Electrochemistry in and of the fly brain

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Abstract

Studying brain function and neuronal communication has been always crucial due to the complexity of these systems. A great deal of technology and model systems has been developed to study this subject. Yet, very small invertebrate systems such as the fruit fly, *Drosophila* are excellent models and often have better defined and more easily manipulated genetics. This review focuses on in vivo and in vitro measurements by electrochemical techniques on the fly nervous system. Better understanding of brain function in model systems should aid in finding solutions to biological and bioanalytical challenges related to human brain function and also neurodegenerative disease.

Keywords: *Drosophila*, Electrochemical methods, Brain, …

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1. Introduction

After being introduced more than 100 years ago, *Drosophila melanogaster*, fruit fly, has been used as a research subject significantly contributing to several medical and biological fields, such as gene biology, developmental biology and cell biology [1]. Owing to the chromosomal theory of Mendelian heredity developed by Thomas Hunt Morgan in the last century, the utility of the fly in scientific studies has led to many important approaches to understand the mechanisms of human developmental and physiological processes. A combination of various key reasons makes the fly a powerful model for scientific studies: *Drosophila* has a short life cycle and is easily maintained in the laboratory in large numbers; gene manipulation in *Drosophila* is quite fast and simple. Additionally, a relatively rapid lifespan and an ability to generate a large numbers of progeny make it realistic to generate large-scale genetically identical mutant genes, thus offering a useful tool for research. Several of these genes have later been shown to be highly conserved and functional in mammals [2].

Drosophila provides an invaluable resource for genetic research. Although fly and human lineages diverged evolutionary more than 600 million years ago, the sequencing of the fly and human genomes revealed them to be impressively conserved [3]. In the past few years, the analysis of *Drosophila* genomes has discovered 177 fly genes related to human disease gene[4]. It has also been found that cellular mechanisms, such as regulation of cyclin-dependent kinase and insulin signaling regulating cell growth, are evolutionarily conserved between *Drosophila* and mammals[5]. With the genome information, the expression of the disease form of human gene in flies has been introduced and developed [6]. The fly model of human diseases can then be analyzed in detail to understand the modulated genes associated with human diseases and define genetic and biological pathways that cause the disease. Therefore, *Drosophila* models have successfully provided a better understanding of the mechanisms underlying disease pathology and even identified therapeutic agents for the treatment of human diseases.

The life cycle of the fly consists of four developmental stages: embryo, larva, pupa, and adult. While larvae and adult flies are attractive models for neurological studies, the fly embryo is used for investigating the cellular and molecular development [7]. The fly nervous system alters during development. In the late embryo and first instar larva, the neuroblasts of larval brain are developed [8]. During pupation, the larval neuroblasts develop into neurons [9]. Although the anatomic structures of the fly brain are simpler compared to the human counterparts,

many fundamental cellular and biological processes associated with neurobiology remain similar, including synapse formation, membrane trafficking, neuronal communication, and cell death [10]. Given the number of similar fundamental mechanisms of neuronal signaling between flies and humans, several models of human disorders have been developed in flies providing important insights into the possible neurobiological mechanisms of these poorly understood disorders.

Because the chemical interactions between neurons are complex in mammals, the use of the fly model system makes the analysis of these interactions simpler with its less complex nervous system. The nervous system of the adult fly also consists of neurons and glia and displays high-order brain functions including learning and memory [11]. Similar to mammals, the communication between neurons in the fly brain is primarily based on the chemical signaling produced from exocytotic release of neurotransmitters at synapses. This chemical signaling modulates cell activity in different ways and relies on the neurotransmitters and receptors recruited. Neurotransmitters including biogenic amines and amino acids are also found in the *Drosophila* central nervous system (CNS). In both fly and mammalian systems, these neurotransmitters are essential for different physiological processes [11a]. Amino acid transmitters, such as glutamate, glycine and γ-aminobutyric acid, offer the majority of excitation and inhibitory in the nervous system [12]. Biogenic amine transmitters including serotonin, dopamine, histamine, tyramine, and octopamine also play important roles in the fly brain. For instance, dopamine plays a key role in movement, while serotonin regulates a wide range of behaviors including mood, memory, and cognition [13]. Other monoamines, tyramine and octopamine, which are represented as the invertebrate counterparts of mammalian epinephrine and norepinephrine, function to regulate several behaviors and locomotor activity [14]. Since the roles of most of these neurotransmitters are highly conserved between *Drosophila* and mammals in the CSN, neurochemical research on mammalian systems can be broadened to the smaller and more controllable fly model.

The dysregulation of neurotransmitter signaling and abnormalities in their biosynthesis are correlated with severe psychiatric and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, depression, stress, as well as drug addiction [15]. Due to the important roles of neurotransmitters in many vital functions related to these diseases, the ability to analyze these molecules in *Drosophila* promises to lead to greater understanding the physiological mechanisms that underlie human behaviors, addictions, and neurodegenerative diseases. Several analytical techniques have been employed to identify neurotransmitters in *Drosophila*. Traditionally, immunohistochemistry and histochemistry have been carried out to localize catecholamines, as well as octopamine and histamine in fly CNS [16]. However, the techniques capable of providing quantitative information are relatively new. In the early 1970s, Ralph Adams and coworkers demonstrated that electrochemistry could be used to directly measure the alteration of neurotransmitters and modulators in the CNS [3b]. In recent years, separations-based methods have been used to quantify several biogenic amines in biological samples. High performance liquid chromatography coupled with electrochemical detection (EC) has been used to analyze small electroactive molecules in adult flies, whole larval bodies, and in isolated larval brain [17]. Another method, gas chromatography with mass spectrometry, has also been used to show the presence and quantify transmitters in *Drosophila* head homogenates [18]. However, this technique requires complex sample preparation with derivatization and volatilization of the fly sample. More recently, capillary electrophoresis (CE) coupled with EC has been used to investigate and measure the chemical contents of individual fly samples. With CE-EC, highly efficient, rapid separations, and excellent detection limits can be achieved for small volume samples.

Given the many benefits of *Drosophila* as a model animal, it is not surprising that the focus eventually turned to the brain of the adult fly and *in vivo* measurements. Some benefits of *in vivo* measurements are that chemical changes can be followed in as natural environment as possible, reducing potential effects from sample preparations or losses in temporal resolution. It also makes it possible to estimate the kinetics and dynamics of reactions, an extremely important aspect of physiology and neuroscience. Many of the neurotransmitters present in the human nervous systems are also present in *Drosophila*, with the exception of epinephrine and norepinephrine. Instead, *Drosophila* has octopamine, a minor biogenic amine in humans that plays the physiological role of epinephrine and norepinephrine in the fly[19]. Due to the small sample volumes involved in *in vivo* measurements in fly brains, a sensitive technique is needed for adequate detection.

Electrochemical measurements have high sensitivity and can detect several neurotransmitters, as long as they are electroactive (Fig. 1). Various electrochemical techniques differ in their strengths as some have a higher temporal resolution (e.g. amperometry) and others have higher selectivity (e.g. fast scan cyclic voltammetry, FSCV). When studying the complex brain, selectivity usually is prioritized in order to decrease ambiguity caused by interferents. In fact, one study did try both amperometry and FSCV for *in vivo* detection in the fly brain but a high variability in the amperometric data made FSCV the method of choice for continued studies[20].

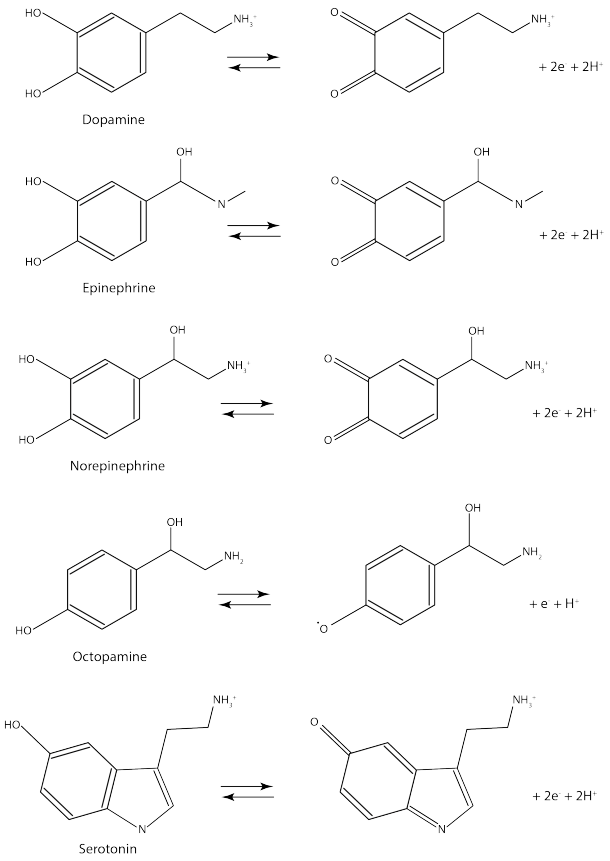

Fig. 1. Examples of electroactive neurotransmitters relevant for human and/or *Drosophila* nervous systems.

Herein, we discuss the use of electrochemical analysis for neurotransmitter detection, especially biogenic amines, from *Drosophila* adult and larva leading to neurological information. To get a better understanding of the cell-to-cell communication in the fly brain, this review then centers on measurements of exocytosis in *Drosophila*.

2. Capillary electrophoresis – electrochemical detection of fly heads and brains

After being introduced to scientific research last century, the fruit fly continues to be a valuable tool for many researchers. *Drosophila* mutants have been successfully used as models for several disorders and diseases associated with the disruption of the neurotransmission system. In order to understand the role of neurotransmitters on neurotransmission and brain functioning in humans, the development of analytical techniques capable of probing and quantifying chemical composition is important for accelerating process in biological and medical sciences. However, the small size of *Drosophila* brain, approximately 5 nL in volume, makes chemical quantification a challenge. Due to the requirement of relatively small injection volume ranging from nanoliters to femtoliters, CE is ideally suited for separations to study fly brain metabolites. Electroactive substances, such as biological amines and their metabolites can be sensitively detected by EC. Another advantage of CE is that the small dimension of the capillary column allows rapid dissipation of Joule heat though the capillary wall, and thus reduces band broadening. This then leads to higher peak capacity in rapid time. Finally, the small capillary diameters in CE allow smaller injection volumes and reduce solvent waste.

A variety of detection methods have been coupled with CE, including electrochemical, laser induced fluorescence, and mass spectrometry (MS) detection [21]. Laser-induced fluorescence is one of the most sensitive CE detection methods, but its application is limited for fluorescent molecules. Although derivatization with active fluorophores can be used, such procedures add more complexity to the CE analysis. Another method, CE-MS, separates compounds based on their differences of electrophoretic mobilities and structure. The majority of CE-MS applications have been in the areas of biological sciences, pharmaceutical and drug metabolism, and environmental analysis [22]. The coupling of CE and MS provides advantages of high separation efficiency and fragmentation information. Besides that, several drawbacks have been reported such as poor concentration sensitivity, high detection limit, fluctuation in analyte retention time, and limitations in electrolyte selection.

The design and use of CE-EC systems for single cells was used early on to determine determination of catecholamine neurotransmitters [21a]. In early work, two sections of fused silica capillary column covered with a

porous glass joint were joined near the cathodic end of the capillary (Fig. 2). This glass joint was immersed in the buffer reservoir along with the cathode. The difference in the applied potential across the capillary resulted in separation of molecules based on their sizes and charges due to electrophoretic mobility. Detection is performed at the end of the capillary without the effects of the applied electric field. The combination of CE and EC detection offers significant advantages for studying the electroactive neurotransmitters because this technique offers low detection limits and high selectivity against background signals in biological samples.

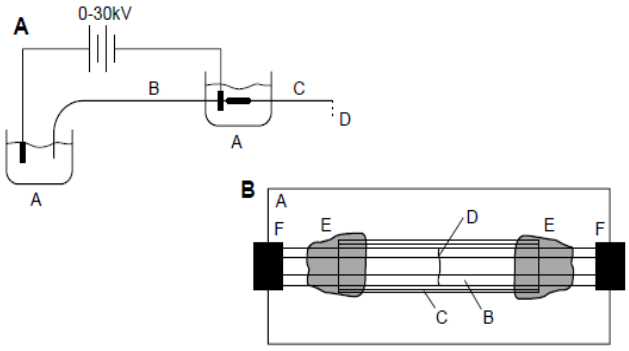

Fig. 2. A) Schematic of coupled capillary zone electrophoresis system: A, Buffer reservoir; B, Separation capillary; C, Detection capillary; D, Eluent. B) Detailed schematic of porous joint: A, Microscope slide; B, Fused silica capillary; C, Porous glass capillary; D, Joint; E, Epoxy; F, Polymer coating. Adopted with permission from ref. [21a]

CE-EC can be used to simultaneously quantify biogenic amines with high sensitivity, high selectivity and low sample consumption. CE-EC opens new opportunities to gain important insights into the neurotransmission roles in physiological and behavioral processes. There are a variety of EC detection methodologies for coupling with CE. Amperometric electrochemical detection is one of the most sensitive methods and does not require sample derivatization. It also enhances selectivity owing to elimination of interferences of nonelectroactive species. Sloss *et al.* originally described the design for CE with end-column amperometric detection providing enhanced reproducibility and low detection limits [23]. However, amperometry is only useful for a limited number of electroactive chemical substances. Additionally, the detection of biogenic amines is often difficult owing to interference with substances having similar electroactive behavior [24]. In contrast to traditional amperometric detection not providing chemical identification, CE with FSCV allows peak determination by both migration time and the half wave potential of the cyclic voltammogram, and has been introduced for neurotransmitter quantification. Denno *et al.* have shown the simultaneous determination of different neurotransmitter contents in tissues of various *Drosophila* life stages [25]. Pupae contain large amounts of tyramine but almost no octopamine. This can be explained as there is no movement or feeding during pupation; with a high content of tyramine possibly serving to inhibit movement in the pupa. Additionally, there is no requirement for octopamine which is responsible for locomotion. The use of EC coupled to FSCV in this work gives sensitive and accurate determination of tyramine, serotonin, octopamine, and dopamine with low detection limit in the 5-16 pg range. Recently, Fang et al. described the application of CE-FSCV to determine dopamine, serotonin, and octopamine with low detection limit in the range of 1-4 nM from a single *Drosophila* larva [26]. Also, they show the effects of genetic and pharmacological manipulations on the neurotransmitter content in fly CNS. For example, Ddc-GAL4, a genetically altered driver line, has lower serotonin and dopamine contents than wild type flies.

However, conventional CE has the drawback of not being capable of differentiating between neutral molecules as they do not interact with the electric field and migrate with the electroosmotic flow. This problem can be alleviated by using an alternative CE approach, called micellar electrokinetic capillary chromatography (MEKC). MEKC is a suitable technique to analyze the mixture of monoamines in complex biological samples. The addition of surfactant to the electrophoresis buffer results in the formation of charged micelles that interact with neutral molecules. Consequently, the separation is obtained due to differential interactions and partitioning. Moreover, analytes with similar charges and electrophoretic mobilities, such as catecholamines, can also be separated [27]. Recently, Ewing and coworkers have developed MEKC with amperometric EC to identify biogenic amines and their metabolites in homogenates of small populations of flies [28]. The use of amperometry as a detection method provides a high sensitivity for monoamines while the interferences from non-electroactive molecules are removed. This method does not require a complex procedure for sample preparation. Hence, the coupling of MEKC-EC becomes a great tool for identification as well as quantification of neurotransmitters in *Drosophila*. Using MEKC-EC,

electroactive neurotransmitters, neuromodulators, and metabolites in individual heads, head homogenates, and individual brains of adult fly have been detected [29]. For example, Powell *et al.* implemented MEKC-EC to simultaneously determine biogenic amines and their metabolites in single fly heads at the picomole level (Fig. 3) [29b]. Peak identification was obtained by migration times from standards. Innovative construction of miniature tissue homogenizers from glass capillaries was also presented in this paper with high reproducibility.

EC-CE offers an analysis tool for probing and measuring neurotransmitter compositions providing significant information for fly research. Even variations in biological neurochemicals among individuals with distinct traits can be detected. EC coupled to CE has also been employed to examine fly models of disease and behavior providing correlations between biogenic amines and neurological dysfunction.

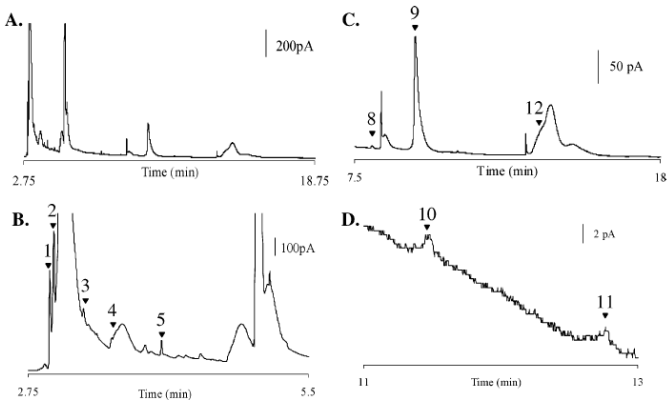

Fig. 3. Electropherogram of a single fly head homogenate in 250 nL of homogenates solution. A) MEKC-EC separation of single male head homogenate. B) Enlargement of the first 5 min of the separation highlighting L-DOPA (1), N-acetyloctopamine (2), N-acetyldopamine (3), N-acetyltyramine (4), and N-acetylserotomin (5). C) Enlargement of the latter half of the separation highlighting octopamine (8), internal standard dihydroxybenzylamine (9), and serotonin (12). D) Enlargement emphasizing dopamine (10) and tyramine (11). Field strength for the 13-µm-i.d. capillary is 568 V/cm. Working electrode was held at +750 mV vs a Ag/AgCl reference electrode. Adopted with permission from ref. [29b]

3. *In vivo* electrochemistry in *Drosophila* fly larvae

A great deal of work has been accomplished in the area of *in vivo* electrochemistry in the fly larva. This has led to the development of new methods to study release and fundamentals in neuroscience. An overview of these studies is presented here in approximate chronological order showing the development of this area.

Venton's group developed and characterized a method to measure dopamine release in isolated ventral nerve cord (VNC) from *Drosophila* larvae [30]. The entire CNS was isolated and the optic lobes were removed by a horizontal cut across the anterior thorax region (Fig. 4A). A cylindrical carbon-fiber microelectrode (CFME) was inserted into the neuropil region using a micromanipulator (Fig. 4B). They genetically expressed Channelrhodopsin-2 (ChR2) specifically in dopaminergic neurons, and this was used with blue light stimulation to elicit release from only one type of neuron. Dopamine release was evoked by activation of ChR2 and detected using FSCV at the electrode. In FSCV, the voltage is rapidly scanned to oxidize and then reduce the molecule of interest. In this technique a background current must be subtracted and the resulting background-subtracted cyclic voltammogram (CV) can be used to identify the compound detected.

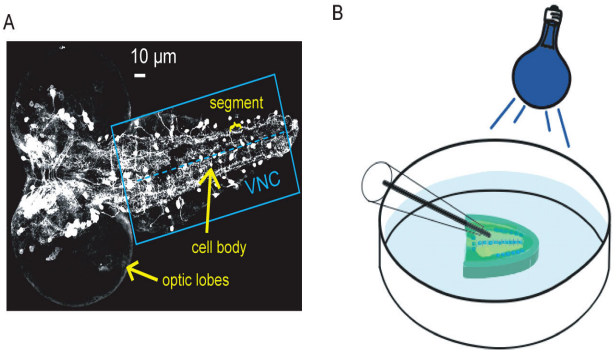

Fig. 4. A) Fluorescence microscopy image of dopaminergic neurons in larval CNS. The blue box indicates the ventral nerve cord (VNC). B) Schematic of microelecrode placement into the neuropil region of the ventral nerve cord with blue-light stimulation. Adopted with permission from ref. [30]

They found an average concentration of evoked dopamine of 810 ± 60 nM, which is similar to concentrations observed in mammalian experiments. They also studied the effect of stimulation time on evoked dopamine. The results show that as the duration of activation increases, the amount of dopamine released also increases until a saturation point was reached after approximately 10 s. To confirm that the detected signal is in fact from dopamine release, they inhibited dopamine synthesis pharmacologically by feeding larvae for 2 days prior to the experiment with NSD-1015 mixed with yeast. NSD-1015 blocks the aromatic L-amino acid decarboxylase (AADC) enzyme. AADC catalyzes the decarboxylation of L-DOPA into dopamine, which is the second step in the dopamine synthesis pathway. Results showed the amount of evoked dopamine decreases significantly when larvae were fed with NSD-1015. They

also examined the effect of blocking uptake in *Drosophila* with cocaine. Cocaine is a dopamine transporter (DAT) inhibitor. By adding cocaine to the VNCs during dissection the peak height of the stimulated release peak was similar for the cocaine and control traces, but the signal took longer to return to baseline after cocaine. Thus, the half decay time (t_{50}) for cocaine-exposed nerve cords increased significantly compared to buffer-incubated controls. These results indicated that uptake plays a role in dopamine clearance in *Drosophila* VNC.

The Venton group also quantified changes in extracellular serotonin and dopamine in the isolated fly larva (VNC)[31]. Again they used flies which had ChR2 expressed, although this time in the serotonergic neurons. Following blue light stimulation serotonin was detected by FSCV while signals were not detected from control VNCs (without ChR2 expression). A similar experiment was performed for dopamine release. To confirm that release was from vesicular serotonin, they incubated VNCs in 100 mM reserpine for 30 min to inhibit the packaging of serotonin into vesicles by the vesicular monoamine transporter (VMAT). Subsequent light stimulation did not elicit detectable serotonin release (Fig. 5a), which confirms that serotonin release is vesicular in this system. They also investigated whether released serotonin is eliminated through reuptake by serotonin transporter (SERT) in *Drosophila* by blocking SERT function with cocaine and fluoxetine. The results confirmed that transporter inhibition does not affect the evoked serotonin concentration (Fig. 5b), even though it does significantly increase the value of t_{50} (Fig. 5d). D2 receptors (D2Rs) are known as a predominant dopamine autoreceptor and dysfunction of D2 autoreceptors is involved in disease etiology. Therefore, D2 receptors are important drug target sites [32]. Venton's group determined if *Drosophila* D2 receptors act as autoreceptors by studying the effect of D2 agonists and antagonists on dopamine release [33]. Similar to the other work from this group, dopamine release was evoked by optogenetic stimulation and recorded with FSCV. Bromocriptine and quinpirole were administrated in the bath around the nerve cord as D2R agonists. They observed dopamine release significantly decreased after 15 min and 30 min compared to control (Fig. 6).

In addition, they administrated flupenthixol, butaclamol, or haloperidol as dopamine antagonists and observed significantly increase in dopamine release. In summary, they found a similarity between *Drosophila* and mammals in the control of dopamine release by both D2R agonists and antagonists which suggests *Drosophila* D2R functions as an autoreceptor similar to mammalian systems.

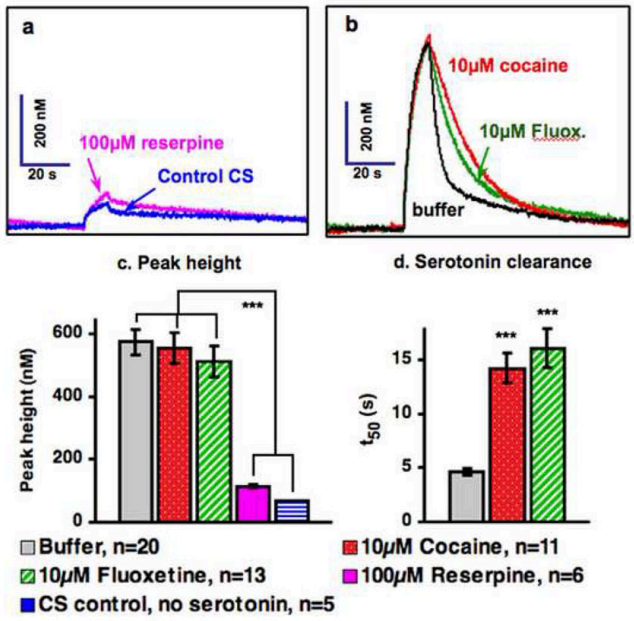

Fig. 5. a) Representative trace from a VNC incubated in reserpine b) Representative traces from VNCs incubated in buffer, cocaine, or fluoxetine c) Peak height is not affected by incubation in cocaine or fluoxetine but reserpine leads to a significant reduction in released serotonin d) Cocaine and fluoxetine-incubated VNCs exhibit significantly longer time to half maximal signal. Adopted with permission from ref. [31]

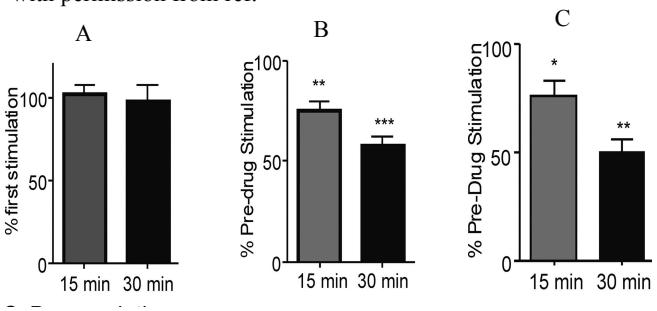

Fig. 6. Effect of D2 agonist bromocriptine (B) and quinpirole (C) on evoked dopamine versus control (A) after 15 and 30 min after the initial stimulation. Adopted with permission from ref. [33]

After measuring the catecholamine from *Drosophila* VNC by optogenetic stimulation, Venton's group measured dopamine clearance by implanting a carbon-fiber microelectrode in *Drosophila* larval CNS and characterizing Michaelis−Menten kinetic parameters [34]. They injected picoliter volumes of dopamine into the neuropil with a micropipette and measured the clearance

of extracellular dopamine with FSCV at the microelectrode. Different concentrations of dopamine were applied and the time course of the decay from the maximum dopamine concentration was fitted with a single exponential decay. The initial velocity of dopamine clearance was calculated by using $V = K[DA]_{max}$. They also calculated the maximum rate of uptake, V_{max}, and the affinity, K_m, by using the Michaelis–Menten equation. To study the effect of diffusion and nonspecific clearance on the K_m and V_{max} values, they investigated dopamine uptake in *fumin* (*fmn*) mutants, in which the dopamine transporter (DAT) is not genetically expressed. They observed a slower clearance in *fmn* larvae. They also incubated VNC with cocaine to inhibit DAT and studied the clearance of exogenously released dopamine. Slower clearance was observed after cocaine in wild type flies but was similar to that in *fmn* mutants, indicating that the majority of transporters were blocked by cocaine.

Venton's group have also fabricated carbon nanopipette electrodes (CNPEs) with 250 nm diameter tips, and controllable lengths of exposed carbon [35]. These electrodes were inserted into the VNC of *Drosophila* larva to detect dopamine release stimulated with a more newly developed red light sensitive cation channel CsChrimson by FSCV. The CNPEs developed in this work had the robustness to be implanted in tissue. Also, the sensitivity per unit area for CNPEs with FSCV was slightly less than that of traditional CFMEs for dopamine, but they were still able to measure endogenous dopamine in *Drosophila* larvae. In addition, they showed by reducing the length of this electrode that it can be used for high spatial resolution measurements in *Drosophila*. They also investigated the effects of pulsed optical stimulation trains on serotonin and dopamine release in larval VNC and kinetic parameters calculated by fitting those data with the Michaelis−Menten model [36]. They found serotonin release increased with increasing stimulation frequency and then plateaued. Also, after administering fluoxetine, a serotonin transporter inhibitor, the steady-state response and the frequency dependence disappeared. According to the model, V_{max} was 0.54 ± 0.07 μM/s and 0.12 ± 0.03 and K_m was 0.61 ± 0.04 μM and 0.45 ± 0.13 μM for serotonin and dopamine, respectively. Also, the amount of serotonin and dopamine released per stimulation pulse was found to be 4.4 ± 1.0 nM, and 1.6 ± 0.3 nM.

Another important transmitter, endogenous octopamine, was detected by Venton's group in the *Drosophila* larval VNC by applying FSCV following release [37]. In this work, they optimized a FSCV waveform so that the potential for octopamine oxidation was not near the switching potential, thus minimizing interferences. CsChrimson, the red-light activated ion channel, was again inserted into neurons expressing tyrosine decarboxylase (Tdc2), the enzyme used to synthesize the octopamine precursor. CsChrimson was selected because it has no background shift close to the octopamine oxidation peak due to the photoelectric effect. Endogenous octopamine release was measured electrochemically following a 2-s light stimulation and they observed a concentration of 0.22 ± 0.03 μM for octopamine release in the VNC. Repeated stimulations were stable with 2 or 5 min inter-stimulation times whereas pulsed stimulations resulted in frequency dependent release.

Approximately 90 dopaminergic neurons in the larval *Drosophila* CNS, including the clusters in the protocerebrum, subesophageal zone, and the VNC, have been found. Dopaminergic neurons mediate specific physiological responses and behaviors in each region. For example, in the VNC, dopaminergic neurons function in motor behaviors such as grooming, whereas in the protocerebrum, they control olfactory learning and memory. Also, dopaminergic neurons in the subesophageal ganglion trigger proboscis extension as part of the primary taste relay. Although dopamine release and uptake have been studied in the *Drosophila* larval VNC, other areas of the CNS remain uncharacterized. Therefore, Venton's group compared the kinetic of dopamine release in the VNC and protocerebrum of third instar wandering *Drosophila* larvae [38]. CsChrimson, red-light activated ion channel, was selectively expressed in the same tissue as the enzyme used to synthesize dopamine. A CFME inserted into the protocerebrum (Fig. 7a) and neuropil section of the VNC (Fig. 7b) using a micromanipulator. They observed dopamine release after only a single, 4 ms duration light pulse; however, stimulated dopamine release was larger in the VNC than the protocerebrum. They used Michaelis-Menten modeling to understand release and uptake parameters for dopamine in both regions. They found smaller initial and average amount of dopamine released per stimulation pulse in the protocerebrum than in the VNC. Also, they calculated the average V_{max} and K_m in both regions. They concluded that even though there is a lower amount of dopamine released in the protocerebrum than in the VNC, the

maximum rate of clearance is higher. These results suggest that since protocerebrum is active in sensory learning and memory, it may not require as much dopamine for activation because of receptor sensitivity in this region. Also, sensory learning and memory might be less important in this wandering stage of larval development. On the other hand, dopamine neurons in the VNC have been shown to be primarily involved in motor regulation. Thus, locomotion might require more dopamine signaling and might be more active in this larval stage.

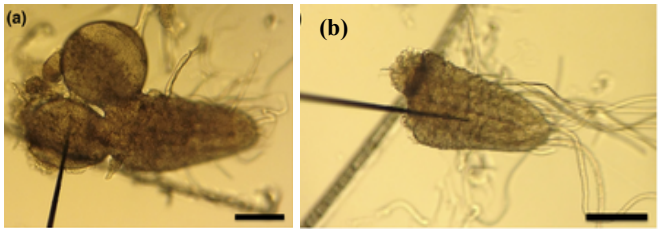

Fig. 7. A 7 μm carbon fiber microelectrode inserted into the a) medial protocerebrum and b) VNC. Scale bar is 50 μm. Adopted with permission from ref. [38]

Venton's group also used ATP/$P2X_2$-mediated dopamine release to evaluate the roles of synthesis and uptake in maintaining the releasable dopamine pool in *Drosophil*a [39]. They measured dopamine release with FSCV in the neuropil of an isolated larva from VNC, which was genetically modified to express $P2X_2$ in dopaminergic cells. $P2X_2$ is a ligand-gated cation channel, which is activated by extracellular ATP. They implanted a carbon fiber electrode into the neuropil of a larval VNC and a capillary micropipette filled with ATP inserted from the other side approximately 15–20 μm away from the electrode. They observed dopamine release by injecting 2 pmol ATP into larval VNC. (Fig. 8A) while they did not observe any changes in dopamine release in larva without expressing $P2X_2$ (Fig. 8B).

This methodology was then used to measure dopamine release after pharmacologically inhibiting the synthesis or uptake with 3-iodotyrosine or cocaine, respectively. They then evaluated the contributions of these drugs to the maintenance of the releasable dopamine pool. They observed a significant increase in evoked dopamine concentration for the first stimulation after cocaine application due to inhibition of dopamine uptake (Fig. 9A) but no depletion was observed after 3-iodotyrosine (Fig. 9B).

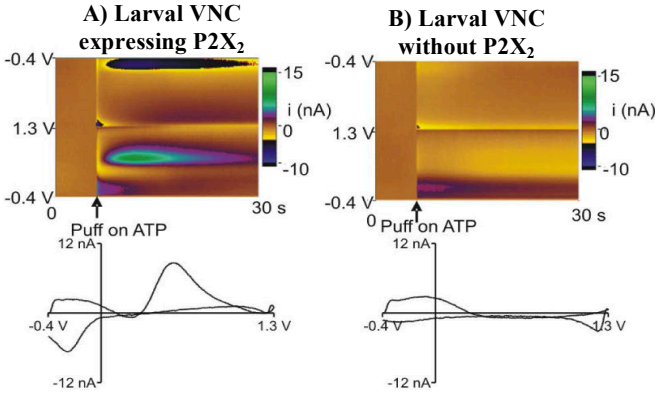

Fig. 8. 2 pmol ATP was injected into a larval VNC expressing $P2X_2$ (A) and a control larval VNC without $P2X_2$ expression (B) The color plot and CV show that dopamine is released upon ATP stimulation in larval VNC expressing $P2X_2$ and even though in control larval the color plot shows minor fluctuations upon ATP injection corresponding to pressure changes but the CV does not show any characteristic dopamine peaks. Adopted with permission from ref. [39]

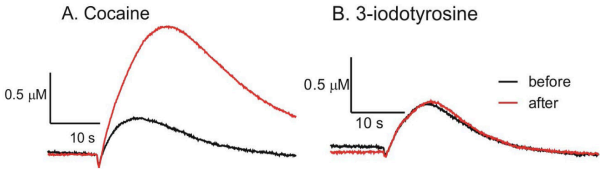

Fig. 9. Concentration vs time traces for dopamine after ATP (2 pmol)/$P2X_2$ stimulation before and after incubation with 60 μM cocaine (A), and 100 μM 3-iodotyrosine (B). Adopted with permission from ref. [39]

They also studied the effect of these drugs on dopamine release during repeated stimulations. For repeated stimulations at 1 min intervals, the dopamine release was depleted by the 5th stimulation after blocking uptake with cocaine. The depletion effect of 3-iodotyrosine was about the same for 1 min and 5 min stimulation intervals. They also observed a significant decay with repeated stimulations in both cocaine and 3-iodotyrosine. The data for the 1 and 5 min stimulation intervals showed that the decay is very similar for 3-iodotyrosine, whereas for cocaine the decay at 1 min stimulation intervals is significantly more than at 5 min intervals. This led them to conclude that cocaine and 3-iodotyrosine have different effects on dopamine release based on the interval during repeated stimulations. This might reflect the storage of dopamine for release and the fraction released during stimulation.

The neuromuscular junction (NMJ) in *Drosophila* larvae is located at the periphery and can be readily accessed. Using this advantage, Majdi et al. studied octopamine release from small varicosities in live, dissected larvae. They genetically expressed the fluorescent protein m-Cherry and the earlier mentioned ChR2 in type II varicosities (octopaminergic terminals). The varicosities were visualized with the help of red light (Fig. 10) and a CFME was placed on top of the octopaminergic varicosities. Octopamine release was evoked by activation of ChR2 with blue light and detected using amperometry at the electrode. They found ~22,000 molecules to be released in each recorded event. Interestingly, different types of release were observed with varying shapes which was suggested to be related to the mechanism of pore opening of the vesicles. This potential regulatory mechanism would allow the vesicle fusion pore to open just the right amount and release the necessary fraction of neurotransmitter at differential rates, possibly affecting plasticity presynaptically[7c].

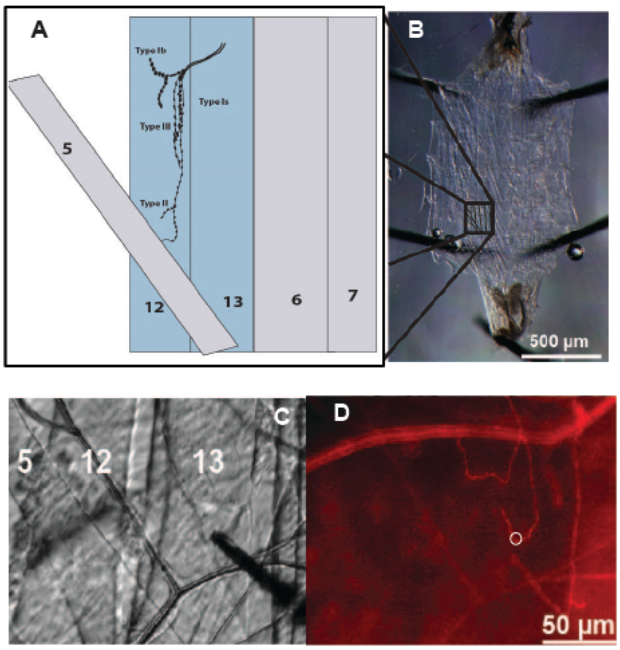

Fig. 10. Amperometric measurement of octopamine release from *Drosophila* larvae. A) The muscle fibers and nerve terminals of the *Drosophila* larval system. B) A filet of the muscle wall of the larva. C) The muscle structure in one hemisegment of the body wall with the microelectrode placement on the type II varicosities in muscle 13. D) The same view as that in panel C, but with the red fluorescent protein m-Cherry at octopaminergic terminals (type II varicosities), which are visible as red lines (the white ring shows the location of the microelectrode). Adopted with permission from re. [7c]

4. *In vivo* electrochemistry in the adult fly brain

Studies have shown that the brain region of adult flies contains the electroactive transmitters dopamine, serotonin, octopamine, and tyramine[25]. Ethanol and nicotine are stimulants that cause an increase in extracellular dopamine, as do substances of abuse such as cocaine and amphetamine. The latter are known to be potent blockers of the plasma membrane dopamine transporter, which is thought to be the key feature of their ability to induce addictive behavior[40]. In order to study the dopaminergic system and the function of the dopamine transporter in *Drosophila* adult fly brain, Makos et al. developed a method for measuring dopamine uptake *in vivo* with FSCV[20]. This was done by positioning a cylindrical carbon-fiber electrode (length 40-50 µm) in the protocerebral anterior medial (PAM) brain area. The PAM area contains the largest number of dopaminergic neurons is the fly brain and can be accessed through microsurgery where the top part of the cuticle is removed, exposing the brain (Fig. 11A,B). However, prior to surgery the flies were sedated and immobilized on ice. Interestingly, a study of adult fly hemolymph collected under different conditions (cold-anesthetized, unanesthetized, and unanesthetized and pinched) revealed changes in amino acid content that were seemingly related to stress levels in the animal[41]. Knowledge of the sedation technique used and the potential effects it has on the animal is an important parameter to consider when performing *in vivo* measurements, and further studies are needed to fully understand physiological effects of anesthesia on

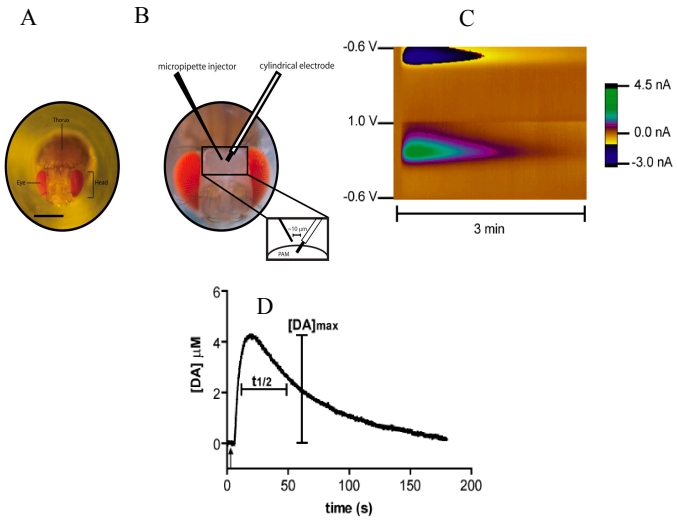

Fig. 11. In vivo measurement of *Drosophila* dopamine uptake. A. An immobilized fly (scale bar 500 um). B. Fly after cuticle was removed showing positioning of electrode and injection pipette in PAM area. C. Example of false color plot representation of dopamine measurement. D. Extracellular dopamine concentration over time in PAM area. Adopted with permission from ref. [20]

Drosophila adults. After surgery, micromanipulators were used to position the electrode inside the PAM area and an injection pipette containing dopamine solution was placed in proximity of the electrode (Fig. 11B). Through this pipette, small amounts of dopamine could be applied to the brain and the clearing of the exogenous dopamine was followed.

Dopamine (DA) was detected with FSCV. The electrode potential was scanned between -0.6 V to +1.0 V

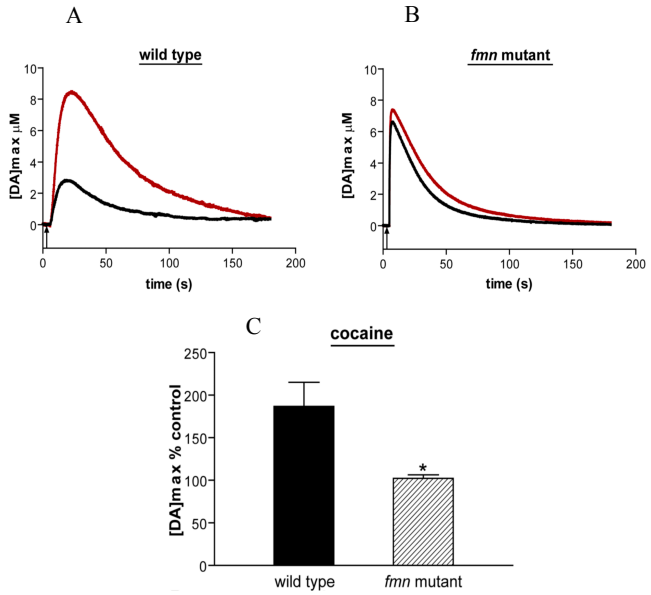

Fig. 12. Effects of transporter mutation and cocaine on exogenous dopamine uptake. A) Representative measurement of dopamine concentration in wild type fly before and after cocaine incubation (black and red, respectively). B) Representative measurement of dopamine concentration in *fmn* mutant before and after cocaine incubation (black and red, respectively). Black arrows in A and B signify 1 s injection of 1.0 mM dopamine. C) Analysis show an increase of dopamine after cocaine application in wild type flies, whereas the dopamine levels in *fmn* mutants stay the same. Adopted with permission from ref. [20]

versus Ag|AgCl at a scan rate of 200 V/s. During this potential scan, an electroactive species can be both oxidized and reduced, given that the reaction is reversible. As the scan was repeated at a frequency of 10 Hz, sizable amounts of data were produced. To facilitate interpretation, the resulting voltammograms were combined in a false color plot showing oxidation in green and reduction in blue (Fig. 11C). By identifying the redox peaks along with the specific shape of single voltammograms, dopamine can be verified as the substance measured. In order to assign measured currents any physiological meaning, *in vitro* calibration was performed after each measurement. The calibration and the *in vivo* measurements were combined to provide a plot showing extracellular dopamine concentration in the brain over time. In Figure 11D a typical concentration versus time plot is shown indicating the parameters maximum dopamine concentration, $[DA]_{max}$, and width at half maximum value, $t_{1/2}$. Due to differences between flies and small variations in pipette positioning, two baseline measurements were recorded in a fly before any pharmacological administration and subsequent measurement. This procedure allowed the measurements to be normalized based on the average baseline for each fly.

To see whether this method was capable of evaluating dopamine transporter function, two different fly genotypes were used. One was the wild type fly with regular dopamine metabolism, and the other was the *fumin* (*fmn)* mutant with a genetic defect in the dopamine transporter. The effects of this mutation were observed as an increase in $[DA]_{max}$ in the *fmn* flies after exogenous application of dopamine (Fig. 12A,B). This indicates a higher rate of uptake and clearance of dopamine in the wild type fly compared to the *fmn* mutant, as was expected. Further verification was done with application of cocaine, a known dopamine transporter blocker. After collection of baseline measurements, cocaine was applied to both wild type and *fmn* flies. Wild type flies showed larger dopamine concentrations after cocaine administration, whereas *fmn* fly response did not change (Fig. 12 A,B,C). Again, this is evidence that cocaine blocks the *Drosophila* dopamine transporter and where there is no functional transporter, i.e. *fmn* flies, cocaine has no effect.

Besides cocaine, the effects of other pharmaceuticals have been tested with this method. One of those was tetrodotoxin (TTX), a blocker of voltage-gated sodium channels. As incubation with TTX led to a reduction in dopamine uptake, there is a possibility that the dopamine transporter is dependent on neuronal activity and membrane potential [3]. Other stimulants were studied as well. Amphetamine, methamphetamine, and methylphenidate are, like cocaine, known substances of abuse although they vary in their potency of leading to abuse. By using the aforementioned technique of measuring and normalizing baseline dopamine uptake before drug incubation, Makos et al. determined which of the stimulants had the largest effect on uptake of exogenous dopamine, i.e. which was a more potent inhibitor of the dopamine transporter[42]. Ordering the stimulants after their ability to decrease dopamine uptake places cocaine as the most potent, followed by

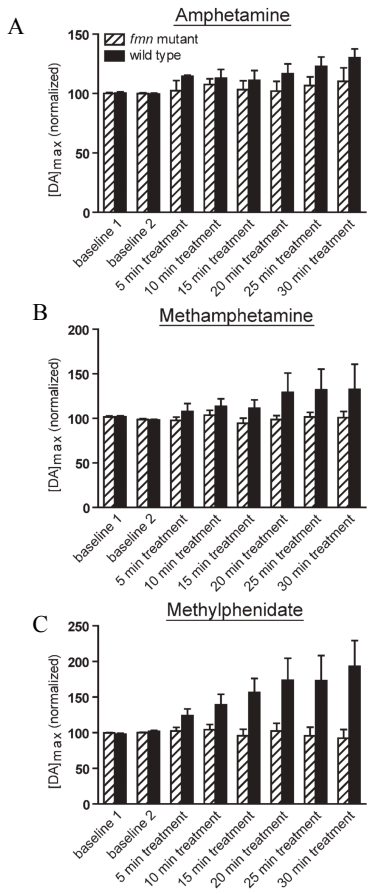

Fig. 13. Effects of stimulant drugs on exogenous dopamine uptake. A) Wild type flies treated with amphetamine show a 117% increase in dopamine compared to baseline levels. B) Wild type flies treated with methamphetamine show a 129% increase in dopamine compared to baseline levels C) Wild type flies treated with methylphenidate show a 174% increase in dopamine compared to baseline levels. Adopted with permission from ref. [42]

methylphenidate, methamphetamine, and lastly, amphetamine (Fig. 13). By varying the treatment times of the stimulants, information concerning the time needed for maximum transporter blockage was also obtained. This is useful for estimating pharmacokinetics *in vivo*, which can be quite different from *in vitro* studies. Compared with cocaine and amphetamine, methamphetamine tended to take longer to maximally block the dopamine transporter. This is an interesting aspect that warrants further research into *in vivo* transporter kinetics in *Drosophila* models[42].

Although the previously mentioned studies all have concerned acute exposure of the brain directly to the drug of interest, the method described above was also adapted to study the effects of oral administration of methylphenidate (MPH). Instead of submersing the brain in saline containing MPH, adult flies were fed a yeast paste containing MPH for 3-5 days prior to the experiment [43]. After oral administration, the flies were subjected to microsurgery as previously described and the uptake of exogenously applied dopamine was measured using FSCV. By combining and comparing oral (semi-chronic) and acute exposure of MPH, it was discovered that flies fed paste containing MPH had reduced inhibition of dopamine uptake upon acute MPH treatment (Fig. 14A). Due to the potentially similar pathways and mechanisms of action of MPH and cocaine, studying their combined effects and interactions was desired. With the methodology described before, flies were fed various concentrations of MPH for days, and then were acutely exposed to cocaine for different durations of time. A concentration-dependent decrease of the cocaine inhibition of the dopamine transporter was observed. The MPH from the oral administration might already have blocked or desensitized the transporter, rendering cocaine incapable of further inhibition. The role of the dopamine transporter in this mechanism was further supported by the lack of effect in the *fmn* mutant flies, without a functional dopamine transporter (Fig. 14B) [43]. More in-depth kinetic analysis of the fall time from $[DA]_{max}$ to the end of the trace made it possible to model dopamine uptake and tease out two terms called k_{slow} and k_{fast}. The first was most likely related to diffusion as it had no dependence of cocaine concentration, whereas k_{fast} was concentration dependent and might therefore be of future use in describing the kinetic effects of various drugs on dopamine uptake.

5. Conclusions

The combination of the *Drosophila* model with electrochemical analysis is clearly advantageous and allows many different aspects of the nervous system to be studied. With CE-EC the electroactive compounds in

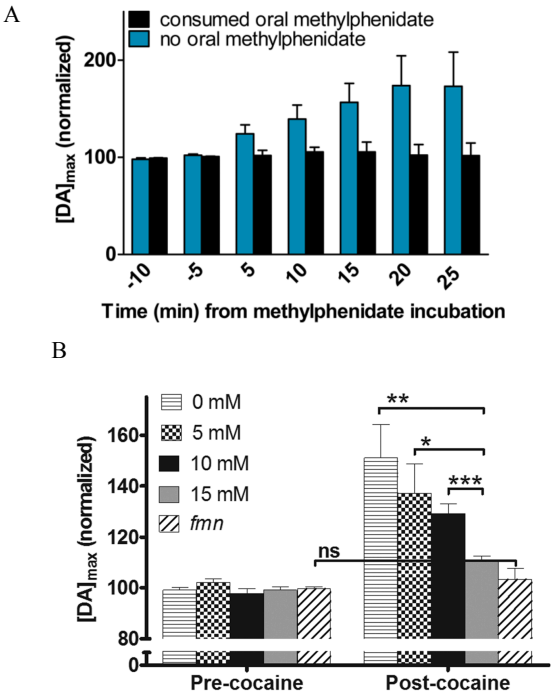

Fig. 14. Competitive inhibition of methylphenidate and cocaine. A. After oral administration of methylphenidate, acute exposure of methylphenidate has no further effect on dopamine uptake. B. Oral administration of methylphenidate diminishes the effects of cocaine on the dopamine transporter in a concentration dependent manner. Legend denotes methylphenidate concentrations delivered in yeast paste or *fmn* mutant. Adopted with permission from ref. [43]

whole tissue can be separated and quantified, providing a general idea of substrates and metabolites present at a certain time point. In contrast, *in vivo* measurements in adult fly brains are limited with regards to the lack of separation and the number of analytes that can be studied. However, these approaches can provide insights into neurotransmitter metabolism with fairly high temporal resolution which is vital for understanding the effects caused by e.g. protein mutations or pharmacological treatments. The larval model clarifies yet another component of the nervous system, making it possible to study neurotransmitter content in an earlier developmental stage. Due to the relative transparency of the larvae, optogenetic stimulation can be easily performed. This has the advantage of stimulating only a small, specific subset of cells and thus endogenous transmitter release can be detected. *Drosophila* as a model animal is a powerful approach system. The future is likely to bring new genetic mutants to the forefront. With models for most neurodegenerative diseases, this could be extremely valuable. In addition, the concept of measuring release at varicosities or in synapses of fully living systems is highly intriguing.

6. References

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