DV -7.0 mm for the second infusion, 6° angle (Paxinos and Watson 2014). We infused virus at 100 nL/min and left the injector in place for 5 min following each infusion. Once the two infusions were completed, we raised the needle by 0.1 mm and waited 2 additional min prior to removal. Following the virus infusion, we implanted fiber optic cannula (AP +1.3 mm; ML ±2.4 mm; DV -6.8 mm; 6° angle). We anchored the cannula to the skull with 1/8" pan head sheet metal screws (Fastenere #842176107226) and dental cement (Stoelting #51458) and covered the fiber optic cannula with dust caps (Thor Labs, CAPF) for protection.

Post-operative Procedures: Rats received 5 mg/kg subcutaneous meloxicam (Covetrus, 6451602845, SKU #49755) as a post-operative analgesic. Rats then recovered for 7 days prior to beginning food restriction (see Fig. 13A for timeline).

<u>Operant chambers</u>

We trained and tested rats in Med-Associates (Fairfax, VT) operant chambers (#ENV-008-VPX) enclosed in sound attenuating chambers. Each operant chamber was equipped with a non-retractable lever that served as the inactive lever (#ENV-110M) to the left of the magazine (ENV-200R2M-6.0). The magazine contained a photobeam array (ENV-254-CB) to detect head entries and was connected via polyethylene tubing (Everbilt, HKP001-PVC012) to a pellet dispenser (ENV-203M-45) outside the operant chamber. To the right of the magazine was a retractable lever (ENV-112CM) with a LED stimulus light (ENV-221M) above it that served as the active lever. We used retractable levers to give us more experimental control over behavioral responding and force a single isolated response from the rat. This prevents multiple temporally proximal responses, which make photometry data analysis more challenging.

<u>Behavioral Procedures</u>

Magazine training: We trained rats to retrieve food from a magazine in the operant chamber for 3 days for 40 trials (~20 min/day). The start of training was indicated by the onset of white noise. After a variable amount of time (ITI average = 35 sec) two highly palatable food pellets (LabDiet 5TUL) were delivered into the magazine paired with a 4-sec light cue. The inactive lever was extended throughout, but responses were not recorded during this phase. The active lever was never extended during magazine training. During magazine training, water was provided ad libitum in the operant chamber.

On the last day of magazine training, we habituated the rats to the fiber optic cable. We cleaned the cannula for each rat with electronic grade 99.9% anhydrous isopropyl alcohol (MG Chemicals, #824-1L), and then placed the rat into the operant chamber. They were connected to a fiber optic patch cable (Thor labs Custom: FP-400URT, 0.5NA, 0.4m length, FT023SS tubing with a 2.5mm stainless steel ferrule) via a ceramic connector (Thor Labs, ADAF1-5) and the cable was passed through the top of the box and attached to a steel arm with a counterweight (Med Associates, PHM-110-SAI). We recorded during this last session to prescreen rats. Prescreening was conducted to verify that a DA and GCaMP signal was detected and identify the hemisphere (for bilaterally implanted rats) with the best signal prior to advancing to behavior. For unilaterally implanted rats, only rats that had detectable signal comparable to the selected hemisphere from bilaterally implanted rats proceeded in the study.

Food self-administration (SA): We trained rats to self-administer food pellets for four sessions (SA1-SA4, ~55 min/day for 4 days) with each session containing 75 trials. Like magazine training, the start of the session was indicated by the onset of white noise. Following this, the start of a trial was indicated by the extension of the active lever into the operant chamber. The lever was kept extended for 20 sec or until the rat pressed. If the rat responded on the active lever, it immediately retracted and resulted in the delivery of 2 pellets and start of a 4-sec light cue above the lever. If a rat failed to respond within 20 sec the lever was retracted and there was no consequence. The inter-trial time was variable with an average of 25 sec (minimum 20 sec, maximum 30 sec). Both active lever presses and magazine entries were recorded as our measure of food SA acquisition. Responses on the inactive lever were recorded but had no consequence. During SA

training, water was provided ad libitum in the operant chamber. Photometry measures were made throughout all sessions as detailed below under _Fiber photometry recordings._

Extinction training: Extinction training (Ext) began the day after SA4 and was identical to food SA, except that active lever presses no longer resulted in pellet delivery or cue light presentation. Six sessions were performed (Ext1-6).

Reinstatement tests: All rats received two reinstatement tests after 6 days of extinction training. The first test (cue-primed reinstatement; performed on the day after Ext6) began with the onset of white noise and a 4-sec cue light presentation. Subsequently, the trial began with lever extension; during the trial, active lever presses resulted in cue light illumination but no pellet delivery. On the next day, rats underwent a pellet+cue-primed reinstatement test. We included this to see if reward+cue prime would produce a more robust effect. In Chapter 1, we did not perform analogous drug-primed seeking tests (only cue-induced seeking tests) as cocaine-primed cocaine-seeking does not incubate (Lu, Grimm et al. 2004). This test was identical to the first reinstatement test except that the initial priming event at the start of the session entailed delivery of a pellet plus presentation of the light cue (pellet+cue reinstatement).

Behavioral analysis: Both active and inactive lever presses on the last day of SA (SA4), last day of extinction (Ext6), cue reinstatement, and pellet+cue reinstatement were compared via mixed effects analysis performed in GraphPad Prism (version 10.2.2) with an assumption of sphericity (equal variability of differences). To test for an effect of session on lever pressing, we set fixed effects of session (SA4, Ext6, Cue, Pellet+cue) and lever (active or inactive), with a random effect of subject. We made simple effect comparisons comparing active and inactive responding across sessions separately and corrected for multiple comparisons using a Holm-Šídák correction. Family-wise alpha threshold for

confidence was set to 0.05. Full model output can be found in tables 11-14. Sex differences was not analyzed as this study only contained 11 total subjects and therefore was not powered to detect sex effects.

Fiber photometry recordings

Recording parameters: Prior to all recordings, fiber optic cables were bleached at 200 mA for 8 h overnight. On the morning of the recording day, the LED power level and DC were adjusted so that the 488 nm LED, 405 nm LED, and 560 nm LED all had a power output of 40 µW at the end of the fiber optic patch cable as measured using a Thor labs power meter (PM100D+). For recordings, we passed excitation wavelengths (488 nm,405 nm, and 560 nm) from the TDT RZ10x system via TDT patch cables (200 µm core, 2 m length, 0.5 NA). These TDT cables then connected to a Doric Minicube (FMC6) which in turn was coupled to two Thor fiber optic patch cables (FP400URT-Custom: FP400URT, FT0.5SS tubing, 4 m length coupled to a smaller 0.4 m patch cable via a TDT cable coupler) and then connected to the rat as described in *Magazine training.* Emissions (555-570 nm,460-490 nm, and 580-680 nm) were received via the same patch cable and then decoupled at the Minicube and returned to the RZ10x via TDT response cables (600 µm, 2 m length, 0.5 NA). We acquired these emissions via the RZ10x photodetectors, digitized at 6 kHz, and recorded in the TDT Synapse Software (version 95-44132P) on WS4 at frequency 330 Hz for 465 nm, 210 Hz for 405 nm, and 450 Hz for 560 nm. All recordings had a 6 Hz lowpass filter and a 9.5 V clip threshold.

Recording during behavior: For recordings during behavior, each rat was removed from the home cage, weighed, and had their fiber optic cannula cleaned. We connected the fiber optic patch cable via a ceramic connector as described in *Recording parameters*. The patch cable was passed through the operant chamber on a counterweight system as

described in *Magazine training*. Rats were placed into the operant chamber and the photometry recording began. The Med Associates program (to record nose-pokes and infusions) was started 3 s later to avoid light artifact obscuring any start of session signals. The photometry recording was continuous for the entire session. During each session, rats were also recorded via a Logitech webcam (#C615) and checked every 10 min for possible disconnection or tangling of the fiber optic cable. If an intervention was required, it was done as quickly and unobtrusively as possible and the time of the intervention during the test session was noted. After the session ended, the photometry recording was halted and the rat was returned to the home cage.

Data analysis: Fiber photometry data were analyzed using the analysis suite GuPPy created by the Lerner Lab (Northwestern University), which can be downloaded on their [GitHub page](). The details of the data manipulations used to generate the GCaMP8s and rGRAB_DA3m signal traces are described in (Sherathiya, Schaid et al. 2021). In brief, for each recording session, we applied least-squares linear fit to align the same 405 nm isosbestic to both DA and calcium signal channels and used fluctuations in the isosbestic channel to account for florescence changes in the signal channels that are not a result of ligand binding. Any disconnects during the recording, defined by a rapid substantial shift (>10 mV) in average mV, were snipped prior to fitting the traces and any nose pokes within those snips were excluded from further analysis. Then we calculated a change in fluorescence measure ($\Delta F/F$=Signal-Fitted Control/Fitted Control) and applied z-score normalization to control for between-session and between-rat differences in virus expression or recording. Finally, we averaged normalized traces across behavioral event replicates for each rat, and then averaged by session.

For DA and calcium transients associated with lever presses, first we extracted z-scored ΔF/F traces within a 15-sec window around each active lever press and averaged all traces to generate an average response for the rat during each test. We identified significant DA and calcium transients using continuous threshold bootstrapping methods. Bootstrapping is a statistical method in which multiple stimulated data sets are generated from an original data set to enable hypothesis testing and calculation of confidence intervals. When applied to fiber photometry data we consider each timepoint in the trace to be a data set, with each rat contributing 1 data point from their individual averaged trace. We then generate 1000 replications of this data set by resampling with replacement from our original 11 data points (one for each rat). This generates a normal distribution of values for each timepoint, which we can then use to generate confidence intervals. This enables us to say with 95% confidence what the z-score would be at that timepoint. This is used as a method to detect likely transients (i.e., z-score ≠ 0) in the entire 6-sec time window analyzed. However, rapid non-physiological changes in fluorescence can occur, for example due to a cable hitting a wall; to account for these non-physiological events, we set a consecutive threshold where the 95% confidence interval cannot not contain 0 (baseline) for a consecutive number of samples set in the code parameters. We selected a consecutive threshold of 1000 for our 6 kHz acquisition, which equates to 0.167 s, based on recommendation from the code developer given our sampling rate. Bootstrapping is thought to provide an unbiased way to identify relevant transients in photometry without experimenter selection of a time window and can be considered a data driven approach. Using this analysis, we identified instances in our recorded traces where the z-score is 95% likely to not be at baseline and marked these epochs with horizontal bars above the traces. In addition to this analysis, we performed permutation tests on the bootstrapped distributions. This is a statistical method in which we compare the mean of the simulated

data for each timepoint and assess if it is significantly different from the mean of another bootstrapped sample distribution (i.e., timepoint 0-sec for SA1 vs timepoint 0-sec for SA4).

In tandem with this analysis, we also performed the classical hypothesis driven approach in which we selected time windows before and after our behavioral event of interest and calculated the Area Under the Curve (AUC) for the z-scored ΔF/F trace within this time window. Using these AUC values, we performed two-tailed paired t-tests comparing SA1 vs SA4, Ext01 vs Ext06, and Ext06 vs both reinstatement tests. We assumed a Gaussian distribution for all t-tests and set our confidence level to 95%.

Histology

Upon completion of experiments, rats were euthanized via lethal injection of Fatal Plus (Covetrus, #35946) diluted to 80 mg/kg with sterile saline. Once animals no longer displayed reflexive motor responses, they were perfused transcardially first with 1x phosphate buffer saline (PBS) followed by 4% formaldehyde/1% methanol in 1x PBS, pH ~7, at a rate of ~100 mL/s over 5 min. Brains were extracted and allowed to rest in the formaldehyde solution for up to 24 h. Brains were transferred to 1x PBS with 0.01% sodium azide and then sliced on a vibratome (Leica VT1000s; frequency 8, speed 70, blade DORCO plat. 5T300) at 60 μm. Slices were kept in a 24 well plate in 1x PBS with 0.01% sodium azide at 4°C until the start of immunohistochemistry.

Immunohistochemistry: We performed immunohistochemistry (IHC) on tissue from photometry experiments to amplify the GFP and mApple signals prior to imaging to confirm virus expression and placement. We began IHC with three 30-min washes, first in 1x PBS (diluted from 10x PBS, Quality Biological, 119-069-151) and then twice in 1x PBS with 0.5% (v/v) Triton-X100 (Electron Microscopy Sciences, #22140). After this we

permeabilized the tissue for 2 h in 1x PBS with 0.5% (v/v) Triton-X100, 20% (v/v) DMSO (Sigma Aldrich, #276855), and 2% (w/v) Glycine (Sigma Aldrich, #G8898) at room temperature (RT) on a rocking shaker. Next, we blocked tissue in 1x PBS with 0.5% (v/v) Triton-X100, 10% (v/v) DMSO, and 6% (v/v) Normal Donkey Serum (NDS, Jackson Immuno Research, 017-200-121) for 2 h at RT on a rocking shaker. After blocking we incubated the tissue overnight at RT in 1:1000 Anti-GFP (Aves, GFP-1010) and 1:1000 Anti-DsRed2 (Santa Cruz Biotech, SC-101526) in 1x PBS with 0.5% (v/v) Tween-20 and 0.01% (w/v) Heparin (Sigma Aldrich, #H3393-100KU) with 3% NDS and 10% DMSO on a rotator. The following day, we washed the tissue 3 times for 30 min in 1x PBS with 0.5% (v/v) Tween-20 (Thermo Scientific, #J20605-AP) and 0.01% (w/v) Heparin before incubating in 1:250 Anti-chicken-488 (Jackson Immuno Research, 775-546-155) and Anti-mouse-647 (Jackson Immuno Research, 715-606-150) in 1x PBS with 0.5% (v/v) Tween-20 and 0.01% (w/v) Heparin with 3% NDS overnight at RT on a rotator. The third day, we performed two 30-min washes in 1x PBS with 0.5% (v/v) Tween-20 and 0.01% (w/v) Heparin before beginning a final wash in 1x PBS (30 min). Slices were kept in 1x PBS with 0.01% sodium azide at 4˚C until being mounted onto Superfrost Plus slides (Fisher Scientific, Cat# 1255015) and coverslipped (Fisher Scientific, 12541026) using Vectashield Vibrance with DAPI (Vector Labs, H-1800-10).

Imaging: Images were acquired using a Leica DMi8 inverted microscope equipped with an ORCA-Flash4.0 LT+ Digital CMOS camera (Hamamatsu). LASX Premium Software was used for image acquisition and ImageJ was used for analysis. Exposure was determined using the software to avoid saturation while maximizing the pixel distribution in the histogram. We acquired images at 2.5x magnification with 3 s exposure for DAPI, 1 s exposure for FITC, and 5 s exposure for Cy5.

Results

Two weeks prior to the start of behavioral testing, rats received either bilateral (n = 8) or unilateral infusions (n = 3) of a viral cocktail of GCaMP8s and GRAB_rDA3m into the nucleus accumbens core. In the same surgery, a fiber optic cannula was implanted above the virus injection site. Rats were allowed to recover for 1 week and then experienced 1 week of restricted chow access in the home cage. A representative image of virus co-expression of GCaMP8s and GRAB_DAr3m (showing the cannula track) is provided in Fig. 13B, whereas cannula placements are summarized in Fig. 13C.

We trained rats to lever press for delivery of 2 highly palatable pellets paired with a 4-sec light cue. Rats readily acquired this behavior, with the majority reaching 100% trial completion (75 trials) after 4 days of SA training (Fig. 13D). We then put rats through extinction training in which active lever presses no longer resulted in a food reward or a light cue (Fig. 13D). After 6 sessions of extinction rats had suppressed their responding on the active lever compared to the last day of self-administration (Fig. 13E; t_{59} = 14.77, p< 0.0001). They also reduced responding on the inactive lever, a permanently extended lever associated with no consequence throughout all training and testing (Fig. 13E; t_{59} = 3.779, p = 0.0022). Once lever pressing had been extinguished, we tested the rats for cue-primed reinstatement. During this ~55 min test, lever presses delivered the same 4-sec light cue previously paired with food reward. The session began with a single non-contingent presentation of the light. Rats increased responding for the cue compared to the last day of extinction (Fig. 13E; t_{59} = 2.980, p = 0.0125) with no effect on inactive lever responding. On the next day, we performed a second reinstatement test identical to the first except that the initial light cue also was paired with non-contingent delivery of a pellet, acting as a reward prime. As in the previous reinstatement test, rats also
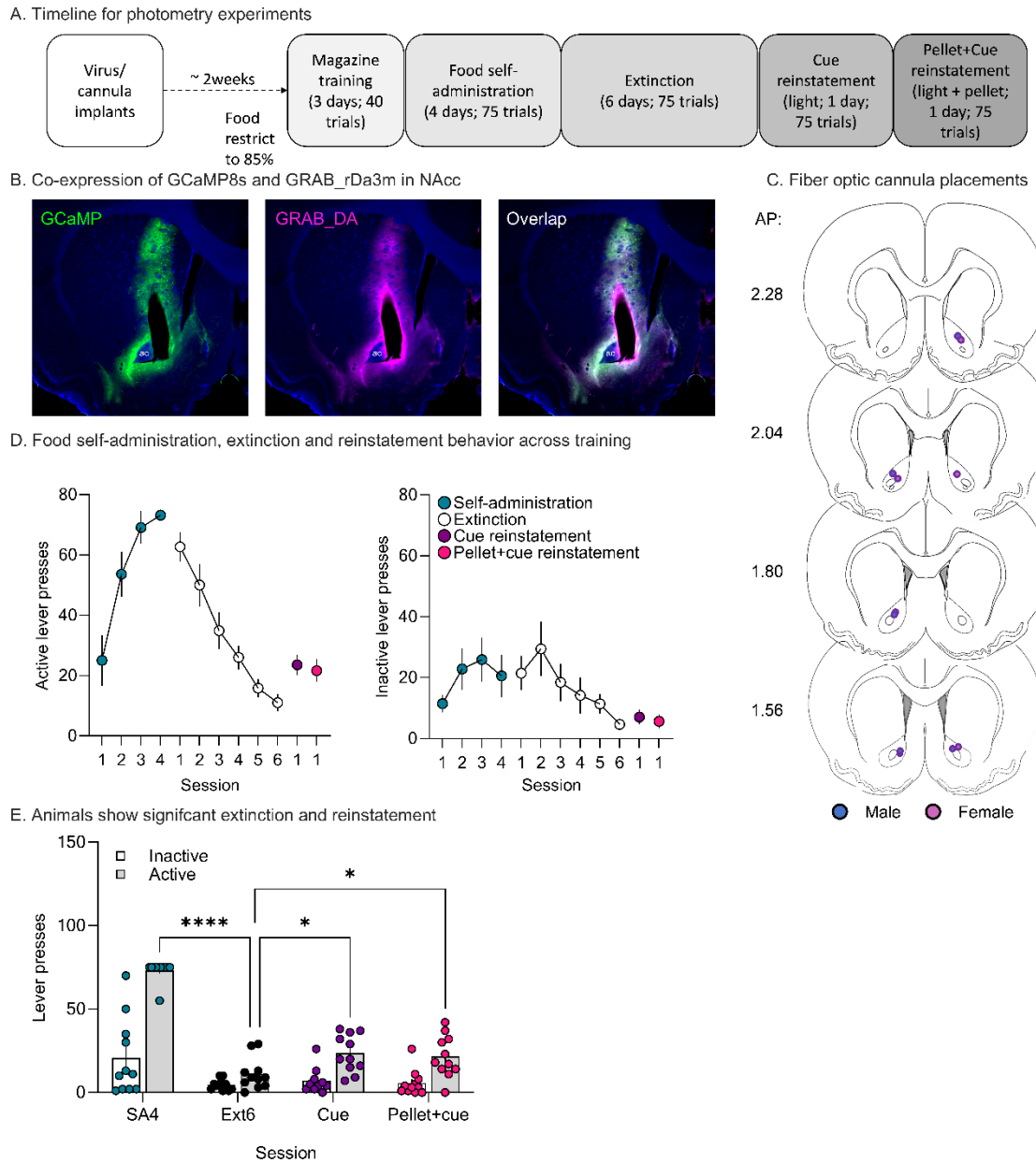
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Figure 13. Behavior and virus expression. **A**. Timeline for experiment. **B**. Representative image of virus co-expression: *left,* AAV9-syn-jGCaMP8s-WPRE0, *middle,* AAV2/9-hsyn-rDA3m, and *right,* Overlap of viral expression. The cannula track appears as an oblong shape terminating to the right of the anterior commissure. **C**. Fiber optic cannula placement for rats. The dot signifies the end of the cannula (females, pink; males, blue). Placement was determined after immunohistochemistry for GFP and mApple to confirm virus expression at the end of the cannula. Sections adapted from Paxinos & Watson 7th edition. **D**. Food self-

administration sessions (SA1-4), extinction sessions (Ext1-6), and reinstatement tests. *Left:* Active lever

presses for all rats (n = 11) across all sessions for all behavioral phases. *Right:* Inactive lever presses for the

same sessions. **E.** Data from D graphed to show direct comparison of SA4, Ext6, Cue-primed reinstatement

and Pellet+Cue-primed reinstatement (***p<0.0001, *p<0.05). Bars indicate mean (±SEM) for each session

while dots represent individual rats. AP, anterior posterior.

Table 11. Statistical output for behavioral data in Fig. 13

Expt phase	Measure	Fixed effects	F-value	P-value	Significant?	Figure
SA4, Ext6, Cue, Pellet+cue	Lever presses (n = 11, 6M/5F)	Session x Lever				1E
		Session	$F_{3,30}=68.67$	<0.0001	****	
		Lever	$F_{1,10}=88.86$	<0.0001	****	
		Session x Lever	$F_{3,29}=23.85$	<0.0001	****	
		Holm-Šídák†				
		Inactive				
		SA4 vs. Ext6	$t_{59}=3.779$	0.0022	**	
		SA4 vs. Cue	$t_{59}=2.995$	0.0159	*	
		SA4 vs. Pellet+cue	$t_{59}=3.542$	0.0039	**	
		Ext6 vs. Cue	$t_{59}=0.6811$	0.8739	n.s.	
		Ext6 vs. Pellet+cue	$t_{59}=0.2376$	0.8805	n.s.	
		Cue vs. Pellet+cue	$t_{59}=0.4500$	0.8805	n.s.	
		Active				
		SA4 vs. Ext6	$t_{59}=14.77$	<0.0001	****	
		SA4 vs. Cue	$t_{59}=11.79$	<0.0001	****	
		SA4 vs. Pellet+cue	$t_{59}=12.24$	<0.0001	****	
		Ext6 vs. Cue	$t_{59}=2.980$	0.0125	*	
		Ext6 vs. Pellet+cue	$t_{59}=2.527$	0.0282	*	
		Cue vs. Pellet+cue	$t_{59}=0.4535$	0.6518	n.s.	

†adjusted P-values reported for post hoc comparisons. M, male, F, female.

exhibited increased responding on active but not inactive levers (Fig. 13E; t_{59} = 2.572, *p* =

0.0282). See Table 11 for the full statistical output.

We simultaneously recorded both MSN calcium transients (Fig. 14) and DA

release (Fig. 15) in the nucleus accumbens core on all days of the regimen. For all traces,

we examined a 15-sec window around the active lever press. GCaMP and DA traces are

plotted to compare the first and last day of food SA (Fig. 14A and 15A), the first and last

day of extinction (Fig. 14B and 15B), the last day of extinction and the cue-primed reinstatement (Fig. 14C and 15C), and the last day of extinction and the pellet+cue-primed reinstatement (Fig. 14D and 15D). Above all the traces are horizontal lines of matching color that indicate periods where the 95% confidence interval does not cross baseline (i.e., z-score = 0) for the consecutive threshold period determined with a continuous threshold bootstrapping method (Jean-Richard-Dit-Bressel, Clifford et al. 2020, Liu, Jean-Richard-Dit-Bressel et al. 2020, Yau and McNally 2022). Time periods during which permutation testing revealed differences between sessions is indicated by a light gray line.

Focusing on comparison of food SA sessions 1 and 4 (SA1 and SA4), analysis of GCaMP traces showed the development of a significant suppression of MSN calcium levels as training progressed (Fig. 14A left). We quantified the area under the curve (AUC) for the time period 0 to 3-sec and compared SA1 to SA4 with a paired t-test to further confirm this decrease (Fig. 14A right, Table 12; t_{10}=5.143, p=0.0004). A different pattern emerged for DA traces. Upon lever press, we observed a small increase in SA1 that appeared to become more robust by SA4; in SA4, this DA peak was followed by a persistent dip below baseline (Fig. 15A left). Despite the apparent increase in magnitude of the initial DA peak, when quantifying via AUC over the same 0-3 s time period used for GCaMP traces, we found a significant decrease (Fig. 15A right; t_{10}=3.183, p=0.0098, Table 12). We suspected this was due to the initial DA transient moving earlier in time to precede the lever press, as suggested by visual inspection of Fig. 15A. We therefore performed a mixed effects analysis on average GRAB_DA peak height in both the -5 to 0 sec bin and the 0 to 3 sec bin (Fig. 16A and B, respectively) and found a significant effect of session in the -5 to 0 SA data ($F_{3,30}$=3.667, p=0.0231) with no effect in the 0 to 3 sec data ($F_{3,30}$=0.7458, p=0.5333). Full model output can be found in Table 13. Furthermore,
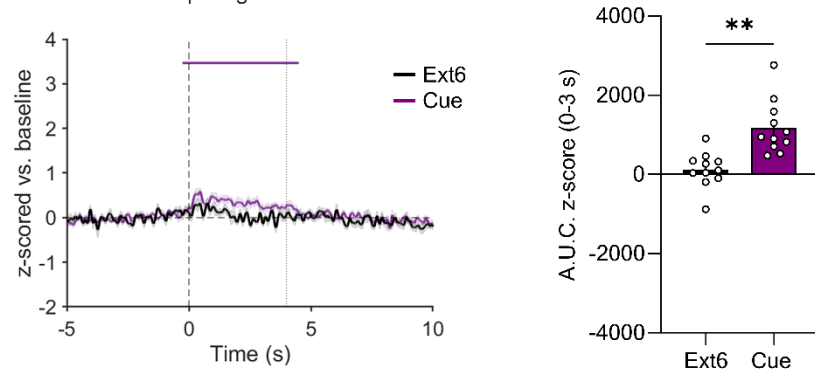
A. GCaMP traces during food self-administration (SA) training
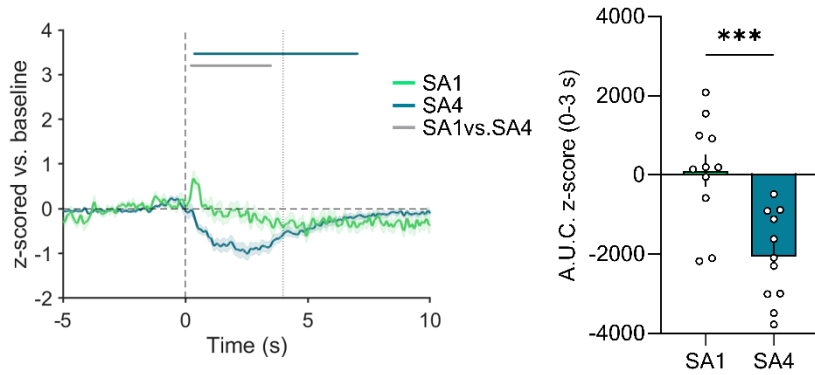
B. GCaMP traces during extinction (Ext) training
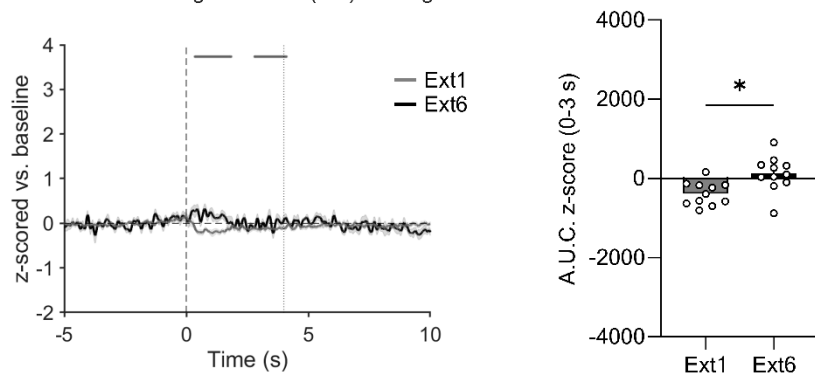
C. GCaMP traces comparing extinction and cue reinstatement

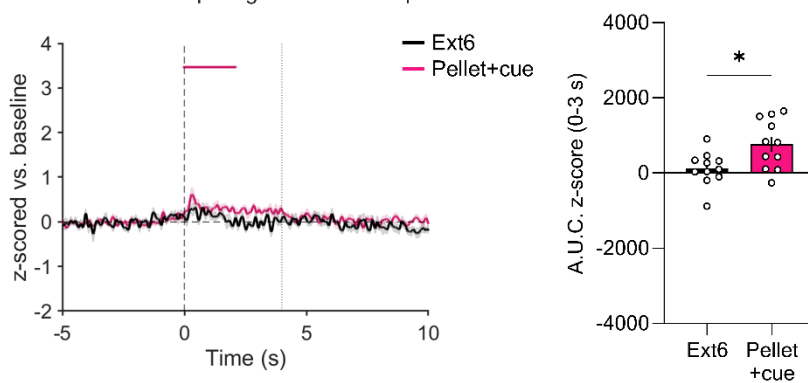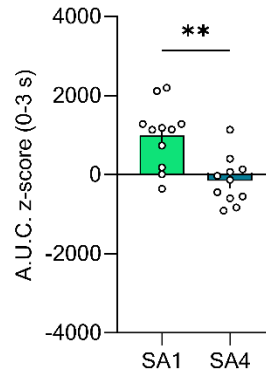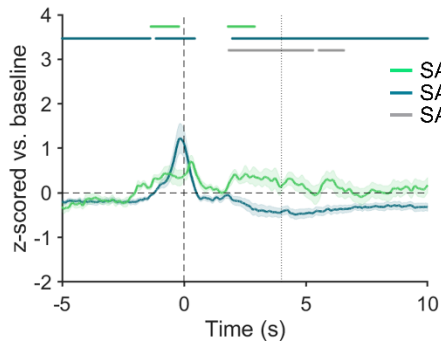D. GCaMP traces comparing extinction and pellet+cue reinstatement

Figure 14. Fiber photometry recordings of nucleus accumbens core neuronal calcium across self-administration, extinction, and reinstatement. **A.** *Left:* z-scored mean GCaMP traces time-locked to active lever presses in first session of self-administration (SA1) and the last session of self-administration (SA4) normalized to a baseline period (-5 to 0 s). SEM is shown in shaded area around the mean. The matching-colored lines above the traces show periods in the 15-s window during which bootstrapping indicates 95% confidence that the mean is not equal to zero (baseline level). *Right:* Area under the curve for the traces shown in *Left*. Bars show mean (±SEM) for 3 s after the lever press while dots indicate individual rats. **B-D.** These panels show GCaMP traces, as described in **A,** during the first session of extinction (Ext1) and the last session of extinction (Ext6) (**B**), the last session of extinction (Ext6) and cue-primed reinstatement (Cue) (**C**), and the last session of extinction (Ext6) and pellet+cue-primed reinstatement (Pellet+cue) (**D**).

**Table 12. Statistical output for GCaMP fiber photometry data in Fig. 14**

Expt phase	Measure	Comparison	T-value	P-value	Significant?	Figure
SA	AUC (n = 11)	SA1 vs. SA4	t_{10}=5.143	0.0004	***	2A, right
Extinction	AUC (n = 11)	Ext1 vs. Ext6	t_{10}=3.017	0.0130	*	2B, right
Extinction/ reinstatement	AUC (n = 11)	Ext6 vs. Cue	t_{10}=3.766	0.0037	**	2C, right
Extinction/ reinstatement	AUC (n = 11)	Ext6 vs. Pellet+cue	t_{10}=2.631	0.0251	***	2D, right

when performing post hoc analysis on the -5 to 0 sec data we found a significant increase on SA3 (t_{30}=2.526, *p*=0.0338) and SA4 (t_{30}=2.755, *p*=0.0294) compared to SA1, supporting the idea that the supporting the idea that the DA transient now precedes the press. Additionally, we graphed AUC values for all sessions to better track progressive changes in DA and calcium responding (Fig. 17). Overall, fiber photometry results obtained during SA training suggest that MSN activity, as measured by calcium, decreases from basal levels upon a lever press that results in food reward and that this develops over the course of training. In contrast, DA release associated with active responding rises from baseline levels and this peak becomes more robust and moves to precede the lever press. A potential explanation is that DA release begins to track lever entry (which precedes the lever press) as the rats learn to perform the task.
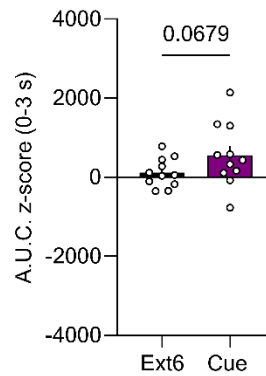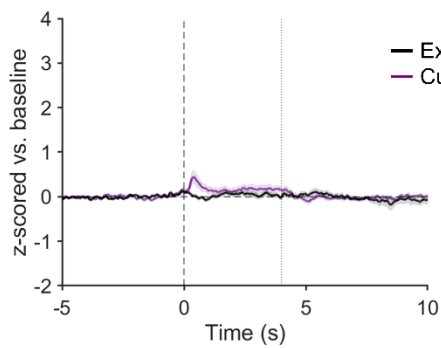
A. GRAB_DA traces during food self-administration (SA) training

B. GRAB_DA traces during extinction (Ext) training
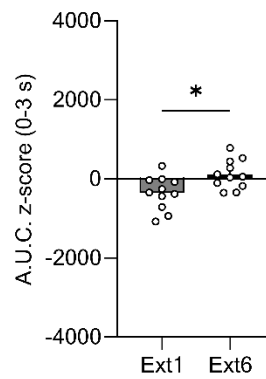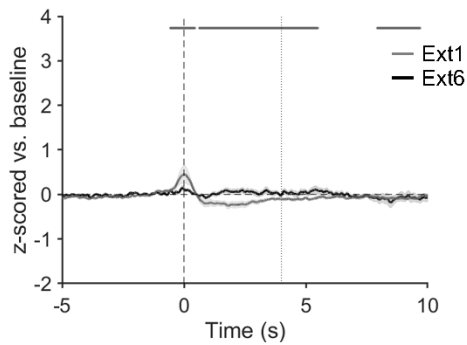
C. GRAB_DA traces comparing extinction and cue reinstatement

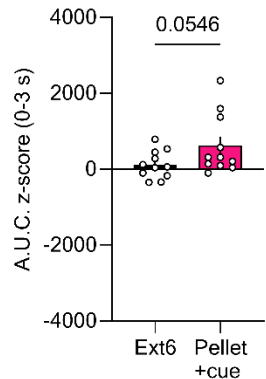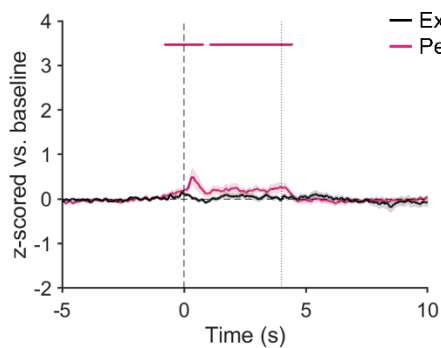D. GRAB_DA traces comparing extinction and pellet+cue reinstatement

Figure 15. Fiber photometry recordings of DA transients in the nucleus accumbens core across self-administration, extinction, and reinstatement. **A.** *Left:* z-scored mean DA traces time-locked to active lever presses in first session of self-administration (SA1) and the last session of self-administration (SA4) normalized to a baseline period (-5 to 0 s). SEM is shown in shaded area around the mean. The matching-colored lines above the traces show periods in the 15-s window during which bootstrapping indicates 95% confidence that the mean is not equal to zero (baseline level). *Right:* Area under the curve for the traces shown in *Left*. Bars show mean (±SEM) for 3 s after the lever press while dots indicate individual rats. **B-D.** These panels show GRAB_DA traces, as described in **A,** during the first session of extinction (Ext1) and the last session of extinction (Ext6) (**B**), the last session of extinction (Ext6) and cue-primed reinstatement (Cue) (**C**), and the last session of extinction (Ext6) and pellet+cue-primed reinstatement (Pellet+cue) (**D**).

Table 13. Statistical output for GRAB_DA fiber photometry data in Fig. 15

Expt phase	Measure	Comparison	T-value	P-value	Significant?	Figure
SA	AUC (n = 11)	SA1 vs. SA4	t_{10}=3.183	0.0098	**	3A, right
Extinction	AUC (n = 11)	Ext1 vs. Ext6	t_{10}=2.452	0.0341	*	3B, right
Extinction/ reinstatement	AUC (n = 11)	Ext6 vs. Cue	t_{10}=2.046	0.0679	n.s.	3C, right
Extinction/ reinstatement	AUC (n = 11)	Ext6 vs. Pellet+cue	t_{10}=2.176	0.0546	n.s.	3D, right

We performed the same analysis for the first and last sessions (Ext1 and Ext6) of extinction training. For GCaMP, bootstrapping analysis indicated a small post-press decrease in Ext1 that is eliminated by Ext6 (Fig. 14B). In fact, there was very little GCaMP response at any point in Ext6. When comparing the AUC for these two sessions with a paired t-test this change is significant (Fig. 14B right, Table 12; t_{10}=3.017, *p*=0.0130). We observed a similar pattern for DA, with bootstrapping analysis showing a post-press dip below baseline during Ext1 that is no longer present in Ext6. This change, similar to results for calcium recordings, was significant (Fig. 15B right, Table 13; t_{10}=2.452, *p*=0.0341). We note that there also appeared to be a small positive peak preceding the response on Ext1 but not Ext6, as observed during SA4, but it was not significant according to bootstrapping analysis. These data suggest that the observed calcium and dopamine responses are not due to the motor action of the lever press (as the motor action is equivalent across

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A. GRAB_DA average peak from -5 to 0 sec
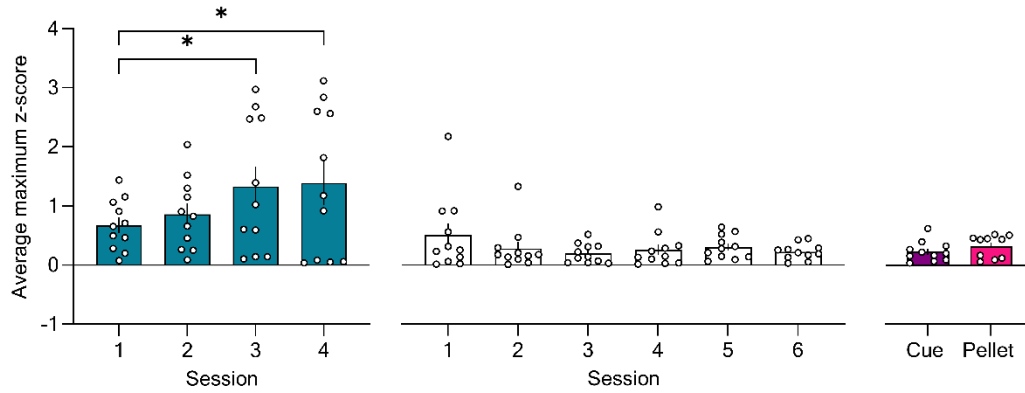
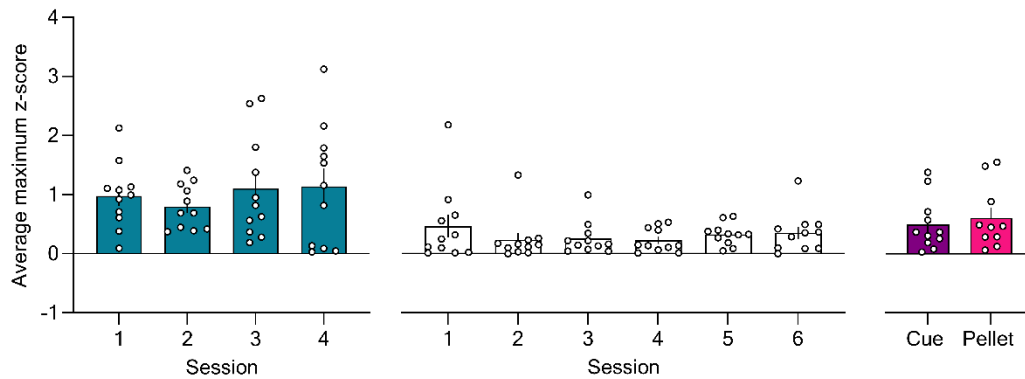B. GRAB_DA average peak from 0 to 3 sec

Figure 16. Average maximum z-score (positive peak) data for rGRAB_DA across all recorded sessions. **A.** *Left:* Average positive peak of rGRAB_DA in the -5 to 0 s time period for all sessions of SA. Average positive peak increases in this time window with training. *Middle:* Same data for all extinction sessions. *Right:* Average peak data for the two reinstatement tests. Bars show mean (±SEM) while dots indicate individual rats, *p<0.05. **B.** Same data shown in A, but for the 0 to 3 s time period. Average positive peak remains the same in this time window with training.

Table 14. Statistical output for GRAB_DA peak analysis in Fig. 16

Expt phase	Measure	Fixed effects	F-value	P-value	Significant?	Figure
SA	Average positive peak, -5 to 0 s (n = 11)	Session	$F_{3,30}=3.667$	0.0231	*	S1A
		Holm-Šídák†				
		SA1 vs. SA2	$t_{30}=0.7101$	0.4831	n.s.	
		SA1vs. SA3	$t_{30}=2.526$	0.0338	*	
		SA1 vs. SA4	$t_{30}=2.755$	0.0294	*	
SA	Average positive peak, 0 to 3 s (n = 11)	Session x Lever				S1B
		Session	$F_{3,30}=0.7458$	0.5333	n.s.	

†adjusted P-values reported for post hoc comparisons
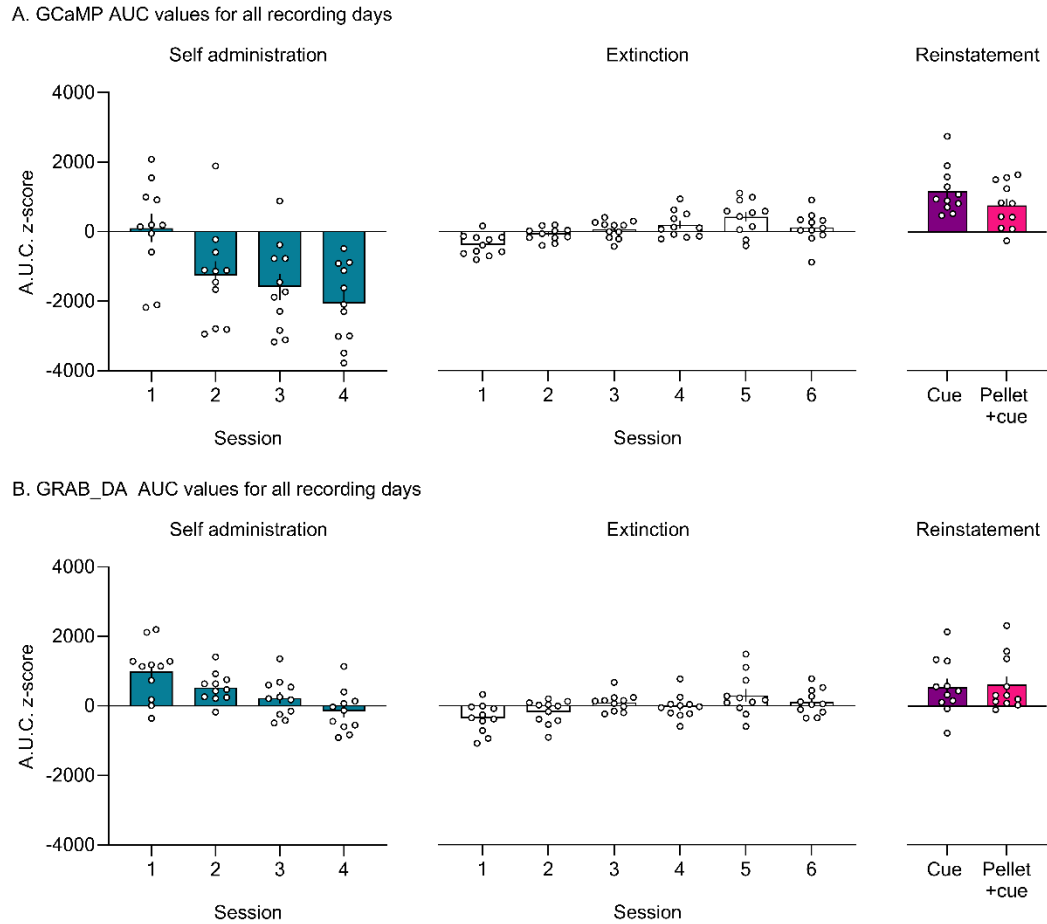
Figure 17. Area under the curve (AUC) for GCaMP and rGRAB_DA traces across all recorded sessions. **A.** AUC for calcium traces (0-3 s after the lever press). *Left*. Self-administration. *Middle:* Extinction. *Right:* Reinstatement tests. Bars show mean (±SEM) AUC while dots indicate individual rats. **B.** DA traces analyzed as described for in A but for rGRAB_DA.

extinction sessions) but rather due to the reward and learned operant response outcome. However, when comparing these two sessions it is important to keep in mind that the number of trials completed in Ext6 is greatly reduced compared to Ext1, which could potentially have affected our analysis.

Reinstatement tests allowed us to further examine nucleus accumbens core MSN calcium and DA responses in a reward seeking context. When examining MSN calcium

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via GCaMP, bootstrapping analysis indicated a small increase from baseline that overlapped with the light cue presentation in both cue-primed (Fig. 14C) and pellet+cue-primed tests (Fig. 14D). This increase was significant when assessed with AUC and compared to the last extinction session using paired t-tests (cue: t_{10} = 3.766, p = 0.0037; pellet+cue: t_{10} = 2.631, p = 0.0251). For DA transients, there was an apparent increase for cue-primed reinstatement (Fig. 15C) that was not considered a likely peak by bootstrapping analysis. For pellet+cue-primed reinstatement, bootstrapping analysis detected a DA peak that overlapped with cue presentation (Fig. 15D). However, analysis with AUC did not find significant increases in either reinstatement test compared to the last extinction session (p=0.0679 and p=0.0546 for cue- and pellet+cue-primed reinstatement, respectively).

Discussion

Previous work using *in vivo* electrophysiology in the nucleus accumbens found that a subset of neurons increased their firing rate during operant responding for food but then paused during consumption (Carelli, Ijames et al. 2000, Roitman, Wheeler et al. 2005, Krause, German et al. 2010). We have expanded on this work by recording intracellular calcium transients in nucleus accumbens neurons during food self-administration, extinction, and reinstatement. Our finding of a large dip in the z-scored GCaMP signal during food self-administration agrees with these prior observations of decreased nucleus accumbens neuronal firing rate. However, we see this dip develop over the course of training. This could reflect increased trial completion and therefore more consumption. Alternatively, it may also reflect task learning and not solely food consumption. The observation that a dip in calcium is also observed in the first extinction session and then disappears over subsequent sessions lends credence to the latter theory. Furthermore, in

the reinstatement test we see an elevation of MSN calcium paralleling the increased cue-induced responding after extinction. In addition to investigating MSN activity, we simultaneously recorded DA transmission given its role in reward seeking and food reinstatement (Berridge and Robinson 1998) in an effort to further connect DA transmission and MSN responding. We found a DA peak that initially follows lever pressing but moves earlier in time, and appears to increase in size, as training progresses. This peak is reduced in the first extinction session and eventually disappears with extinction learning. During reinstatement triggered by non-contingent presentation of a food pellet with the light cue, bootstrapping analysis identified a small DA response that overlaps with cue presentation; there appeared to be a similar DA response when reinstatement was triggered by the cue only, but it was not identified by bootstrapping. Overall, the finding that both MSN activity and DA transmission change across the three phases of this experiment suggests that both MSN cell activity (driven largely by glutamatergic input) and DA release (which modulates MSN responding) are involved in acquisition of food self-administration, extinction, and reinstatement.

Methodological Considerations

Sensor multiplexing studies are complex and require methodological considerations which we shall detail here. First, despite simultaneously recording both MSN calcium and DA, we never make direct quantitative comparisons due to the inherent differences in these biosensors. We selected these two particular sensors due to their reported brightness and similar kinetics, but there are small differences in kinetics which could impact our results (Zhang, Rózsa et al. 2023, Zhuo, Luo et al. 2023). Furthermore, such measurements of these parameters are made typically in cultured neurons with controlled amounts of ligand. All our recordings are made *in vivo*, and of particular note,

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measure changes taking place in either in an intracellular (calcium) or extracellular (dopamine) environment. The relative abundance of calcium or DA can change the kinetics of these sensors, so we avoided comparing onset and offset of calcium and dopamine directly. Another consideration is that we normalized to baseline in all our peri-event analyses and the level of calcium or DA at our defined baseline could affect subsequent analyses. For instance, if extracellular DA is very low during baseline then any phasic release will show as a prominent peak, whereas if there is more baseline activity for calcium then any phasic increase or decrease may be harder to detect. This makes direct comparisons of the two types of transients difficult to interpret, leading us to limit our interpretations of the relationship between DA and calcium signals.

In addition to biological considerations, one of the main challenges of sensor multiplexing, particularly with green and red channels, is that many red fluorophores show emission when excited with blue light. We used a blue light channel to record an isosbestic signal for our green channel, but this light was consistently applied throughout recording and therefore no transient changes should be due to blue light excitation. However, another consideration with the isosbestic channel is signal bleed from the green GCaMP into the red GRAB_DA. We applied the same blue isosbestic recording (405 nm excitation wavelength) to both green and red channels, and it is known that, with a strong green signal, negative peaks may appear in the isosbestic recording as 405 nm is not the true isosbestic point of most GFP biosensors. Thus, when creating the $\Delta F/F$ signal, it is possible that peaks or dips are slightly amplified. When comparing isosbestic and signal channels for the same sensor this is typically not an issue but given that we applied a blue isosbestic recording to a different sensor it is possible some of the GCaMP signal may be reflected in our GRAB_DA traces. However, we believe this to be unlikely given how

different these transients are in shape and in magnitude across different phases of the experiment. Furthermore, when piloting this analysis, a subset of data were processed without the inclusion of an isosbestic channel and the data appeared largely unchanged.

Both unilateral and bilaterally implanted animals were used in this study. We selected from both groups only animals with proper placements and sufficient signal, and did not see any behavioral differences between these animals. We included both sexes in our data set, but given that our study only included 11 subjects we cannot assess potential sex differences.

Role of the nucleus accumbens core in food consumption

The nucleus accumbens has been shown to play a key role in extinction-reinstatement paradigms involving both drugs and food (Calu, Chen et al. 2014, Scofield, Heinsbroek et al. 2016). More work has been done with drugs, especially cocaine, and here evidence suggests that both DA and glutamate transmission are required (Scofield, Heinsbroek et al. 2016, Liu and McNally 2021). Some studies have found differences in mechanisms and pathways involved in drug and food reinstatement (McFarland and Kalivas 2001, McLaughlin and Floresco 2007), potentially reflecting cellular and circuit adaptations in response to cocaine. Earlier studies established that a portion of nucleus accumbens neurons increased firing with food reward predictive cues in a discriminative stimulus (DS) task, and that either direct intra-nucleus accumbens core DA receptor antagonism or ventral tegmental area inhibition reduced that firing and task performance (Yun, Wakabayashi et al. 2004, du Hoffmann and Nicola 2014). These studies suggest that DA facilitation of nucleus accumbens neuron firing is essential for operant responding for food. Our studies show both DA and calcium events time-locked to a lever press for

food reward, which suggests an involvement of both DA and glutamate inputs to the nucleus accumbens core, but this is only correlational evidence.

These two systems have often been examined in isolation from each other, particularly dopamine in operant reward tasks. DA has long been thought to be essential for reward learning, but the exact nature of its role is debated. The classic view of DA is that it encodes discrepancies between the prediction of reward as a result of behavior and the actual consequences, termed "reward-prediction error" (Keiflin and Janak 2015). In this model of DA as a learning signal, it would be expected that DA responses would be elevated early in training, as the consequence of reward from a lever press is new to the rat and would decrease as this outcome becomes more predicted. This would then be followed by an increased DA response in extinction when the response-outcome contingency changes. That pattern of DA responding is not reflected in our data. This could be due to our analysis of session averages, and perhaps if we investigated within SA1 and Ext1 we would find evidence of classical RPE signaling. A more recent theory of DA's significance proposes that it acts as a value signal and, particularly in the nucleus accumbens, high DA indicates that effortful work will be beneficial (Berke 2018). This proposed role of DA signaling tracks more with our observations, with DA increasing as the rat completes more trials and becomes more assured of the value of the lever press, and decreasing in Ext1 when the value of that work reduces. Recent work presents additional thoughts of DA as a signal of perceived saliency (Kutlu, Zachry et al. 2021) or as a tool for retrospective learning (Jeong, Taylor et al. 2022), both of which would track with our observations of DA transients during food SA and extinction. Overall, there is a consensus in the literature that DA signaling in the nucleus accumbens during operant

reward tasks plays an important role in learning, but the nature of that exact role is still debated.

The role of MSN activity in the nucleus accumbens core has been investigated, typically with *in vivo* electrophysiology, in regard to reward-associated cues and a role in food intake. As previously discussed, it has been established that during consumption, nucleus accumbens neurons exhibit a pause in firing that is necessary for consummatory behavior (Carelli, Ijames et al. 2000, Nicola, Yun et al. 2004, Roitman, Wheeler et al. 2005, Krause, German et al. 2010). Our calcium data seems to parallel this work with a negative transient occurring in our traces after the lever press. This indeed could be partially due to reward consumption, but there is also likely a learning component. We would predict that if this calcium decrease was simply due to reward consumption it likely would be unchanged across SA sessions, but instead we find that this decrease in calcium becomes more pronounced with training. This could reflect higher trial completion, and therefore a higher number of rewards eaten, but as we only analyzed completed trials, we believe this to be unlikely. Supporting a role for learning, a study that also found a pause during consumption found that it was modulated depending on the presentation of a predictive cue (Nicola, Yun et al. 2004). Likely, the lever extension at the start of the trial acts as our predictive cue or 'DS' and could be modulating the decrease in calcium signaling as the task is learned. Further supporting this, in the first session of extinction training a small decrease from baseline upon lever press remains evident in the average trace. In this session there is zero consumption, indicating that some portion of this decrease in activity is due to other aspects of this behavior. Future studies manipulating nucleus accumbens core function will be required to isolate the behavioral component encoded by this

decrease from basal calcium. For example, it would be interesting to determine the effect of exciting these neurons during the extinction phase of this task.

Role of the nucleus accumbens core in food seeking

The reinstatement portion of the study is unique in that it allows for assessment of reward seeking in the absence of reinforcement, specifically isolating the value of the reward-associated cue and its ability to invigorate behavior. We found significantly increased calcium transients in our reinstatement tests compared to the last session of extinction. This was the case both in the cue-primed and the pellet+cue-primed tests. For DA, bootstrapping analysis detected a transient during the pellet+cue-primed test. There appeared to be a similar pattern during the cue-primed test but bootstrapping did not identify a likely peak. In both reinstatement tests, DA levels did not differ significantly from Ext6 when AUC was assessed, although strong trends were observed. These data suggest a different relationship between DA and calcium in the reinstatement tests than during training. Many studies design their task to investigate both the cue in isolation and the reward response by having the cue precede the operant action. These studies found increased activity of nucleus accumbens neurons during the cue period which was facilitated by DA (Yun, Wakabayashi et al. 2004, du Hoffmann and Nicola 2014). This increased activity is likely due to an interaction of DA signaling and input from glutamatergic afferents into the nucleus accumbens MSN. Furthermore, intra-nucleus accumbens DA receptor antagonism with SCH23390 or raclopride attenuated cue-induced food reinstatement (Guy, Choi et al. 2011). Overall, these results suggest DA and glutamate act in concert during food seeking.

Role of D1 and D2 MSN in observed responses

Our recordings sampled all neurons in the nucleus accumbens core, but it is important to consider the cell types that are contributing to this general population signal. The NAcc is primarily composed of GABAergic MSNs that express either dopamine type1 (D1R) or dopamine type 2 (D2R) receptors and a much smaller interneuron population (Le Moine and Bloch 1995). These receptors exert neuromodulatory effects that generally enhance (D1R) or reduce (D2R) MSN firing and therefore increase or decrease motivated behavior (Nicola, Surmeier et al. 2000, Bamford, Wightman et al. 2018). It is likely that both of these cell-types are represented in our population signal, but one may be contributing more than the other. For instance, during the self-administration phase we see a decrease from baseline in the calcium signal that is paired with an increase in the DA signal. Given what we know about the physiology of these cells we can speculate, as has been done before (Frazier and Mrejeru 2010), that this dip in calcium activity is occurring primarily in D2-MSNs. There is some support for this from recent photometry studies investigating food behavior in a cell-type specific manner. One study using a Pavlovian food task reported that D2-MSN activity, as measured by GCaMP6s, increased just before magazine entry, and then was suppressed during food consumption. In the same task, D1-MSNs showed increased calcium during food consumption that decreased with experience (Guillaumin, Viskaitis et al. 2023). They further demonstrated with optogenetic studies that D1-MSN activation increased the number of magazine entries, while D2-MSN activation increased the length of time spent in the magazine, suggesting that both cell types coordinate in this task. In contrast, another group also used cell-type specific GCaMP recordings in a Pavlovian task and found that both cell types show a rise in response to the food cue, but diverge during food consumption with D1-MSNs showing a dip and D2-MSNs showing a peak and then a dip (Walle, Petitbon et al. 2024). They suggest that this different pattern of calcium activity may bias behavior towards feeding

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and away from locomotion, with higher D2-MSN activation lowering the likelihood of movement.

Conclusions

Nucleus accumbens core neuronal activity as measured by intracellular calcium was progressively reduced during food self-administration training, while the inverse was observed for DA signaling. Both responses were reduced on the first day of extinction before disappearing (no detectable transient) by the last day of extinction. When measured during cue-primed and pellet+cue-primed reinstatement, GCaMP signals showed an increase from baseline time-locked to the lever press. Bootstrapping analysis identified a DA response during pellet+cue-primed reinstatement, although DA levels measured as AUC did not differ significantly from Ext6. This change from negatively to positively associated calcium and DA signals suggests that our calcium population signal may be more reflective of D2-MSN activity in the self-administration phase, with D1-MSN activity becoming more prominent during the reinstatement tests. Future studies will need to assess nucleus accumbens core calcium signals in a cell-type specific manner to test this hypothesis. In addition to providing new information about calcium and DA dynamics in this food reward paradigm, this study also served to validate sensor multiplexing as a technique that can be applied in the future to the incubation of cocaine craving model that was the focus of Chapter 1.

SUMMARY AND CONCLUSIONS

Overall conclusions

This dissertation sought to connect two signaling systems that regulate activity of the nucleus accumbens core in reward seeking. The first Chapter does so by building on previous work that showed the necessity of glutamatergic plasticity in incubation of cocaine craving. In the first study of DA release associated with this phenomenon, I detected DA transients time-locked to cue-induced cocaine seeking in the nucleus accumbens core, but found that their magnitude is unchanged after protracted forced abstinence. However, I also found that the role of DA does change with forced abstinence, that is, D1R and D2R antagonism in the nucleus accumbens core suppresses cocaine seeking only on FAD40-50, not FAD1-2. This suggests that, much like the glutamatergic changes described previously, there is some form of postsynaptic plasticity in the MSNs that alters how these cells respond to DA release. However, the isolation of this plasticity mechanism will present challenges. Detecting and characterizing postsynaptic changes caused by neuromodulators like DA is much more difficult than for neurotransmitters like glutamate and GABA, which act on ionotropic receptors to produce currents which can be directly measured via techniques like *ex vivo* electrophysiology. Neuromodulators typically act via GPCRs which activate second messenger cascades; for instance, D1Rs activate adenylate cyclase which in turn activates protein kinase A (PKA). This can lead to many possible changes in the postsynaptic cell, making determining the exact role of DA in regulation of MSN excitability difficult to determine with traditional techniques (Nicola, Surmeier et al. 2000). However, it should be noted that effects of neuromodulators on the induction of excitatory synaptic plasticity can be detected (e.g., Tsetsenis, Broussard et al. 2022).

Furthermore, while we provide evidence that DA plays a role in incubation of cocaine craving in addition to glutamate's actions via CP-AMPARs, we do not present any evidence directly connecting these two systems. It is likely that they are interconnected, but it is also possible these systems act in parallel to each other. We think the evidence for interplay of glutamate and DA is strong, as previous work demonstrated that the reinforcing nature of glutamatergic input from the basolateral amygdala into the nucleus accumbens core was blocked by D1R antagonism (Stuber, Sparta et al. 2011). Furthermore, work in cultured nucleus accumbens MSNs raises the possibility of a more direct mechanism, with D1R activation facilitating AMPAR synaptic insertion via PKA phosphorylation of GluA1 (Sun, Milovanovic et al. 2008). One alternative mechanism could be PKA acting on other channels to modulate cell excitability in parallel with CP-AMPAR upregulation. For instance, PKA is known to phosphorylate voltage-gated sodium channels, reducing sodium currents, which can impact cell excitability (Neve, Seamans et al. 2004). Furthermore, it has been shown that combined, but not individual, activation of both D1R and D2R can lead to an increase the firing rate of cells in the nucleus accumbens shell due to PKA's actions on potassium channels (Hopf, Cascini et al. 2003). Therefore, given the widespread actions of PKA, it is possible that DA acts to enhance cocaine seeking after forced abstinence through actions on ion channels that modulate cell excitability in tandem with the described AMPAR changes. This might involve previously described actions of DA facilitating the transition of MSNs to the up-state, discussed below and in Chapter 1 (Tseng, Snyder-Keller et al. 2007, Flores-Barrera, Vizcarra-Chacón et al. 2011).

However, another mechanism of action could be D1R effects on gene expression. We present evidence that transient DA signaling during the seeking test is necessary for incubated cocaine seeking. It is possible that these fast actions can be modulating

plasticity solely during the test, possibly through actions on channels described above. However, with DA release unchanged we hypothesize that the requirement for DA signaling for incubated craving is attributable to postsynaptic change. This postsynaptic change is long lasting, occurring between the FAD1 and FAD40-50 seeking tests during the abstinence period, which means it is possible an alteration in gene expression has occurred. A great deal of work has demonstrated that DA action at D1R leads to the activation of the transcription factor cyclic AMP response element-binding protein (CREB) (Neve, Seamans et al. 2004). Furthermore, many studies have shown that CREB regulates the expression of many immediate early genes, which in turn regulate transcription, as well as many other genes that have been implicated in SUD (Berke and Hyman 2000).

Apart from these mechanistic considerations, it should be noted that we used males and females throughout our studies and did not find a main effect of sex or interactions with sex. However, we had low n values so future studies focusing on sex differences could be done to further assess potential sex differences. In Chapter 2, we had only 11 subjects and therefore were unable to perform any analysis with respect to sex. Again, future studies could be performed to further assess sex differences.

Chapter 2 sought to more directly connect glutamatergic and DA signaling in the nucleus accumbens core by piloting sensor multiplexing in a food extinction-reinstatement task. We simultaneously recorded nucleus accumbens core neuronal calcium via GCaMP and nucleus accumbens core DA via rGRAB_DA. During food SA, GCaMP traces time-locked to the lever press exhibit a decrease from baseline activity. This decrease may in part be due to consummatory behavior as has been suggested previously (Carelli, Ijames et al. 2000, Nicola, Yun et al. 2004, Roitman, Wheeler et al. 2005, Krause, German et al. 2010), but given that this decrease becomes more pronounced with training and appears

in the first session of extinction there may also be a learning component to this dip. In our DA traces, we see a time-locked increase from baseline in SA that becomes larger as the task progresses. We also see a shift in timing of DA peak to precede the lever press, possibly tracking lever entry as a cue. This increase with greater trial completion and task performance suggests that DA could be signaling a value of work as has been proposed (Berke 2018). However, it could also be encoding perceived salience (Kutlu, Zachry et al. 2021) or acting to reinforce learning retrospectively (Jeong, Taylor et al. 2022). Both calcium and DA traces show no detectable transient by the sixth extinction session. When recording during both the cue-primed and the cue+pellet-primed reinstatement tests we detected a significant increase of nucleus accumbens core neuronal calcium time-locked to the lever press. Likewise, bootstrapping detected a DA signal during the pellet+cue-primed reinstatement test (a similar peak was visually observed during cue-primed reinstatement but was not detected as a likely peak by bootstrapping). This transition from an inverse relationship between calcium and DA during SA (DA up, calcium down) to a positive association during reinstatement (both DA and calcium up) could reflect a change in the predominant cell type driving this population signal. Other results indicate that the pause in nucleus accumbens core neuron activity during feeding behavior may be driven largely by D2-MSNs, with D1-MSNs showing more activity during physical actions such as in magazine entry or in a wheel running task (Guillaumin, Viskaitis et al. 2023, Walle, Petitbon et al. 2024). We observed a dip from baseline calcium levels during SA, which matches with these recordings of D2-MSNs. In our reinstatement test, we report an increase in calcium from baseline time-locked to the lever press. This could fit with the data from these prior studies that establish a role for D1 in motor and approach behavior.

In our sensor multiplexing experiments we used the calcium biosensor GCaMP as a proxy for glutamate-mediated activation of MSNs. As previously discussed, L-type

calcium channels in MSNs are involved in transitioning to the up state (Vergara, Rick et al. 2003, Tseng and O'Donnell 2005, Tseng, Snyder-Keller et al. 2007), so an increase in MSN calcium levels may be correlated with increased likelihood to respond to excitatory input. Furthermore, when GCaMP is expressed without cell compartment restriction it is more reflective of non-somatic activity, i.e., activity in processes (Legaria, Matikainen-Ankney et al. 2022). In contrast, there are biosensors developed to detect glutamate directly, and these iGluSnFrs accurately reflect cell firing in cultured neurons and in *ex vivo* slice (Aggarwal, Liu et al. 2023). However, as discussed in Chapter 1, when expressing these biosensors one must consider the off-target effects of buffering the substrate being measured. Indeed, one study used both slice experiments and computer simulations to demonstrate that iGluSnFr buffers glutamate diffusion (Armbruster, Dulla et al. 2020). This could have a number of effects; for example, it could reduce the amount of extrasynaptic glutamate available to act on extrasynaptic metabotropic glutamate receptors, which in turn regulate signaling and plasticity, including CP-AMPAR plasticity (Loweth et al 2014; Kawa et al 2022). Due to concerns about buffering a critical molecule in synaptic transmission we used GCaMP as a proxy for glutamate, but buffering intracellular calcium can cause off target effects as well. One study found that GCaMP expression at the calyx Held, a well-characterized central synapse in the auditory system, reduced the amount of synaptic vesicles released both at rest and with electrical stimulation, suggesting that buffering this intracellular calcium could have impacts on synaptic transmission (Singh, Lujan et al. 2018). Additionally, GCaMP can interfere with the gating of the voltage gating calcium channel $Ca_v1.3$, which can interfere with excitability induced changes in gene expression (Yang, Liu et al. 2018). However, we diluted our GCaMP and expressed it in a neuronally restricted manner which is expected to prevent the worst symptoms of buffering. Furthermore, in the studies described here and others in our lab we report no

aberrant behavioral effects with GCaMP expression. This indicates we are not buffering calcium to a problematic level in our studies. Overall, available data indicate that GCaMP is likely a safer measurement of activity driven by glutamate signaling.

While we investigate reward seeking behaviors in both Chapters, we use different reward types, as well as very different paradigms. The most important paradigm difference is the use of the incubation model in Chapter 1 versus extinction-reinstatement in Chapter 2 (see below for discussion), but there are other differences such as nose poke (Chapter 1) versus lever press (Chapter 2). This makes comparisons between these two Chapters difficult. However, there are some similarities. During cocaine SA, cocaine increases baseline DA levels, and rats will self-administer at a schedule that maintains this elevated DA (Wise, Newton et al. 1995). In our photometry studies we cannot measure a change in baseline as we perform repeated normalizations as part of the analysis, but in our recordings during food SA we do see an overall increase in phasic DA across the four SA sessions. We did not perform recordings during cocaine SA due to technical complications. In Chapter 2, we also recorded during multiple extinction sessions, a phase absent from our Chapter 1 experiments. One study performed this same extinction-reinstatement paradigm with cocaine reward in mice and reported a reduction in DA signaling comparing the first and last extinction sessions (Luján, Oliver et al. 2023). This directly mirrors what we found during extinction of food SA. This same study also showed greatly increased DA transients during the reinstatement test for both food and drug, where as our reinstatement associated changes in DA were modest. However, there is evidence that distinct mechanisms underlie food and drug reinstatement. For example, inactivation of the nucleus accumbens core with a GABA agonist was shown to reduce cocaine reinstatement, but not food reinstatement (McFarland and Kalivas 2001).

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As noted above, the main focus of this dissertation is reward seeking, which in Chapter 1 is measured during cue-induced seeking tests during forced abstinence (cocaine-primed seeking was not studied because it does not incubate (Lu, Grimm et al. 2004) and in Chapter 2 is measured during either cue-primed or pellet+cue-primed reinstatement tests following extinction training. While cocaine seeking in the incubation model could be considered similar operationally to a reinstatement test, and making comparisons across these studies can be tempting, it is important to remember that in the incubation model, cue-induced seeking is measured under extinction conditions (absence of reinforcement) but no prior extinction training has occurred. Extinction training causes new learning that changes behavioral responding, and this new learning induces its own set of cellular adaptations (Bender and Torregrossa 2020, Hemstedt, Lattal et al. 2017). As such, it could be more appropriate to compare our cocaine seeking photometry data (Chapter 1; incubation model) to the data recorded during the first extinction session of the food paradigm. However, the absence of the reward associated cue in our food extinction sessions makes this comparison less than ideal as well. Still, to correlate broadly, I show that DA increases from baseline with operant responding in our cocaine seeking tests as well as the first session of food-extinction. Additionally, in the reinstatement tests I found a DA response as detected with bootstrapping (albeit only for cue+pellet reinstatement) and trends for an increase in AUC of the DA response compared to the last session of extinction. Regarding GCaMP changes, I did not perform any calcium recordings during cocaine seeking, but a postdoctoral fellow in the Wolf lab has performed recordings from D1-MSNs and adenosine 2A receptor-expressing MSNs (A2a-MSNs) during the expression of incubated cocaine seeking (Wunch et al., manuscript in preparation). The A2a receptor co-localizes with the D2R in nucleus accumbens MSNs and is preferred as a marker because D2Rs are also expressed on other cellular elements

including cholinergic interneurons (Svenningsson, Le Moine et al. 1997). In this study, there was an increased calcium response in D1-MSNs but not A2a-MSNs associated with cue-induced cocaine seeking. Future cell type specific studies can determine if D1-MSN specific activation also occurs during food reinstatement. Overall, this suggests that, while there are key differences between the studies described in this thesis, broadly we can conclude that during reward seeking both DA and MSN calcium are increased from baseline when time-locked to an operant response.

Future directions

There are many future experiments that would extend the conclusions made in this dissertation. In Chapter 1, we demonstrated that DA in the nucleus accumbens core is necessary for cocaine incubation, but the question of sufficiency remains. One potential future experiment could be to elevate intra-nucleus accumbens core DA just before a cue-induced seeking test, via a DA agonist or optogenetic stimulation of DA terminals originating from the ventral tegmental area, at an early FAD timepoint when we do not normally see cocaine incubation. If the actions of DA are interconnected and interdependent with the changes in glutamatergic plasticity already described we would not expect to see any effect of DA stimulation as previous work shows that the insertion of CP-AMPARs does not occur until past FAD35 (Wolf and Tseng 2012). However, if these systems are acting in parallel through separate mechanisms, then perhaps we would see potentiated cocaine seeking in the absence of CP-AMPARs.

For Chapter 2, we previously discussed employing cell type specific techniques in order to better understand our GCaMP traces in relation to the DA signal. In our lab we already have transgenic rat lines that express Cre-recombinase under the control of the Drd1 (D1) or Adora2a (A2a) promoter (Pettibone, Yu et al. 2019). These transgenic rat

lines combined with a Cre-dependent AAV-GCaMP would allow us to selectively measure the calcium transients in either D1 or D2-MSNs and could help further elucidate if a particular population plays a more prominent role during certain phases of our extinction-reinstatement task. With our DA sensor, there is no Cre-dependent version to allow for more cell type specificity, but as extracellular DA is being detected the value of localizing the sensor to a particular cell type is likely low. There is evidence that DA is released in particular hot spots (Wightman, Heien et al. 2007) with many projecting dopaminergic axons being 'silent' (Pereira, Schmitz et al. 2016), so there could be differential DA release in certain areas of the nucleus accumbens, but cell type specificity is unlikely to clarify that.

The most exciting future experiment would involve the integration of these two chapters by attempting sensor multiplexing with GCaMP and rGRAB_DA in the cocaine incubation model. This study could be carried out in the same way as the photometry studies described in Chapter 1, but with injections of the viral cocktail used in Chapter 2. It would be necessary to first conduct a preliminary study to ensure that dual virus expression remains stable at the much longer timepoint that would be used to measure incubated craving (10 weeks), as our studies in Chapter 2 only lasted 4-5 weeks. Furthermore, quantification would need to be done to ensure both GCaMP and rGRAB_DA expression levels are not significantly changed between the two seeking tests as was done for GRAB_DA2m in Chapter 1. This experiment would represent an exciting step forward in connecting DA and glutamate signaling during expression of incubated cocaine craving, perhaps showing a change in how they correlate with one another as a function of abstinence time, similar to our results in different phases of the food self-administration task in Chapter 2 experiments. With specific information in hand about the

relationship and timing between DA transients and changes in calcium in specific MSN subtypes, improved electrophysiological experiments might be designed to test mechanisms underlying their interaction.

Final conclusions

My work in Chapter 1 shows that DA transmission at D1R and D2R in the nucleus accumbens core is required for incubated cocaine seeking, as shown previously for CP-AMPAR transmission (Conrad, Tseng et al. 2008, Loweth, Scheyer et al. 2014, Kawa, Hwang et al. 2022). Since the magnitude of DA release is the same in early and late withdrawal seeking tests, I hypothesize that a postsynaptic change has occurred in the nucleus accumbens core to alter how the MSNs respond to the DA released. In Chapter 2, I piloted sensor multiplexing in a food extinction-reinstatement task. I recorded changes in both calcium and DA transients across the different phases of this paradigm, with the results suggesting hypotheses (e.g., differing roles for D1 and D2-MSNs in this task) that could be tested in the future. In both of these Chapters, I sought to further our understanding of how DA and glutamate may interact to facilitate reward seeking. Due in large part to technical limitations, most work has studied these systems in isolation. With the advent of biosensors and other genetic tools, we are better equipped to address how these two systems operate to simultaneously guide motivated behavior.

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