

## Advances in studying phasic dopamine signaling in brain reward mechanisms

Robert J. Wickham<sup>1,2</sup>, Wojciech Solecki<sup>2</sup>, Liza R. Rathbun<sup>2</sup>, Nichole M. Neugebauer<sup>2</sup>, Robert Mark Wightman<sup>3,4</sup>, Nii A. Addy<sup>1,2,5</sup>

<sup>1</sup>Interdepartmental Neuroscience Program, Yale University School of Medicine, New Haven, CT, USA, <sup>2</sup>Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA, <sup>3</sup>Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill North Carolina, USA, <sup>4</sup>Neuroscience Center, University of North Carolina at Chapel Hill, Chapel Hill North Carolina, USA, <sup>5</sup>Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT, USA

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### 1. ABSTRACT

The last sixty years of research has provided extraordinary advances of our knowledge of the reward system. Since its discovery as a neurotransmitter by Carlsson and colleagues (1), dopamine (DA) has emerged as an important mediator of reward processing. As a result, a number of electrochemical techniques have been developed to measure DA in the brain. Together, these techniques have begun to elucidate the complex roles of tonic and phasic DA signaling in reward processing and addiction. In this review, we will first provide a guide for the most commonly used electrochemical methods for DA detection and describe their utility in furthering our knowledge about DA's role in reward and addiction. Second, we will review the value of common *in vitro* and *in vivo* preparations and describe their ability to address different types of questions. Last, we will review recent data that has provided new mechanistic insight of *in vivo* phasic DA signaling and its role in reward processing and reward-mediated behavior.

### 2. REWARD CIRCUITRY: A BRIEF HISTORICAL PERSPECTIVE

The neural system that has come to be known as the “reward pathway” in brain was serendipitously discovered by Olds & Milner when they made the observation that rats would spend more time in the corner of a chamber where they had previously been given non-contingent intracranial electrical stimulation (2). Additionally, they demonstrated that rats would perform an operant response to receive intracranial self-stimulation (ICSS) only in specific brain regions and would extinguish responding when the stimulation was no longer available, establishing that ICSS is positively reinforcing (2). Further experiments aimed at mapping the reward pathway indicated that brain regions along the medial forebrain bundle, a fiber tract known to connect the midbrain tegmentum and limbic regions, supported the most robust ICSS (for review see (3)). Specifically, several nuclei along the mesocorticolimbic dopaminergic pathway were shown to support ICSS including the ventral tegmental area, dorsal striatum,

nucleus accumbens and medial prefrontal cortex (4-7). Alterations in these nuclei have subsequently been shown to play a role in many psychiatric disorders, including drug addiction.

Early research demonstrated that pharmacological manipulations of catecholamine signaling within the mesocorticolimbic pathway altered ICSS such that compounds that increase extracellular catecholamines, like amphetamine and cocaine, facilitate ICSS and those that block catecholamine signaling at the receptor level decrease ICSS (8-10). While it was initially thought that these findings indicated a significant role for norepinephrine neurotransmission in maintaining ICSS, a more critical role for DA neurotransmission soon emerged (11). While the pharmacological manipulations used to characterize the effects of catecholaminergic neurotransmission on ICSS do alter norepinephrine signaling, they also affect the dopaminergic system. Evidence indicating that norepinephrine is not a critical component for ICSS was provided in studies showing that in rats displaying stable ICSS, depletion of norepinephrine behavior had no effect on ICSS nor did it affect amphetamine-induced enhancement of ICSS (12, 13). However, selective decreases in DA levels reduce ICSS and decrease amphetamine-induced enhancement of ICSS (12-14). Further studies demonstrated that lesions of ascending noradrenergic pathways did not disrupt ICSS, while lesions of ascending dopaminergic pathways reduced ICSS (15, 16). While other neurotransmitter systems likely contribute to ICSS behavior, these studies indicated a prominent role for DA.

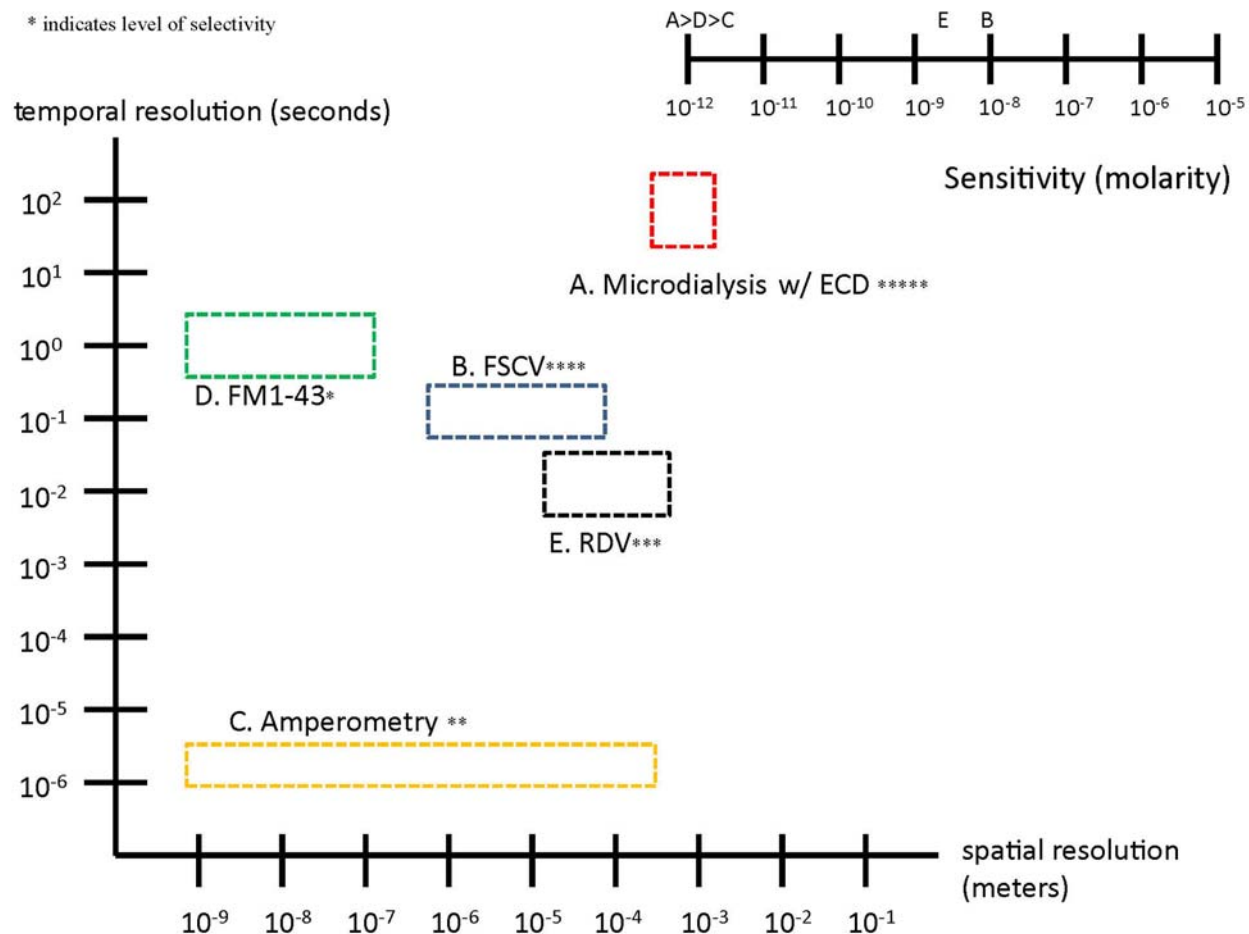
Research indicating that both ICSS and modulation of ICSS behavior by psychostimulants are dependent upon dopaminergic mechanisms provided the basis for the DA hypothesis of drug reward, which posits that drugs of abuse are rewarding because they increase mesolimbic dopaminergic neurotransmission (17). As further evidence, self-administration of psychostimulants such as amphetamine, cocaine and nicotine increase extracellular DA in the nucleus accumbens (NAc) (18-21). In addition, studies revealed that animals will self-administer drugs of abuse into specific brain regions of the mesolimbic pathway. For instance, direct infusion of cocaine or amphetamine into the NAc shell and nicotine infusion into the ventral tegmental area (VTA) are sufficient to support self-administration behavior (22, 23). In light of these findings, particular attention has been given to the dopaminergic neurons of the VTA and their target regions including the NAc and prefrontal cortex and their role in drug reward. The NAc is particularly implicated in drug-seeking behavior as the region where information from limbic regions and the prefrontal cortex is integrated and translated into behavioral output (24, 25). Thus, changes in tonic (on the order of minutes) or phasic (on the order of seconds) DA have been analyzed under various experimental conditions in order to understand the role of DA dynamics in reward. In the next section, we will review a number of methodologies and preparations that have been used in these research efforts.

## 3. METHODS FOR ANALYSIS OF DA DYNAMICS

DA neurons can fire in either a tonic or phasic-like mode. Tonic activity is characterized by 3 to 8 Hz firing frequencies while phasic firing is characterized by bursts of action potentials with a frequency of ~12 to ~20 Hz (26-28). The firing patterns of DA neurons are minimally modulated during sleep or anesthesia, although different stages of wakefulness can modulate their activity (29, 30). This neuronal firing leads to the release of DA from presynaptic terminals into the extracellular space, where it binds to receptors. DA receptors are also present in the extrasynaptic space and DA signaling is characterized by "volume transmission" where DA diffuses from the synapse and binds to extrasynaptic receptors to initiate downstream processes (31). The levels of extracellular DA in target regions are regulated by four main processes: diffusion, neuronal firing, vesicular release from pre-synaptic terminals and reuptake by the DA transporter (DAT). Further, drugs of abuse typically exert their effects by altering one or more of these processes (32). Thus, various methods have been used to measure changes in DA concentration as well as specific changes in DA release and uptake. Among these approaches are microdialysis, electrochemical-based techniques using carbon fiber electrodes (CFE's) (amperometry, chronoamperometry, fast-scan cyclic voltammetry (FSCV)), rotating disk voltammetry (RDV), and radiolabeling methodologies (Figure 1). Furthermore, a number of additional preparations have been combined with the above techniques. Advantages and disadvantages of using *in vitro* (synaptosomes, single cell, tissue) and *in vivo* (anesthetized and awake-behaving animals) techniques will be discussed below, using specific examples from the literature highlighting their advantages, as well as their compatibilities, with DA detection methods.

### 3.1. Microdialysis

Microdialysis is one of the most commonly used methods to measure neurotransmitter levels in the extracellular space of the brain. The microdialysis technique evolved from the push-pull cannula, an arrangement of two concentric tubes that allowed fluid to be directed into the brain and then removed. First described in the late 1960s and practically implemented in the early 1970s (33, 34), over 10,000 papers have been published examining DA levels in the brain using some form of microdialysis (key word search: DA and microdialysis, Web of Knowledge database). Microdialysis itself is a collection method and is not to be confused with methods that are often used in conjunction with microdialysis to detect analytes of interest (i.e. DA). The microdialysis probe consists of a semi-permeable membrane that allows small molecules to pass through (<20 Kd). Typically, a physiological salt solution, such as artificial cerebral spinal fluid (aCSF), is infused through the microdialysis probe. Since most analytes of interest, such as DA, are not in aCSF, they will diffuse down their concentration gradient and across the dialysis probe to be collected and sent to a detector.



**Figure 1.** Temporal versus spatial resolution plot of five commonly used techniques to monitor DA levels. Values plotted are typical and not theoretical ranges. Inset: Ranking of sensitivity of each of these five techniques. A greater number of asterisks indicate greater sensitivity.

Ultimately, the samples collected via microdialysis must be analyzed. Typically the volumes of samples collected are on the order of microliters, therefore, the amount of analyte is very low, often in the femtomole range. Thus, the methods used to analyze dialysate samples must be very sensitive. The most common detection methods used in conjunction with microdialysis are chromatographic-based techniques, such as gas (GC) and high-performance liquid chromatography (HPLC). GC is generally too insensitive for measuring neurotransmitters, therefore, HPLC is typically employed. HPLC uses stationary phases that are contained in columns. The mobile phase and sample are pumped into the HPLC column. Each analyte in the sample will interact differently with the stationary phase, which will produce different retention times, or time it takes to emerge from the column. The retention time typically serves as a unique characteristic of an analyte and therefore provides selectivity for this technique. HPLC is usually coupled with a sensitive detection scheme such as electrochemical detection (EC) (35), fluorescence (36), ultraviolet (UV) (37), or mass spectrometry (MS) (38, 39).

Microdialysis with HPLC-EC is one of the most common analytical methods for the detection of DA *in vivo*, and is best suited to determine long-lasting changes in extracellular DA concentration. To determine absolute DA concentration, the “no-net flux” method is used where several known concentrations of the analyte (such as DA) are perfused through the dialysis probe until equilibrium is reached. When equilibrium is reached, the concentration of analyte at the sampling site equals the concentration of analyte in the perfusate thus allowing estimation of the analyte at the sampling site.

The limits of temporal resolution for microdialysis are usually limited by the detection method employed. Finer temporal resolution requires better mass detection methods. HPLC’s detection limit requires sample times of 5-10 minutes. However, recent advancements in HPLC has allowed temporal resolution to decrease to 1-2 minutes by decreasing the inner diameter of the HPLC column (from 4-5 mm to 0.3-1 mm), resulting in less dilution of the analyte prior to reaching the detector. A more recent advance in detection methods utilized with microdialysis is capillary liquid chromatography (cLC).

cLC columns have very small inner diameters (25-150  $\mu\text{m}$ ) and, when combined with electrochemical detection methods, can provide temporal resolution as high as 10 seconds (40).

Microdialysis, coupled with even the most sensitive detection methods, has low temporal resolution (tens of seconds to minutes) compared to the techniques discussed below. However, low temporal resolution is advantageous in some instances. For example, basal, steady-state levels of DA are best assessed using a method with lower temporal resolution. Imperato and Di Chiara used microdialysis to show that ethanol, nicotine, amphetamine and cocaine increase DA levels in the NAc leading the authors to suggest that stimulation of DA transmission in the mesolimbic system may be a pertinent characteristic of drugs of abuse (41, 42). Although the aforementioned techniques have advanced our knowledge about neurotransmitter systems, the temporal resolution limits the questions they can address. Changes in DA levels on the scale of minutes and hours surely contribute to behavior. However, subsecond changes in DA levels correlates with several fundamental aspects of behavior, such as learning, reward and cue processing, saliency, and motivation. Furthermore, measurements of subsecond DA levels allow estimation of DA transporter functioning (DAT), which may play a role in the pathology of addiction. Below, several common techniques to assess subsecond DA levels will be discussed.

### 3.2. Amperometry

Two types of amperometry have been used to monitor DA. Constant potential amperometry, typically referred to as amperometry, is an electrochemical technique that provides high temporal resolution with limited selectivity (Figure 1). In this method, a constant, continuous potential is applied to the working electrode and the current produced is directly proportional to the number of molecules undergoing oxidation and reduction (43, 44). If maximal temporal resolution is desired, then amperometry is ideal as it can be used to detect individual exocytotic events and provide estimates of quantal release in single cells (45). The limitation of amperometry is that the current produced by the oxidation or reduction of all molecules at the applied potential will also be detected, limiting specificity. However, the technique can be used for monitoring electrically-stimulated DA in various preparations, by confirming the validity of the signal with anatomical, pharmacological and physiological data (46). Additionally, constant potential amperometry can be combined with enzyme-based detection systems to detect non-electroactive analytes, such as acetylcholine. In chronoamperometry, the potential is periodically pulsed to a value sufficient to oxidize DA (47). Some chemical selectivity is obtained by monitoring the ratio of the current when the potential is returned to its initial value relative to that measured during the potential step. This current ratio is determined by the stability of the electrogenerated product. Additionally, Nafion, a cation exchange membrane, can be coated on the electrode to limit access of anionic species. Coating the carbon fiber with Nafion can also be applied to other voltametric techniques (discussed

below). Microdialysis coupled with HPLC, however, allows for enhanced chemical selectivity compared to chronoamperometry. Amperometry may also be used in conjunction with Michaelis-Menten based modeling in order gauge functioning of the DA transporter (DAT).

### 3.3. Fast-Scan Cyclic Voltammetry

Fast scan cyclic voltammetry (FSCV) provides high chemical selectivity and temporal resolution. During FSCV, the potential at the working electrode is applied as a triangular waveform, and the time between scans is about ten times as long as the time of the scan itself. This allows the cyclic voltammograms to be recorded with high temporal resolution, typically repeated at 10 Hz. While this is very good temporal resolution, it is lower than the resolution possible with constant potential amperometry (Figure 1). In FSCV, current is generated at different potentials and the electrolysis of different species is manifested as distinct peaks. The background current in FSCV is stable over short periods of time, allowing it to be digitally subtracted. The resulting background-subtracted cyclic voltammogram (CV) provides an electrochemical "fingerprint" revealing the identity of the analyte detected (44). FSCV is further advantageous in that the cyclic voltammogram is able to separate the signal of interest from most interferents such as pH through the use of principal component regression to statistically identify DA events (48). However, brain regions for DA detection must be carefully selected as the CV for DA is almost indistinguishable from the CV for norepinephrine (NE). Thus, FSCV experiments are typically performed in areas that contain primarily DA or NE. Despite this limitation, FSCV has been used to characterize phasic DA release during reward-seeking behaviors, specifically, showing that the learning of reward-predicting cues correlates to cue-evoked DA release in the NAc (49).

FSCV can be used to determine the kinetics of DAT function. Altered DAT functioning can cause three major changes to DA neurotransmission. First, postsynaptic DA receptors may only detect changes in the magnitude of DA release without changes in timing (amplitude-modulated signal). Second, alterations in DAT function can cause a "spread" of the DA signal in time, without changes in magnitude (frequency-modulated signal). Third, both amplitude-modulated and frequency-modulated signal changes can occur (50-52). These changes in chemical neurotransmission can lead to alterations in the typical physiological functioning. For example, acute and chronic cocaine exposure can cause changes in DAT functioning, which may play a role in cocaine addiction (53).

Kinetic modeling has been most useful in slice and anesthetized preparations (54-57). The random walk approach, employed in brain slices, uses a Michaelis-Menten based model of uptake coupled to diffusion and release (58). In both brain slice and *in vivo* anesthetized preparations, non-linear regression and single curve fitting analyses have been used to determine DA release and uptake kinetics (59, 60). Specifically, the major parameters of interest are  $[\text{DA}]_p$ , the concentration of dopamine released per stimulation pulse,  $V_{\text{max}}$ , the maximal rate of

uptake (that reflects the efficiency at which DAT removes DA), and  $K_m$ , the concentration of DA substrate at which half of  $V_{max}$  occurs. Different regions of the brain exhibit different  $V_{max}$  values for DA. However,  $K_m$  and  $V_{max}$  are fairly consistent across FSCV in both slice (61-63) and anesthetized preparations (53, 59, 64-66) and are consistent with values obtained using [ $^3H$ ] DA radiolabeling techniques (described below). For example,  $K_m$  is usually reported in the range of 0.1  $\mu M$  to 0.3  $\mu M$  in the caudate-putamen and nucleus accumbens, with a mean of 0.2  $\mu M$ . (66).

### 3.4. Rotating Disk Voltammetry

Rotating Disk Voltammetry (RDV) provides the most accurate measurements of transport activity. RDE theory is based on the idea of a plane with infinitesimal thickness that is rotating about its axis in solution at a constant rate (50). This motion creates drag, which pulls the solution in a direction perpendicular to the electrode. The analyte of interest is brought towards the electrode and then spun radially away via centrifugal forces. If the analyte is electroactive, then RDV can be applied to oxidize or reduce the analyte and produce a current proportional to the analyte concentration. Typically, the applied voltage is fixed at a value sufficient to electrolyze the analyte. Because of the requirement of a liquid sample, RDV is used in synaptosomal preparations, or cell or tissue suspensions.

In the context of DA uptake, diffusion can confound interpretation of results obtained with electrochemical approaches. However, an advantage of RDV is the solution is in constant flux; therefore, the effects of mass transport and diffusion processes are minimized, enhancing the quality of transport measurements with this technique. In RDV experiments, DAT kinetics are determined using non-linear curve fitting that is based on Michaelis-Menten kinetics (52). A drawback of RDV is that it cannot be used *in vivo* since the methodology is centered around rotation of the solution (50).

### 3.5. [ $^3H$ ] DA radiolabeling and DA radioligand binding

Although not an electrochemical technique per se, radiolabeling techniques are important given their extensive use in the measurements of DA uptake. The general approach is to incubate synaptosomes or tissue slices with [ $^3H$ ] DA, wash off the extracellular fluid, and measure via liquid scintillation counting, the radioactivity accumulated in the tissue (67, 68). The amount of labeled DA that enters the tissue can provide an estimate of DA uptake. An alternative approach to studying DAT is to use a radioligand that binds to DAT. This approach provides a quantitative estimate of DAT number and location, rather than DA function.

### 3.6. Optical methods for monitoring presynaptic DA release

Fluorescent styryl dyes, such as FM1-43, are useful tools to monitor the endocytosis and exocytosis of neuronal synaptic vesicles. FM1-43 can be packaged and released with endogenous neurotransmitters. Therefore,

neurotransmitter release can be indirectly measured by detecting changes in the intensity of fluorescence produced by FM1-43, typically detected by two-photon microscopy. This approach is most often used with *in vitro* preparations such as tissue slices and cell cultures (69-72). There are two major stages for optically monitoring neurotransmitter release using FM1-43: staining and destaining. Staining is accomplished by bath application of FM1-43 to the preparation followed by electrical stimulation of cells or terminal fields to provoke endocytosis of the dye followed by a washout period. Destaining is the process caused by a second electrical stimulation, which should release vesicles that contain FM1-43. After stimulation, the intensity of fluorescence produced by FM1-43 decreases, indicating vesicular release. The change in fluorescence is a proxy for neurotransmitter release.

Deciding which particular DA detection method can best achieve an experiment's desired goals is crucial. The utilization of each of the methods described above in the various *in vitro* and *in vivo* preparations is detailed below.

## 4. EXPERIMENTAL PREPARATIONS

### 4.1. Synaptosomal Preparations

Synaptosomes are the simplest preparation to study neurotransmitter release and uptake (73). Synaptosomes are isolated nerve terminals or varicosities whose axonal attachments have been removed by shearing the tissue in an isosmotic solution (73). In addition, synaptosomes can be used to isolate synaptic vesicles allowing the direct study of release and uptake of DA at the vesicular level. Using synaptosomes, two common techniques have been employed to measure release and uptake kinetics with similar levels of accuracy. These are [ $^3H$ ] DA radiolabeling and RDV, the former being the most popular.

Several important questions about DA uptake and release have been resolved through synaptosomal preparations. Using synaptosomes in conjunction with radiolabeling, the kinetics of DA uptake have been investigated in rat caudate and NAc (74-78). Radiolabeling was initially used for uptake characterization and provided evidence for a transporter-mediated uptake system. Subsequent work using RDV extended this work to determine how drugs of abuse modulate DA kinetics (51, 75-78). More direct measurements of DA uptake were subsequently performed using both RDV and radiolabeling through the isolation of synaptic vesicles (79, 80).

In addition to studying basic mechanisms of DA release and uptake, synaptosomal preparations have also revealed information about specific presynaptic receptors mediating DA release. For example, Grady et al. demonstrated with [ $^3H$ ] DA radiolabeling that  $\beta 2$  containing nicotinic acetylcholine receptors (nAChR) were required for acetylcholine-induced DA release from the striatal, NAcc, olfactory tubercle, and prefrontal cortex synaptosomes (81). These studies highlight uses for synaptosome preparations in studying uptake and release in

both intact axon terminals as well as synaptic vesicles (79, 80, 82).

In comparison to RDV, radiolabeling is more sensitive and can be used to measure DA in sparsely innervated regions. In the experiment by Grady and colleagues (81) radiolabeling was most likely used because of the sparse dopaminergic innervations to the prefrontal cortex. However, it would be difficult to detect such low amounts of DA using RDV. On the other hand, radiolabeling techniques can be confounded by metabolic processes which may contaminate the signal measured. For example, DA conversion to DOPAC can give false readouts of DA, since the tritium will also be in DOPAC. Therefore, radiolabeling has higher sensitivity, while RDV has better selectivity (83).

### 4.2. Single cell preparations

Single cell experiments using voltammetry provide excellent spatial and temporal resolution for measuring neurotransmitter release mechanisms (84, 85). This preparation is suitable for use with all electrochemical, electrophysiological, and radiolabelling techniques which are often used in complement. The power of using single cell preparations is that measurements can be made with high spatial accuracy in conjunction with an inverted light microscope. Historically, single cell preparations using electrochemical detection methods have been utilized to investigate catecholamine release from vesicles (84, 86-89). Original investigations used bovine adrenal cells, which contain large vesicles with slow release kinetics on the order of milliseconds (87, 88), although neuronal cells such as the giant DA neurons in *Planorbis cornutus* have also been used (90-93). The contents of these cells can be measured beforehand via high pressure liquid chromatography to verify dopaminergic content, which then allows the use of amperometry for high temporal resolution measurements. However, when the contents of a cell have more than one major electroactive species, FSCV is required (89) for chemical selectivity.

Single cell preparations have been used extensively with electrochemical techniques to study the kinetics of vesicular release in cells (84, 86, 88, 89, 94, 95). One notable contribution of single cell preparations coupled with electrochemistry is the finding that catecholamine-containing vesicles may partially fuse with the membrane, releasing a trace amount catecholamine (94, 95). This finding was critical since it ruled out an "all or none" mechanism of neurotransmitter release. In addition, extensions of these types of experiments have been performed in neurons (96, 97).

A special consideration for neuronal measurements of single event release is that neuronal release is much faster than non-neuronal release. Therefore, the use of amperometry or FSCV depends largely on the question being asked. Investigations of single event release in neurons must be measured using amperometry to capture its fast release kinetics, 0.5 ms to 2 ms for dense core vesicles in ganglia (96) and about 100  $\mu$ s for small synaptic vesicles (97, 98), while FSCV is necessary to identify the released substances (89). Finally, amperometry cannot be

used to directly determine absolute concentrations in DA release. As discussed above, concentrations are determined using a flow chamber. Since, in single cell preparations, the electrode is placed on the membrane, the diffusion layer is constrained by the distance from the electrode to the membrane, rather than by the properties of the electrode itself. This altered diffusion layer is difficult to reproduce in the flow chamber, hence concentrations are rarely reported from single cell or tissue (see below) amperometric studies. However, the number of molecules released in a single event can be quantified by integrating the current. The integral has units of charge, which, by Faraday's law, allows direct calculation of the number of released molecules that were detected. FSCV can be used to measure local concentrations since its diffusion layer is very small. Therefore, there is negligible difference between the diffusion layer near a cell membrane and that in a flow chamber (85).

### 4.3. Slice preparations

Thin (200-400  $\mu$ m) slices of brain tissue that are continually perfused with oxygenated artificial cerebrospinal fluid are viable for several hours (44). There are several advantages for using slice preparations. Slices can be placed under a microscope for more precise anatomical placement of the recording electrode, which gives this technique greater spatial resolution than that obtained *in vivo*. Slices are also suitable for studying the effects of local application of drugs without interference from systemic processes and without surgical placements of cannulae. Therefore, a known concentration of drug can be applied to the slice. Measurements are also easier to make in slices due to enhanced DA release upon electrical stimulation of slices when compared to *in vivo* stimulation. The reason for higher concentrations of DA is unclear. One potential explanation is that electrical stimulation of the slice itself is more likely to activate the terminals of dopaminergic neurons, whereas *in vivo* stimulation may activate a smaller population of terminals and a larger population of cell bodies, which do not depolarize easily under most stimulation protocols which utilize as small pulse width (~4ms) (99-101). An alternative contribution could be due to the demonstrated lack of basal dopamine tone and basal D2 autoreceptor activation in the slice preparation (102), resulting in little inhibitory tone. Therefore, a combination of a lack of inhibition coupled with active excitatory input may explain greater evoked DA release in slices. Additionally, since DA cells are also silent in the slice preparation (102, 103), there may be a build-up of presynaptic vesicles in the slice, leading to greater evoked DA release.

Due to the larger DA release in slices, it is easier to measure uptake kinetics. Multiple brain regions and multiple slices within a brain region can be used from a single animal, thereby enhancing the utility of each subject and reducing variability, respectively. The use of brain slices precludes the use of anesthetics, which may interfere with DA signaling. Finally, the experimenter has control over the experimental conditions to reduce interference from temperature and pH fluctuations, unlike *in vivo* preparations (104).

FSCV has been the most common electrochemical technique in brain slices, although brain slice preparations do not preclude chronoamperometric techniques. Unlike single cell preparations in which potential analytes can be determined ahead of time, there is no clear way to determine the analyte species present in the slice. Hence, FSCV is the most straightforward approach to studying DA uptake in brain slices because of its ability to identify specific analytes of interest. Caron and coworkers used FSCV in striatal slices to determine changes in DA uptake mice with genetic deletions of the DAT (105, 106). The uptake rate was 300 times faster in striatal tissue from wild type mice than in tissue from the DAT knockout animals whereas the rate of DA uptake in heterozygous animals was approximately one half of the rate in wild type mice. One goal of this experiment was to also verify that DAT was the only transporter responsible in the striatum for DA uptake. Hence, there are three major reasons why Giros *et al.* chose slice preparations. First, since there is continually flowing fluid over the slice, the authors were able to verify that the removal of DA in knockout slices was attributed to diffusion facilitated by the perfusion fluid. This verification cannot be done easily *in vivo*. Second, tissue preparations allow application and wash out of drug. Therefore, when NE and 5-HT uptake inhibitors were applied and washed out of the tissue, there was no change in uptake or release in either condition. Ruling out the role of NE and 5-HT transporters is important because they can also transport DA (107, 108). Third, as previously stated, larger DA release in slices allows for easier characterization of the kinetics compared to other preparations. However, some precautions must be taken with slice preparation as the higher DA release in slices versus *in vivo* are likely due to differences in stimulation areas. Whether this is physiologically relevant is unclear. Overall, however, brain slices with electrochemistry provide a good model for studying release and uptake in complex systems.

#### 4.4. *In vivo* preparations

*In vivo* DA measurements serve two major purposes—to verify *in vitro* measurements in the intact brain, and to allow for simultaneous acquisition of behavioral and electrochemical data. *In vivo* anesthetized preparations are the simpler choice when deciding on an *in vivo* preparation because the surgeries are less involved than freely-moving surgeries and because the data acquisition is done in a more controlled environment, providing a larger signal to noise ratio (47, 109). Urethane has been reported to not interfere with DA signaling, thereby making it a useful drug for anesthetized preparations (110). However, awake, freely-moving animals remove any potential effects of anesthesia on DA signaling and provide behavioral data at the time of DA recording.

Both microdialysis and voltammetric techniques can be used *in vivo*. Microdialysis experiments are conducted most often in awake, behaving animals, but can also be done in anesthetized preparations. Microdialysis is capable of measuring nanomolar amounts of DA in the extracellular environment of the brain. Additionally,

microdialysis is ideal for measuring the relatively slow effects of drugs on extracellular concentrations of DA as drug-induced changes in DA receptor function can be correlated with behavioral changes in freely-moving animals (47). For example, Chefer *et al.* showed that behavioral sensitization to cocaine was correlated with an attenuation of basal DA release and a diminished ability of quinpirole, a D2 receptor agonist, to inhibit DA release (111).

Although microdialysis offers these advantages and excellent chemical selectivity, it cannot offer the high spatial or temporal resolution of electrochemical recording techniques. Carbon fiber electrodes (CFEs) CFEs can be made more than 20-fold smaller than microdialysis probes (~7  $\mu\text{m}$  diameter CFEs vs. ~200 to ~500  $\mu\text{m}$  diameter microdialysis probes). Their small size allows for minimal damage when they are implanted into living brain tissue. The small size also allows for the recording of electrical stimulation events of DA cell bodies with less diffusional distortion (112).

Voltammetric methods with CFEs are used in both anesthetized and freely-moving animals. For example, Gerhardt *et al.* coupled a microinjection cannula to a CFE, allowing them to locally apply DA in the mammalian brain. They employed chronoamperometry in freely-moving animals to compare uptake rates in anesthetized and freely moving animals (113). Chronoamperometry can also be used in anesthetized preparations to analyze baseline differences in DA function and also the effects of drugs that alter DA activity. This is less technically challenging than recording in freely-moving animals, however, data cannot be correlated to behavior (47).

An even more powerful benefit of using FSCV *in vivo* is that discrete, naturally-occurring DA signals can be detected in awake rats during behavior (109). This was first done by Rebec *et al.* in 1997 in the NAc shell (114). These naturally-occurring DA signals, or transients, are not time-locked to external stimuli such as cues and rewards and therefore are likely to reflect either the motivational state of the animal or spontaneous DA release. In the dorsal and ventral striatum, DA transients occur at baseline frequencies, an average of 0.24 per minute (115) and vary from 10 nM to over 1  $\mu\text{M}$  in concentration. They increase in frequency at the presentation of salient stimuli to the animal (114), social interaction (115), pharmacological application (116), and self-administration of drug reinforcers (117). Over the last 10 years, studies employing *in vivo* FSCV in freely moving animals have continued to provide new insights of the complex role of phasic DA signaling in reinforcement learning and reward seeking-behavior.

## 5. THE ROLE OF *IN VIVO* DA SIGNALING: RECENT FINDINGS

### 5.1. Tonic and phasic DA signaling

DA neurons fire in two distinct modes, tonic and burst firing, which leads to different types of DA release at

terminals in target regions. Tonic DA firing occurs at a frequency of 3-8 Hz and leads to DA release that results in steady-state DA levels. In contrast, burst firing occurs with intra-burst frequencies of ~12 to ~20 Hz and leads to transient, phasic increases in DA concentration ranging from 10 nM to 1  $\mu$ M (26, 28, 30, 118-120). Different regulatory systems are capable of selective modulation of tonic and phasic DA signaling. For example, glutamatergic/cholinergic afferents from the pedunculopontine tegmental nucleus (PPT) to the VTA contribute to phasic DA firing/release, whereas GABAergic input from the ventral pallidum (VP) to the VTA modulates tonic DA/release (121). Distinct characteristics of phasic and tonic DA firing/release suggest that these differential temporal dynamics of DA release might have distinct roles in physiology and behavior. Therefore, distinguishing tonic and phasic contributions of DA release may shed light on DA-related central nervous disorders such as Parkinson's, schizophrenia, and addiction.

### 5.2. Physiological consequences of phasic DA firing

DA neurons in the VTA and substantia nigra (SN) project to the nucleus accumbens (NAc), dorsal striatum, prefrontal cortex, amygdala and hippocampus. Phasic firing of DA cells activates excitatory, low affinity DA D1-like receptors, leading to activation of the direct pathway of the basal ganglia and facilitation of long term potentiation (LTP) at excitatory cortico-striatal synapses (122, 123). In contrast, tonic DA activity is proposed to activate DA D2-like receptors, producing long term depression (LTD) of the medium spiny neurons (MNS) within NAc and dorsal striatum and leading to suppressed activity of indirect pathway of the basal ganglia (122, 124). It has been proposed that coordination of D1 and D2 activation, by controlling striatal plasticity, modulates motor and cognitive function and facilitates behavioral flexibility (124). In line with these electrophysiological results is the recent electrochemical data demonstrating that phasic DA signaling in NAc selectively modulates excitatory but not inhibitory responses of NAc neurons during reward-seeking behavior (125). These observations suggest that phasic DA selectively activates discrete NAc microcircuits that influence goal-directed behaviors (125).

### 5.3. Phasic DA signaling is modulated by excitatory afferents

Burst activity of midbrain DA neurons resulting in transient increase in synaptic DA (123) is mediated by large amplitude, slow inactivating excitatory postsynaptic currents (EPSCs). Burst firing of DA neurons and subsequent DA transients are modulated by excitatory afferents from the midbrain pedunculopontine tegmental (PPTg) and laterodorsal tegmental (LDT) nuclei, which process cue-related sensory information to DA neurons (126-128). These afferents have been shown to be both glutamatergic and cholinergic, implicating the involvement of glutamatergic and/or cholinergic signaling in the modulation of DA neuronal activity. Indeed, activation of the PPTg results in burst firing in SNc DA cells and striatal phasic DA release (121) - an effect blocked by intra-SNc infusions of nonselective nicotinic ACh receptors (nAChRs) antagonists (129). In addition, LDT projections

to the VTA have been demonstrated to evoke burst firing of VTA DA cells (130, 131). Specifically, pharmacological activation of the VTA muscarinic ACh receptors (mAChRs) and nAChRs produces increased DA release in DA terminals, whereas blockade of VTA mAChRs and nAChRs attenuates phasic DA signaling in the NAc (121, 126, 132-134). Furthermore, inhibition of nicotinic or muscarinic receptors in the VTA attenuates LDT stimulated phasic DA signaling in the nucleus accumbens (126).

Glutamatergic mechanisms in the VTA have also been demonstrated to influence phasic DA mechanisms. For instance, inhibition of NMDA receptors (NMDAR) in the VTA reduces the frequency of naturally occurring DA transients, decreases cocaine-evoked DA release and attenuates phasic DA encoding of reward-predictive cues during ICSS behavior (135). More specifically, genetic inactivation of the essential NR1 subunit of the NMDAR selectively on midbrain DA neurons attenuates both burst firing of DA neurons in the VTA/SN and phasic DA transients in the striatum (128). Importantly, disruption of burst firing of DA neurons and phasic DA signaling observed in those transgenic mice selectively attenuated learning of many tasks in which cues predicted rewarding or aversive events (128, 136). However, NR1 deletion on DA neurons does not impact the learning of a conditioned approach in response to cue associated with reward (137). Together, the data from these studies suggests that the NMDAR-dependent DA cell burst firing and phasic DA signaling might play an important role in learning contingencies and encoding valence and/or salience of important environmental cues, but is not essential for stimulus-response (S-R) associations (137).

### 5.4. Phasic DA signaling and reward

A growing body of literature examining DA signaling in freely moving rats continues to delineate the complex role of DA in reward mechanisms. For instance, the frequency of naturally occurring phasic DA in rat dorsal and ventral striatum has been shown to increase in response to rewarding events such as novelty, social interaction, and experimenter delivered or self-administered drugs of abuse (114-116, 138-141). Interestingly, cues associated with both natural and pharmacological rewards produce time-locked DA transients in the NAc (49, 138, 142, 143). Similarly, DA transients also occur in the NAc shell during intracranial self-stimulation (ICSS) in response to the intracranial stimulus as well as to the conditioned stimuli that predict reward availability (144). Furthermore, phasic DA contributes to the maintenance of self-administration behavior, as DA transients in the NAc core precede lever depression in rats trained for cocaine self-administration and electrically evoked phasic DA release is sufficient to promote cocaine self-administration behavior (138, 141). These observations suggest that phasic DA may play a crucial role in incentive learning and goal-directed behaviors and are consistent with the idea that DA modulates reward-seeking behaviors (122, 145, 146). Interestingly, recent evidence points to a selective role of phasic DA in NAc in encoding anticipated benefits but not costs to obtain food reward in rats trained on a decision-making task (147). Similarly, the magnitude of the cue-



elicited phasic DA firing and release has been shown to depend on the size of the reward, the time to reward delivery and the probability of receiving a reward (148, 149). In contrast, aversive stimuli such as quinine taste, stimuli conditioned with electric shock, or delayed cocaine availability modulates phasic DA signaling by producing a decrease in phasic DA levels detected with FSCV (136, 150). These observations suggest that phasic DA might encode the attribution of salience and/or valence to the environmentally salient stimuli. Taken together, the data suggest that phasic DA signaling plays an important role in reinforcement learning.

### 5.5. Phasic DA and reinforcement learning

Several lines of evidence point to a role for phasic DA in reinforcement learning. During Pavlovian training for food reward, both burst firing of midbrain DA neurons and phasic DA transients in NAc shift from primary reward to the reward-predictive stimulus (142, 151). This phasic DA plasticity is also apparent during ICSS training with a conditional stimulus (CS). Initially, DA transients are only seen at ICSS delivery. However, with increasing number of repeated pairings with the CS and ICSS delivery, DA transients begin to emerge and increase in response to CS – a process proposed to underlie S-R (stimulus-response) associative learning. Furthermore, ICSS extinction leads to the decrease and eventual elimination of CS-evoked DA transients across trials – an effect accompanied by significant decline in goal-directed behaviors. Furthermore, reinstatement of ICSS behavior is accompanied by the return of DA transients to pre-extinction amplitudes in response to the CS (49). Such results, showing that phasic DA release is associated with a CS that predicts reward, are in line with theories of reward-prediction error, in which DA neurons responsiveness encodes the predictive value of stimuli during associative learning (152). More recently, increased phasic DA encoding during S-R learning has also been demonstrated to reflect the incentive salience of the CS (153).

Additional evidence also points to the role of phasic DA in reinforcement learning as pharmacological and genetic disruption of DA D1R signaling – which is thought to arise from phasic DA – impairs acquisition of S-R associations (123, 154). Interestingly, DA-deficient mice are capable of learning the S-R association but lack the motivation to work for food reward. In addition, hyperdopaminergic mice can learn an instrumental task as fast as controls but exhibit increased motivation to work for food reward (155, 156). Such results suggest the possibility that DA is not necessary for attributing salience and/or valence to environmentally-relevant cues but is instead more important for encoding motivation to obtain the reward. Importantly not all forms of S-R learning require phasic DA signaling. Sign-tracking behavior, where an animal will first approach and seek the CS cue, (the “sign” that is associated with reward) is dependent on phasic DA signaling. In contrast, goal-tracking behavior, where an animal approaches the reward or reward location (the “goal”) after CS presentation and not the CS itself, is not dependent on phasic DA release. In both cases, the CS is a strong predictor of the reward delivery and evokes a

conditional approach behavior. However, a shift in phasic DA signaling to the CS is only present in sign-trackers and is absent in goal-trackers. In addition, the systemic blockade of DA transmission suppresses sign-tracking but not goal-tracking behaviors (153). These results indicate that DA signaling may underlie a form of an S-R learning in which incentive salience is assigned to a CS, as is observed in sign-trackers, rather than encoding a predictive error signal.

### 5.6. Phasic DA signaling, drugs of abuse, and drug-mediated behavior

While microdialysis studies demonstrate that alcohol, nicotine, opiates, psychostimulants, and cannabinoids, increase DA levels (41, 157), recent *in vivo* FSCV studies have highlighted the importance of phasic DA signaling physiological responses to cocaine and in cocaine-mediated behavior. While cocaine self-administration behavior is mediated by cocaine inhibition of the DAT (158), cocaine also increases phasic DA release, in freely moving rats and this is likely a consequence of phasic firing of the VTA DA neurons (159). Interestingly, cocaine-induced increases in phasic DA release are more pronounced in the shell of NAc in comparison to the NAc core (118). This subregion difference is due to DA autoreceptor function, since it is abolished by DA D<sub>2</sub> autoreceptor blockade before cocaine administration. These observations implicate that cocaine directly increases DA release in a regionally specific manner and demonstrates the significance of autoregulation in cocaine-evoked DA transmission (118, 160).

FSCV studies in freely moving rats have also shown that i.v. cocaine produces increases in DA concentration in the NAc as fast as 20-40s after the end of cocaine infusion (161-163). This DA rise in concentration is readily observed in the shell but not core of the NAc (118). This delay is thought to reflect the time-course for cocaine to cross the blood-brain barrier. However, it has been demonstrated that DA transients occur faster in animals which are well trained in cocaine self-administration (163). These DA transients are observed as early as 0-2 s after cocaine i.v. infusion, followed by a second DA rise occurring 2-8 s later (118, 163, 164). The first DA peak after cocaine i.v. infusion reflects DA response to external cues such as the sound of the lever press or the onset of audiovisual cues (such tone or cue light), whereas the second DA peak is thought to be associated with interoceptive cocaine effects in periphery (118, 164). Consistent with the evidence for phasic DA in reinforcement learning, these observations indicate that, after conditioning, both external and internal cues can serve as predictive cues of the later unconditioned cocaine effect on the DA neurotransmission. In contrast, if the CS predicts a delay in cocaine availability, it produces a decrease in DA concentration associated with aversive state, whereas DA concentration rapidly increase in response to cues signaling imminent cocaine delivery in the subsequent self-administration session (150). These findings show rapid, bivalent contextual control over brain reward processing.

During cocaine self-administration behavior, DA transients occur in the NAc core both pre- and post-response (ie lever depression resulting in i.v. cocaine administration and cue presentation). The pre-response DA transients are associated with the lever approach, whereas post-response DA peaks are time-locked to cocaine predictive cues, as discussed above. Furthermore, pre-response DA appears to contribute to the maintenance of self-administration behavior, as electrically evoked phasic DA release is sufficient to promote cocaine self-administration behavior (138, 163). Interestingly, only the post-response DA release is subject to change in response to extinction or reinstatement procedures (141). Therefore, changes in phasic DA signaling appear to reflect associative processes linking cues with drug rewards. These observations suggest that phasic DA may play a crucial role in incentive learning and goal-directed behaviors and are consistent with the idea that DA modulates reward-seeking behaviors (122, 145, 146).

### 5.7. Other mechanisms for phasic DA modulation

Several lines of evidence suggest that norepinephrine (NE) signaling in VTA modulates DA neuronal activity and DA release at terminals. It has been demonstrated that (i) the locus ceruleus – a major source of NE in the CNS - projects to the VTA (165, 166); (ii) NE terminals make synaptic contacts onto midbrain DA neurons (167); (iii)  $\alpha$ -1 and  $\alpha$ -2 adrenoceptors are present within the VTA (168, 169) and (iv) blockade of  $\alpha$ -1 adrenoceptors as well as activation of  $\alpha$ -2 adrenoceptors reduces burst firing in DA cells (170-172). These observations suggest that the NE system may modulate phasic DA signaling. The orexin/hypocretin system is also capable of modulating phasic DA release. Afferent projections from the lateral hypothalamus provide the source of orexin in the VTA (173-175). In addition, orexin release onto VTA neurons activates DA cells and produces DA release at the DA cell terminal fields (174, 176-178). Furthermore, orexin signaling facilitates cocaine-induced glutamate-dependent LTP in the VTA (179). Several lines of evidence suggest a role for orexin in reward-seeking behavior (173-175). Blockade of VTA orexin signaling decreases both basal and cocaine-evoked phasic DA release in NAc core as well as motivation to self-administer cocaine (180). In addition, activation of the orexin system by hypocretin-1 infusion into the VTA increases the effects of cocaine on tonic and phasic DA signaling as well as the motivation to self-administer cocaine (181).

Phasic DA release can also be modulated by facilitation of activity at DA terminals. Glutamatergic afferents originating from basolateral amygdala (BLA) and forming synapses with NAc DA terminals are capable of modulating phasic DA release (182). Such BLA inputs are crucial for processing motivationally salient stimuli during reward-seeking (183). Another system that can modulate phasic DA is the opioid system via the kappa opioid receptors (KOR) located presynaptically within DA terminals or on DA midbrain cell bodies (184, 185). Activation of the KOR by systemic administration of the KOR agonist, salvinorin A, decreased phasic DA release in both NAc core and shell and resulted in attenuated

motivation to obtain reward (186). The role of the cannabinoid system has also been investigated as the CB1 receptor agonist, WIN55,212-2, increases both the number of spikes in a burst and the frequency of burst firing of VTA DA cells (187). Similarly, intravenous administration of the CB1 agonist increases both the rate of DA transients as well as DA concentration detected with FSCV in the NAc, an effect blocked by a CB1 antagonist (116). These observations suggest that phasic DA signaling is controlled and modulated by multiple neurotransmitter systems at the level of DA cell bodies and the DA terminals. Such a complex network is suited to control sub-second DA release in response to salient environmental stimuli, and therefore, is a crucial component of the reward system of the brain. Phasic DA signaling leads to goal-directed behaviors which favor survival in the changing environment. On the other hand, it may also underlie pathological conditions which may lead to psychiatric disorders such as schizophrenia and addiction.

## 6. CONCLUSIONS

The brain DA system and its relation to reward and addiction have been studied extensively since initial findings showing its role for ICSS and reward circuitry. The mesocorticolimbic DA system, consisting of VTA/SN DA neurons which send projections to limbic (ventral and dorsal striatum, amygdala, hippocampus) and cortical sites (medial prefrontal cortex, orbitofrontal cortex, cingulate cortex), has been implicated in processing salient stimuli and influencing goal-directed behaviors. DA signaling has been shown to be involved in both reinforcement learning and habit formation (152). The use of electrochemical techniques (microdialysis, amperometry, chronoamperometry, FSCV and RDV) and as well as radiolabeling techniques, both in the *in vitro* and *in vivo* preparations has established a broad foundation for DA system physiology. Improvements in the electrochemical detection sensitivity have allowed for analysis of 10 nM DA fluctuations with very high spatial and temporal resolution (188), providing an excellent tool to study DA release dynamics. Thus, combined results from electrophysiological and electrochemical studies have allowed for the distinction of tonic and phasic DA activity. Results obtained with electrochemical techniques such as FSCV, strengthened many electrophysiological findings which suggested a role for DA neurons burst firing in the processing of reward and reward associated stimuli. In addition, FSCV allows for the distinction and examination of *in vivo* DA release and uptake kinetics providing insight into the changes of DA signaling caused by presynaptic plasticity (e.g. evoked by psychostimulant treatment) (53, 189). Finally, the use of FSCV in freely moving animals has allowed for functional determination of *in vivo*, real time DA release. These studies point to the crucial role of phasic DA signaling in the NAc – the brain motivation-action interface in the process of reinforcement learning. While phasic DA signaling has been postulated to encode prediction error signals, recent findings also suggested its role in encoding salience of environmental stimuli as well as motivation to work for reward. Importantly, changes in phasic DA signaling in NAc during drug self-

administration, extinction and reinstatement may underlie the mechanisms of how drug-associated cues influence different phases of addiction including drug-seeking behavior during drug-taking and abstinence.

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**Send correspondence to:** Nii A. Addy, 34 Park Street, 3rd Floor Research, New Haven, CT 06520, Tel: 203-737-5646, Fax: 203-737-4037, E-mail: nii.addy@yale.edu