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Carbon nanopipette electrodes for dopamine detection in *Drosophila*

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Abstract

Small, robust, sensitive electrodes are desired for *in vivo* neurotransmitter measurements. Carbon nanopipettes have been previously manufactured and used for single cell drug delivery and electrophysiological measurements. Here, a modified fabrication procedure was developed to produce batches of solid carbon nanopipette electrodes (CNPEs) with ~250 nm diameter tips, and controllable lengths of exposed carbon, ranging from 5 μm to 175 μm. The electrochemical properties of CNPEs were characterized with fast-scan cyclic voltammetry (FSCV) for the first time. CNPEs were used to detect the electroactive neurotransmitters dopamine, serotonin, and octopamine. CNPEs were significantly more sensitive for serotonin detection than traditional carbon fiber microelectrodes (CFMEs). Similar to CFMEs, CNPEs have a linear response for dopamine concentrations ranging from 0.1 to 10 μM and a LOD of 25 ± 5 nM. Recordings with CNPEs were stable for over 3 hours when the applied triangle waveform was scanned between −0.4 and 1.3 V vs. Ag/AgCl/Cl^- at 400 V/s. CNPEs were used to detect endogenous dopamine release in *Drosophila* larvae using optogenetics, which verified the utility of CNPEs for *in vivo* neuroscience studies. CNPEs are advantageous because they are an order of magnitude smaller in diameter than typical CFMEs and have a sharp, tunable geometry that facilitates penetration and implantation for localized measurements in distinct regions of small organisms, such as the *Drosophila* brain.

Carbon-fiber microelectrodes (CFMEs) are traditionally used with fast-scan cyclic voltammetry (FSCV) to study rapid neurotransmitter changes *in vivo*.[1] They allow real-time detection of catecholamines with high sensitivity and selectivity. Traditional CFMEs are 7 μm in diameter[2]; however, smaller electrodes would be useful for neurochemical studies in small organisms such as *Drosophila melanogaster* (the fruit fly). The larval fly central nervous system is extremely small, only about 100 μm wide and the brain is about 8 nL in volume.[3] Individual neuropil, or brain regions, of the adult fly are only a few microns in diameter.[4,5] *Drosophila* is a convenient model organism because it has homologous neurotransmitters with mammals and is easy and fast for genetic manipulation. CFMEs have

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been used to make electrochemical measurements of exogenously applied dopamine in the adult fly mushroom body.[6] In addition, endogenous, stimulated dopamine changes have been measured in a single fruit fly larva.[3] *Drosophila* have glial sheaths surrounding their neuropil that can be tough to penetrate. In larvae, a cut surface is made to insert the electrode[3,7,8,9] and in adults, collagenase has been applied to chemically digest the tissue.[6,10] Therefore, studying the release of endogenous neurotransmitters with better spatial resolution requires a small, dagger-like electrode that can penetrate through the tough glial sheath barrier with minimal tissue damage. Although CNPs have not been previously tested with intact brain tissue, they have been used to penetrate individual mammalian cells while retaining cell viability.[11,12,13] Hence, it is reasonable to expect that the CNPs can penetrate the brain tissue without causing significant damage.

Over the past few decades, nanoelectrodes have been developed for electrochemical applications. Carbon electrodes are preferred for neurotransmitter applications because of their low cost, wide potential window, and good adsorption properties.[14] To make smaller electrodes, carbon fibers can be either flame etched or electrochemically etched to sub-micron tips.[15–18] Recently, flame-etched carbon fibers encased in nanopipettes have been used to make measurements at single synapses, but these electrodes must be individually fabricated.[19] Carbon nanomaterials, such as nanotubes, can also be used as smaller electrodes. Carbon nanofiber microelectrodes have been developed for neurotransmitter detection, but they are on a larger chip and not easily implantable.[20] Small carbon paste electrodes have been made for scanning electrochemical microscopy studies, but are not easy to batch fabricate.[21] Tiny, nanometer-sized electrodes have been made using a single-walled carbon nanotube either sticking out[22] or on a silicon wafer.[23] Alternatively, some methods completely insulate an etched carbon fiber except for the very tip leaving an effective diameter of a few nanometers.[11,24] However, insulation is difficult and a single carbon nanotube or nanometer-sized fiber is not robust enough to be implanted into tissue.

For *in vivo* measurements in *Drosophila*, we desire a sharp, carbon nanoelectrode with high sensitivity to detect nanomolar concentrations that can be easily batch fabricated. Carbon nanopipette electrodes (CNPEs) are nanometer sized electrodes, which have been previously used for electrophysiological measurements and delivering fluids into cells.[25,26,35] CNPEs are fabricated by selectively depositing a carbon layer on the inside of a pulled-quartz capillary. The capillary is then chemically etched to expose the carbon tip. CNPEs are batch-fabricated in a furnace and are rigid because of the quartz insulation. While many of our past designs consisted of hollow pipettes, allowing for drug delivery to cells, extending the carbon deposition time can lead to a sealed, solid tip with a 50–400 nm diameter. Recently, CNPEs with recessed tips have been evaluated as nanosamplers and scanning electrochemical microscopy tips.[27] Here, solid-tipped, cylindrical CNPEs and fast-scan cyclic voltammetry (FSCV) are coupled for the first time.

The objective of this study was to characterize the electrochemical properties of CNPEs using FSCV for the detection of electroactive neurotransmitters and test their suitability for measurements in *Drosophila*. We use three parameters to define the truncated cone geometry of the CNPE tip: the tip diameter, exposed length along the pipette axis, and cone angle. Tip diameter affects invasiveness, cone angle affects sharpness and rigidity, and the

exposed length controls the electrode interfacial surface area. The CNPEs used here were approximately 250 nm in diameter at the tip, with a cone angle of 1.6°, and exposed carbon length ranging from 5 μm to 175 μm, controlled with etch time. CNPEs were successfully used to detect dopamine, serotonin, and octopamine. Dopamine current was stably detected with CNPEs with an optimized triangular waveform of −0.4 V to 1.3 V at a scan rate of 400 V/s and a frequency of 10 Hz. The current was linear with dopamine concentration up to 10 μM. CNPEs are sharp and robust enough to successfully penetrate into a *Drosophila* larva central nervous system without breaking and endogenous, stimulated dopamine release could be measured. CNPEs coupled with FSCV will facilitate fast, real-time measurements of neurotransmitters in specific brain regions of the *Drosophila*.

Methods

Solutions and Chemicals

All reagents were purchased from Fisher Scientific (Fair Lawn, NJ) unless otherwise specified. Dopamine hydrochloride, serotonin hydrochloride, and octopamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Each neurotransmitter was dissolved in 0.1M $HClO_4$ for a 10mM stock solution and diluted daily in phosphate buffered saline (PBS) for testing. The PBS was 131.25 mM NaCl, 3.0 mM KCl, 10.0 mM NaH_2PO_4 monohydrate, 1.2 mM $MgCl_2$ hexahydrate, 2.0 mM Na_2SO_4 anhydrous, and 1.2 mM $CaCl_2$ dihydrate with the pH adjusted to 7.4. Sodium chloride was purchased from VWR International LLC (West Chester, PA), sodium phosphate from Ricca Chemical Company (Arlington, TX) and calcium chloride from Sigma-Aldrich. All aqueous solutions were made with deionized water (Milli-Q Biocel, Millipore, Billerica, MA).

Carbon Nanopipette Electrode fabrication

CNPEs were fabricated with 1 mm outer diameter, 0.7 mm inner diameter, filamented quartz capillaries of 10 cm length (Sutter Instrument Co., Novato, CA) or 1mm outer diameter, 0.8mm inner diameter non-filamented quartz capillaries of 10 cm length (VitroCom, Mountain Lakes, NJ). Pipettes were pulled using a Sutter P-2000 laser-based pipette puller with the parameters: HEAT 800, FIL 4, VEL 60, DEL 128, and PULL 100 (1×0.7mm filamented) or HEAT 750, FIL 4, VEL 50, DEL 150, PULL 55 (1×0.8mm non-filamented). Chemical vapor deposition (CVD) was performed on the pipettes in a 3-zone horizontal tube furnace (Carbolite HVS, Hope Valley, UK) with a 1.3″ inner diameter quartz tube at 900 °C, with flow conditions of 400 sccm methane and 600 sccm argon, for a 3 hour duration. During deposition, the pipette tips were oriented against the flow of the gas, i.e., the tip pointed upstream. Pipettes were cooled under argon flow to prevent the oxidation of the carbon at elevated temperatures. The carbon deposited selectively inside the pipette, not on the outer surface due to the gas confinement effect described by Singhal et al.[28] No catalyst was used.

The carbon-coated pipettes were etched in 5:1 buffered hydrofluoric acid (Transene Co. Inc., Danvers, MA) for 10 to 15 minutes, followed by a 10-minute rinse in deionized water. A friction grip was used to hold pipettes, and a manual manipulator was used to lower the tips into beakers of either HF or water. Short CNPEs for use in the *Drosophila* larval ventral

nerve cord were prepared with an etch time of 60 seconds. The pipettes were inspected under an optical microscope (Olympus Corp. BX-51) and imaged with a SEM (FEI Quanta 600 ESEM, Hillsboro, OR). The tip outer diameter ranged from 50 to 400 nm (average of 250 nm) and the exposed carbon tip length depended on the etch time and tolerances of the pipette puller, but typically was 125 to 175 μm for etch times of 10 to 15 minutes and 5 to 10 μm for a 1 minute etch. For additional details on CNP fabrication we refer readers to the following references.[12,28,29]

To ensure the pipettes were properly sealed, CNPEs were connected to the headstage of a HEKA EPC 10 patch-clamp amplifier using a standard 1.0mm HEKA pipette holder. The pipettes were also connected to a pressure-injection pump (Eppendorf Femtojet, Happauge, NY). The CNPE tip was submerged in phosphate buffered saline (HyClone, PBS1X) and a 0.5 mm diameter silver chloride wire (WPI Inc., Sarasota, FL) was used as a counter/reference electrode in solution in a 2-electrode configuration. The digital lock-in module of the PATCHMASTER software was used to measure the equivalent capacitance of the CNPE interface with a 10mV, 1kHz sinusoidal potential, as the pressure within the pipette was adjusted between 0 and 300 kPa. The tip was first checked for bubbles, which would indicate a broken tip, and then the capacitance was monitored with changing pressure. The capacitance is proportional to the electrode interfacial area, and if it is stable with varying pressure it indicates that there is minimal capillary rise and that the tip is well-sealed. CNPEs that were not well sealed were discarded.

Scanning electron microscopy

Scanning electron microscopy was performed in a FEI Quanta 600 ESEM (FEI, Hillsboro, Oregon) in secondary electron mode. CNPEs were adhered to a standard sample mount with carbon tape such that the CNPE axis was orthogonal to the electron beam. A short working distance (5mm) and low accelerating voltage (2 keV) were used in high-vacuum mode to attain enhanced surface detail and to minimize charging effects.[30] The Environmental SEM provides a large sample chamber that allows CNPEs to be mounted without breaking or modification.

Instrumentation and Electrochemistry

Fast-scan cyclic voltammograms were collected using a Chem-Clamp potentiostat (Dagan, Minneapolis, MN). TarHeel CV software (gift of Mark Wightman, University of North Carolina) was used for data collection and analysis. The hardware and data acquisition were the same as previously described.[31] A triangular waveform was applied to the electrode. The electrode was scanned at a scan rate of 400 V/s from −0.4 V to 1.3 V and back at a frequency of 10 Hz unless otherwise noted. A Ag/AgCl wire was used as a reference electrode. The flow injection apparatus with a six-port, stainless steel HPLC loop injector used is the same as previously described.[32] Electrodes were tested with a 5 s injection time. Because carbon is deposited on the entire length of the CNPEs, a direct electrical connection was made between a silver wire and the inner carbon lining of the CNPE at its distal end in the Universal Pipette Holder (HB180, Dagan Corp., Minneapolis, MN). No backfill solution was used. The holder was connected to a 1 MΩ headstage (Dagan Corp., Minneapolis, MN).

Endogenous Dopamine Evoked by CsChrimson Channelrhodopsin Stimulation

Virgin females with UAS-CsChrimson inserted in attp18[33] (a gift of Vivek Jayaraman) were crossed with th-GAL4 flies (a gift of Jay Hirsh). Resulting heterozygous larvae were shielded from light and raised on standard cornmeal food mixed 250:1 with 100 mM all-trans-retinal. A small amount of moistened Red Star yeast (Red Star, Wilwaukii, WI) was placed on top of the food to promote egg laying. The central nervous system of a third instar wandering larva was dissected in the buffer. Isolated ventral nerve cords were prepared and recorded from as previously described.[34] The electrode was allowed to equilibrate in the tissue for 15 minutes prior to data collection. A baseline recording was taken for 10 seconds prior to stimulation. Red-orange light from a 617 nm fiber-coupled high-power LED with a 200 μm core optical cable (ThorLabs, Newton, NJ) was used to stimulate the CsChrimson ion channel. The light was modulated with Transistor-Transistor Logic (TTL) inputs to a T-cube LED controller (ThorLabs, Newton, NJ), which was connected to the breakout box. TTL input was driven by electrical pulses controlled by the TarHeel CV software.

Statistics

Statistics were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Data are reported as the mean ± standard error of the mean (SEM) for n number of different electrodes. Significance was determined by unpaired *t*-tests and defined as $p \leq 0.05$.

Results and Discussion

The first goal of this study was to electrochemically characterize CNPEs using FSCV. FSCV allows measurements of rapid changes in neurotransmitter concentrations. CNPEs have traditionally been manufactured with open tips[25], but that is not suitable for rapid electrochemistry as sample would wick up into the pipette. Here, the fabrication was modified slightly to grow enough carbon to make an electrode with a solid tip ~250 nm in diameter. The cone angle was 1.6° and the exposed carbon length, controlled with etch time, ranged from 5 μm to 175 μm. The second goal was to test the suitability of CNPEs for dopamine measurements in *Drosophila* larvae, which have a very small central nervous system. This nanoscale electrode would allow for high spatial resolution measurements.

Fabrication of Carbon Nanopipette Electrodes

The carbon nanopipette electrode (CNPE) consists of a pulled-glass/quartz pipette coated with a layer of pyrolytic carbon along its entire inner surface to a thickness sufficient to seal the pipette's narrow opening (Figure 1). Figure 1A shows the fabrication process. First, a quartz capillary was pulled into a fine-tipped nanopipette (Figure 1A (i)). Next, carbon was deposited by CVD until the tip was sealed with carbon (Figure 1A (ii)). Further up, the carbon-coated pipette was still hollow, which facilitates electrical connection via contact with a metal wire. Subsequent to the carbon deposition, the quartz/glass at the tip of the CNPEs was etched in buffered hydrofluoric acid to expose a desired length of a tapered carbon cylinder (Figure 1A (iii)). The exposed length was controlled by the etching time. For the pipette geometry used here, this corresponds to an exposed length of 12.5–17.5 μm/minute as measured with an optical microscope and confirmed with SEM. Figure 1B shows an example CNPE tip with ~170 μm exposed carbon. The interface between the exposed

carbon and the quartz insulation is clearly visible. Figure 1C is an enlarged image of the quartz/carbon interface. The tip diameter of the individual CNPEs used in this work was measured with SEM and the range of tip sizes was 50–400nm (Figure 1D), with an average of 250 nm. Since the glass/quartz template controls the outer dimensions of the deposited carbon, it is likely that the primary source of tip variability stems from the pipette puller parameters. Even with the above variability, this fabrication method consistently yields sub-micron sized tips, an order of magnitude smaller than traditional CFMEs. All electrodes were tested for tip closure prior to use.

Comparison of CNPEs and CFMEs

Dopamine was chosen to analyze with CNPEs because it is an important neurotransmitter, easily oxidized, and adsorbs to carbon surfaces.[35] Dopamine plays an important role in reward, addiction, and motor behaviors.[36] Figure 2 shows examples of background-subtracted cyclic voltammograms (A and B) as well as normalized current vs time plots (C and D) for dopamine at two different waveforms for a CNPE (dashed line) and a CFME (solid line). The first waveform (referred to as the 1.0 V waveform) scanned from −0.4 to 1.0 V vs. Ag/AgCl/Cl^- at a scan rate of 400 V/s and a frequency of 10 Hz (A and C). The second waveform (referred to as the 1.3 V waveform) was the same as the 1.0 V waveform except the switching potential was 1.3 V (B and D). Figures 2B and D show larger currents for both CNPEs and CFMEs with the 1.3 V potential limit than the ones with the 1.0 V potential limit, as expected due to oxidation of carbon.[37] The CFME had more current for dopamine than the CNPE for both waveforms. However, the peak oxidation voltage was lower for CNPEs (Figures 2A and B). CNPEs and CFMEs have a similar time response at the 1.3 V waveform, although CNPEs were slightly slower with the 1.0 V waveform (Figures 2C and D).

Table 1 shows average peak oxidative currents ($i_{p,a}$), background currents, and the difference between the oxidative and reductive peak potentials (ΔE_P) for CNPEs (150 μm in length, ~250 nm diameter) and CFMEs (50–75 μm in length, 7 μm diameter) at the 1.0V and 1.3V waveforms. The average peak oxidative current ($i_{p,a}$) for 1 μM dopamine is about 30% lower for CNPEs than CFMEs at both waveforms; however, the difference was not significant (unpaired t-test, 1.0 V waveform, p=0.1273; 1.3 V waveform, p=0.2353). The background currents were obtained in the absence of dopamine and compared using the maximum values during the forward scans. Background currents for CNPEs were higher than CFMEs, although not significantly (unpaired t-test, 1.0 V waveform, p=0.2484; 1.3 V waveform, p=0.3730). Background current is proportional to electrode surface area. The 7 μm diameter, 62 μm long CFME has a surface area of 1410 μm^2, while the 150 μm long CNPE with a cone angle of 1.6° and a tip diameter of 250 nm has a surface area of 1970 μm^2. Thus, the CNPEs are about 1.4 times larger than CFMEs, which is consistent with background current ratios of 1.5 and 1.4 documented in Table 1 for the 1.0V and 1.3V waveforms, respectively. For *in vivo* measurements, the exposed carbon length was successfully reduced below 10 μm (8.2 ± 1.4 μm) by decreasing the quartz etch duration time. This size was appropriate for producing sufficient current magnitude from a highly localized region.

A lower oxidation current correlated with lower background charging current was expected; however, this was not true for the CNPEs of the sizes used in our experiments. The ratio of background current to peak oxidative current is significantly higher for CNPEs than CFMEs at both waveforms signifying CNPEs are less sensitive per unit area than CFMEs. At the 1.0 V waveform, CNPEs have a background current to peak oxidative current of 75 ± 10; whereas, the CFME ratio is 29 ± 4 (unpaired t-test, $p<0.005$, CNPE $n=8$, CFME $n=6$). For the 1.3 V waveform, CNPEs have a background current to peak oxidative current of 42 ± 5, and the CFME ratio is 21 ± 4 (unpaired t-test, $p<0.005$, CNPE $n=11$, CFME $n=6$). These differences in sensitivity per unit area may due to different types of carbon in CFMEs vs CNPEs or capacitive coupling with the thin quartz near the CNPE tip.

ΔE_p is the difference between the oxidative and reductive peak potentials. The average ΔE_p for CNPEs is significantly lower than CFMEs for the two waveforms (Table 1, unpaired t-test $p<0.0001$). As in the case of the CFMEs, there is no significant difference in ΔE_p for CNPEs for the 1.0 V and 1.3 V waveforms ($p=0.4010$). The decrease in ΔE_p for CNPEs compared to CFMEs implies decreased overpotential for dopamine. However, one would expect to observe higher sensitivity with lower overpotential, and the CNPEs had less sensitivity per unit area. Alternatively, the IR drop may be different for the different materials. The lower ΔE_p might also be due to differences in diffusion and adsorptive behavior due to different carbon types and geometries between the CNPEs and CFMEs. The CNPE carbon is amorphous with graphitic islands, and has surface functional groups that depend on deposition conditions.[38] In contrast, the CFMEs are predominantly graphitic. The lower ΔE_p could be due to different functional groups or the amount of edge plane graphite sites on CNPEs, which play a role in electron transfer and adsorption reactivity.[14]

CNPEs were also used to detect serotonin and octopamine, which are other electroactive signaling molecules in the *Drosophila* central nervous system. Figure 3 shows examples of background-subtracted cyclic voltammograms with inserts of peak oxidation current over time for octopamine (A and B) and serotonin (C and D). The standard waveform (−0.4 to 1.3 V at 400 V/s) was applied at 10 Hz to CNPEs for octopamine detection (Fig. 3A) and serotonin detection (Fig. 3C). In addition, specialized waveforms previously developed for these neurotransmitters were applied, a positive waveform for octopamine detection[39] (0.1 to 1.0 V and back at 600 V/s, Figure 3B) and the serotonin waveform for serotonin detection[40] (0.1 to 1.0 to −0.1 to 0.1 at 1000 V/s, Figure 3D). Both analytes exhibited less electrode fouling when using their respective specialized waveforms, which is indicated by a faster return of the current to baseline after removal of the analyte from the flow cell. For octopamine, a strong secondary peak is observed using the positive waveform at CNPEs (Fig 3B), whereas less secondary peak was observed when using the positive waveform with CFMEs[39]. This could indicate adsorption of the secondary oxidation product of octopamine to the surface of CNPE despite the positive holding potential.

The average peak oxidation current of 1 μM serotonin was 43 ± 11 nA (n=4) for CFMEs and 33 ± 2 nA (n=4) for CNPEs. The average peak oxidation current of 1 μM octopamine was 10 ± 3 nA (n=4) for CFMEs and 2.8 ± 0.2 nA (n=4) for CNPEs. The ratio of background charging current to peak oxidative current for serotonin is significantly lower for CNPEs (2.5 ± 0.4, n=4) than CFMEs (11 ± 2, n=4) (unpaired t-test, $p=0.0117$). However, the ratio

of background charging current to peak oxidative current for octopamine it is not significantly different between CNPEs (52 ± 15, n=4) and CFMEs (31 ± 6, n=4) (unpaired t-test, p=0.2489). Therefore, CNPEs have higher sensitivity for serotonin than CFMEs, while having the same sensitivity for octopamine.

CNPE Stability Over Time

Electrode stability is important for *in vivo* experiments, which can last hours.[41] To test stability, the 1.0 V waveform was applied continuously to the CNPE in buffer and the response to a five-second injection of 1 μM dopamine was measured every hour. Current was normalized for each electrode to the first response to dopamine to take into account differences in individual electrodes. Figure 4 shows that at the 1.0 V waveform, the CNPE sensitivity dropped to 32% of the original current after 3 hours. CFMEs are stable over the same time.[42] The left inset shows example cyclic voltammograms taken at the first injection of dopamine and after three hours for the 1.0 V waveform. The oxidative and reductive peak voltages shifted outward, signifying slower electron transfer kinetics accompanied the decrease in sensitivity. We hypothesize that the surface of the electrode is fouled, which would reduce the sensitivity and slow the transfer kinetics; however, dopamine diffuses to the electrode and some current is still measured from electron tunneling.

This problem of electrode surface fouling is overcome by electrochemically renewing the surface. Scanning to 1.3 V allows for the regeneration of a fresh carbon surface and maintains electrode sensitivity.[37] For the stability experiments using the 1.3 V waveform, the CNPEs were allowed to stabilize with the waveform applied for 30 minutes before taking the initial measurement due to the oxidation of the electrode surface. If not allowed to stabilize, the signal actually increases during this time due to increased surface area from carbon-carbon bonds breaking and increased adsorption due to carbon functional groups.[31] The peak oxidative current was constant over three hours (Figure 4) with the 1.3 V waveform, indicating that CNPEs are stable at this waveform and suitable for longer *in vivo* studies. This is confirmed by the right inset CVs which show the sensitivity and the electron transfer kinetics remained the same after three hours. Three hours is longer than a typical *Drosophila* experiment, and some electrodes were used for much longer or in multiple larvae and showed no degradation in signal. From this stability experiment, we determined that the 1.3 V waveform was most appropriate and we used this waveform for the remaining studies.

CNPE characterization

Figure 5 shows that the CNPE peak oxidation current for 1 μM dopamine is proportional to the scan rate, v (R^2=0.984, *n*=4). The frequency was varied to keep equal time between scans. Current is normalized to the maximum value per electrode to minimize effects due to varying surface areas of different electrodes. For a diffusion-controlled process we anticipate a $v^{1/2}$ proportionality with peak current, arising from the diffusive time scale in the transport equation. For an adsorption-controlled process we expect a proportionality with scan rate, which arises upon the inclusion of adsorption kinetics *via* a Langmuir or linearized Langmuir isotherm.[43] This plot indicates that the kinetics are more adsorption-controlled than diffusion-controlled (plot of i vs $v^{1/2}$ yields an R^2=0.956, *n*=4), similar to carbon-fiber

microelectrodes.[35] Figure 5B shows peak currents for various dopamine concentrations (100 nM to 10 μM). Current is linear with concentration up to 10 μM. The average LOD, calculated from the 100 nM data, was 25 ± 5 nM (*n*=3).

Measurements of Endogenous Dopamine in Drosophila Evoked by CsChrimson Stimulation

To test the use of CNPEs to detect endogenous dopamine in *Drosophila*, dopamine release was stimulated with the red light sensitive cation channel CsChrimson and detected using a CNPE. CsChrimson is a channel that is more red-shifted than the traditional Channelrhodopsin-2 which has been used in optogenetics.[33] Upon red light stimulation, the CsChrimson channels open and cations enter the cell, depolarizing the neuron and causing an action potential. A th-GAL4 driver was used to express UAS-CsChrimson in only the dopaminergic cells of the heterozygous crossed flies.[33] The CNPE does not penetrate cells but measures extracellular changes in dopamine that occur due to volume transmission.

CNPEs with lengths of 125–175 um would be suitable for measurements in mammalian tissues. However, because the *Drosophila* larval VNC is so small (only 200 μm in length), smaller CNPEs were needed. CNPEs with short exposed tips 8.2 ± 1.4 μm in length, were characterized and the average current for 1 μM DA at these electrodes is 0.39 ± 0.08 nA. However, the noise is also small and the S/N values are still good (37 ± 4). Figure 6 shows the cyclic voltammogram for dopamine (Fig. 6A) when a short, 7 μm long CNPE (Fig. 6B) was used to detect stimulated release. The cyclic voltammogram has the characteristic oxidation and reduction peaks for dopamine. A false color plot of the data (Fig. 6C) shows the dopamine release during a 5 second long red light stimulation. Consecutive voltammograms are plotted over time on the x-axis, the y-axis is applied voltage, and current is shown in false color. Green/purple is dopamine oxidation and blue/yellow is dopamine reduction. The concentration versus time plot is made using an *in vitro* calibration to convert maximum peak oxidation current to dopamine concentration. Dopamine is cleared from the extracellular space by dopamine transporters[8] and the concentration begins to decrease after the stimulation is finished. Using this short CNPE, endogenous dopamine was successfully detected, verifying that CNPEs are suitable for dopamine measurements in *Drosophila* tissue.

The batch fabrication of robust, nanosized electrodes suitable for *in vivo* studies is difficult and most methods to fabricate smaller electrodes have involved etching a single electrode by hand. The CNPEs developed here are batch fabricated and have the robustness to be implanted in tissue. The sensitivity per unit area for CNPEs with FSCV is slightly less than traditional CFMEs for dopamine, but CNPEs are able to measure endogenous dopamine in *Drosophila* larvae. Reducing the length to 5 to 10 μm makes these CNPEs useful for high spatial resolution measurements in *Drosophila*. The Mirkin group has recently made disk electrodes from CNPEs with recessed tips for use as scanning electrochemical microscopy tips.[27] Direct fabrication or mechanical polishing of CNPEs to disk electrodes that are flat and not recessed would allow future measurements from discrete regions and at single neuronal cells. The ability to measure endogenous dopamine release in *Drosophila* will allow for studies on how genetics or behavior affects neurotransmission regulation. CNPEs

could also be applied to study other neurotransmitters *in vivo* such as serotonin and octopamine in the future.

Conclusions

We fabricated solid-tipped CNPEs that allow high spatial resolution measurements of dopamine. CNPEs are batch fabricated and the electrode geometry can be easily modified via puller parameters, deposition conditions, and etch duration, to produce electrodes of desired tip size, taper, and exposed surface area. The nanoscopic tip provides for highly localized measurements, and its sharp conical shape makes it ideal for implantation into small regions such as the dopaminergic centers of the *Drosophila* brain. CNPEs were characterized for the first time with FSCV and their electrochemical signals for dopamine were suitable for *in vivo* measurements in *Drosophila*. CNPEs have fast electron transfer kinetics, stability, and good sensitivity. For dopamine, they are less sensitive per unit area compared to CFMEs, but still have sufficient signal for *in vivo* measurements. Interestingly, CNPEs showed improved sensitivity for serotonin compared to CFMEs. Coupled with FSCV, CNPEs could be used to measure real-time dopamine changes in specific regions of the adult fly, where the neuropil are only a few microns in diameter. Future studies in specific brain regions will give a better understanding of neurotransmission underlying discrete physiological processes.

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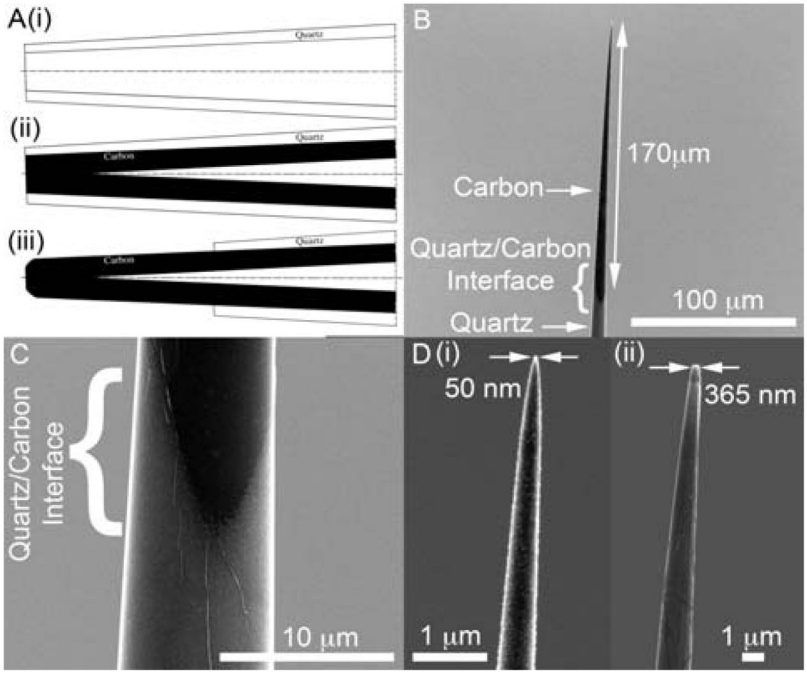

Figure 1.

Carbon Nanopipette Electrodes (CNPE). A) A schematic of the CNPE fabrication process. i) Quartz/glass pipette is pulled to form a template. ii) The pulled pipette is placed in a furnace in the presence of precursor hydrocarbons and carbon is deposited selectively along the pipette's interior surface for a sufficient amount of time until the tip is sealed with carbon. No catalyst was used. iii) The glass/quartz at the tip is wet-etched to expose a desired length of the underlying carbon. B) SEM image of the CNPE tip profile. C) Enhanced SEM view of the quartz/carbon interface. D) Enhanced SEM view of the CNPE tips. i) Tip diameter 50 nm. ii) Tip diameter 365 nm (same CNPE as in B and C). The tip and edges appear brighter due to SEM charging effects.

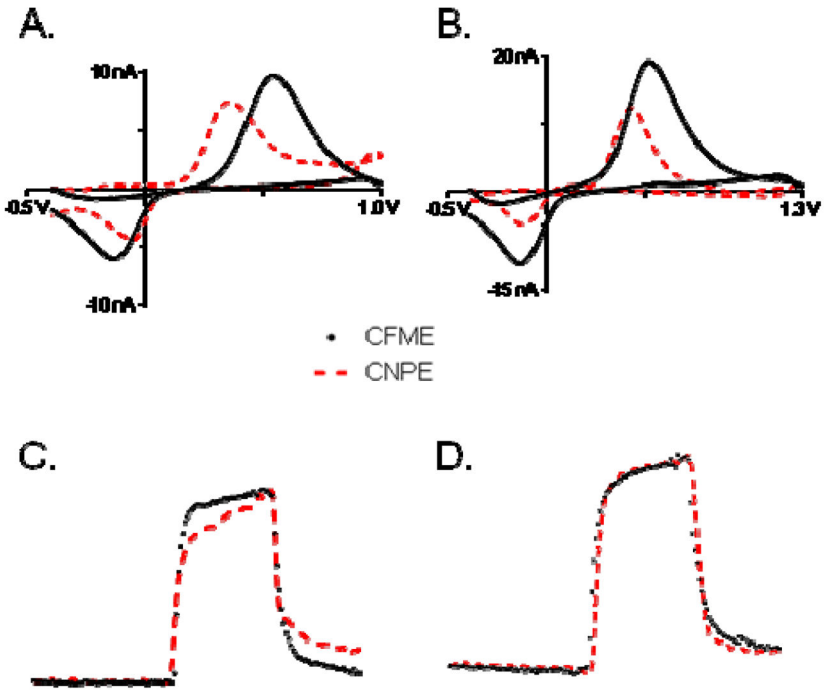

Figure 2.

Example data for a 150 μm long CNPE (red dashed line) and a 50 μm long CFME (black line) with the 1.0 V and 1.3 V waveforms. Background-subtracted cyclic voltammograms for 1 μM dopamine are shown for (A) the 1.0 V waveform and (B) the 1.3 V waveform. Normalized current versus time plots at peak oxidation voltage for (C) the 1.0 V and (D) 1.3 V waveforms.

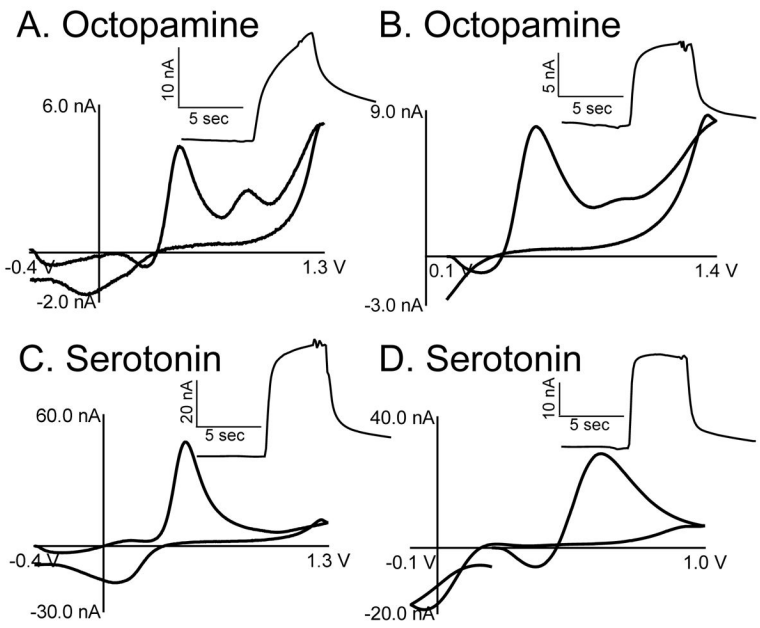

Figure 3.

Example data of octopamine and serotonin detection using a 150 μm long CNPE. Background-subtracted cyclic voltammograms for 1 μM octopamine are shown for A.) the 1.3 V (dopamine) waveform and B.) the positive waveform, 0.1 to 1.4 V and back at 600 V/s. Background-subtracted cyclic voltammograms for 1 μM serotonin are shown for C.) the 1.3 V (dopamine) waveform and D.) the serotonin waveform, 0.1 to 1.0 to −0.1 to 0.1 V at 1000 V/s. The insets show current versus time plots of the main peak oxidation currents for each waveform.

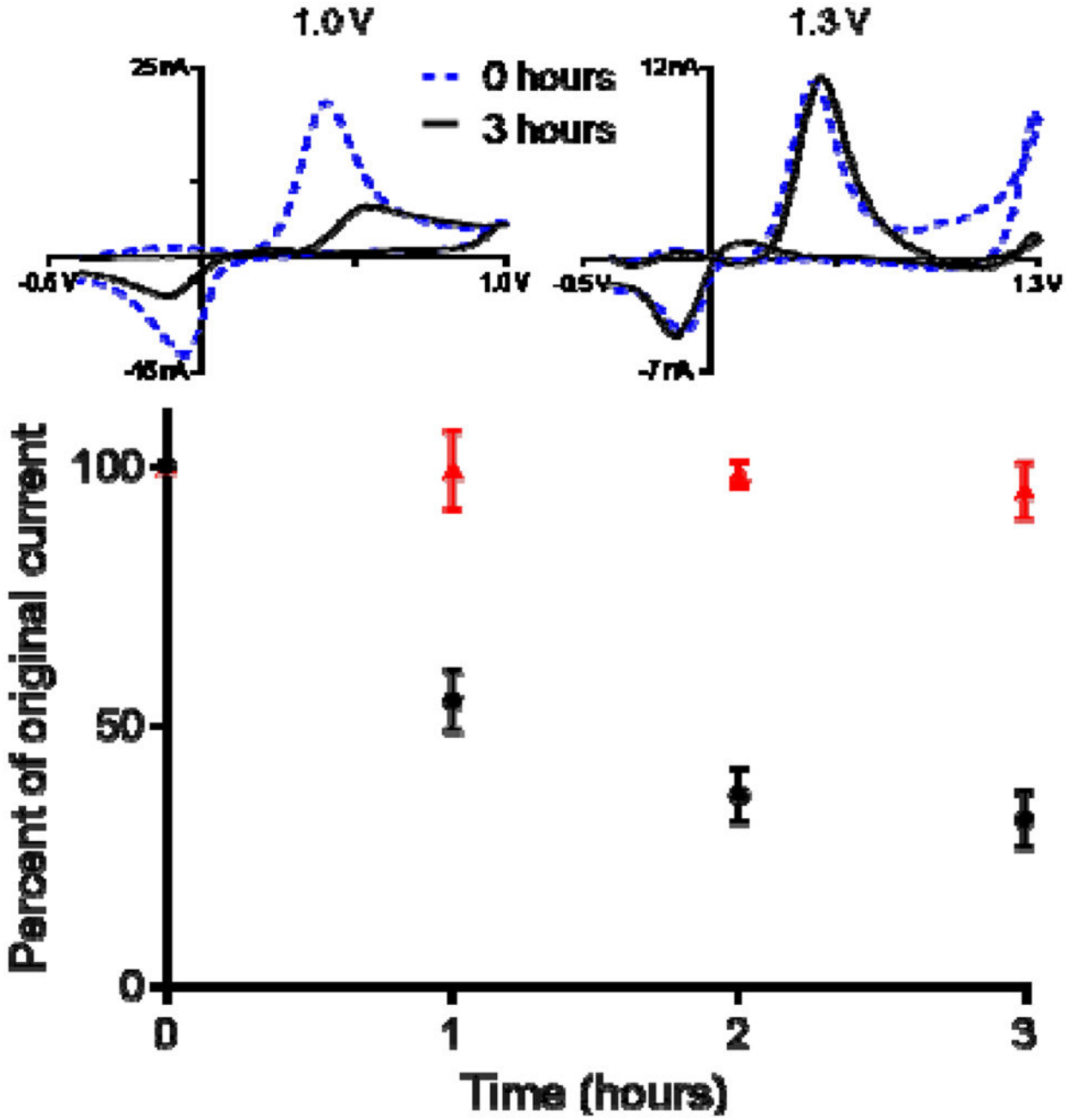

Figure 4.

Stability of CNPEs over three hours at the 1.0 V waveform (black circles) and 1.3 V waveform (red triangles) (n=4 electrodes). Insets show example cyclic voltammograms for 1 μM dopamine with both waveforms at initial measurements and after three hours.

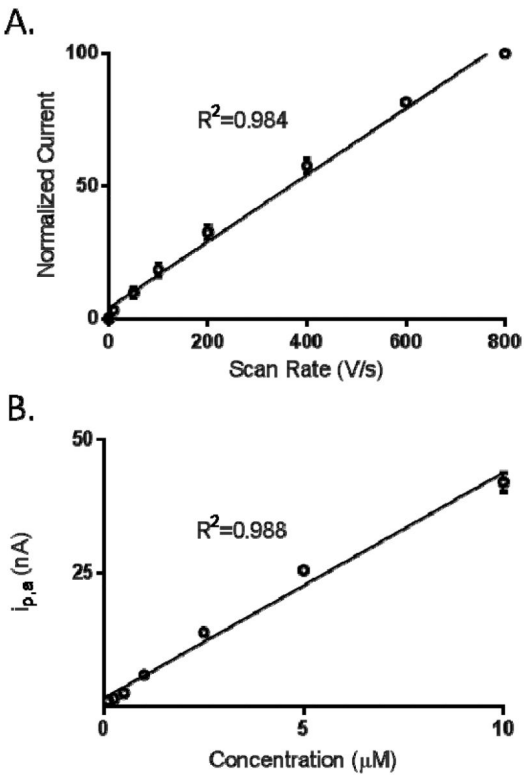

Figure 5.

Electrochemcial characterization of CNPE. (A) Normalized peak oxidative current for 1 μM dopamine vs scan rate. The plot is linear for CNPEs (n=4) showing the kinetics are adsorption-controlled. (B) Peak oxidative current vs concentration (n=3). CNPEs show a linear response in current up to 10 μM. Solid lines are best fits of the data.

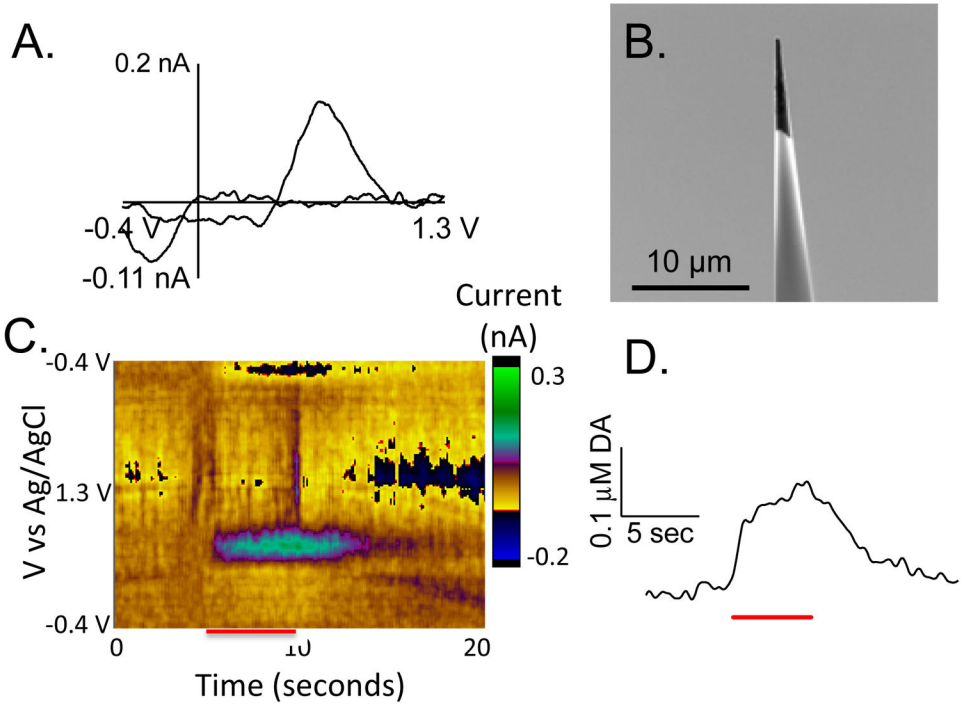

Figure 6.

Example CNPE measurement of endogenous dopamine evoked by a 5 second continuous red light stimulation. (A) Background-subtracted cyclic voltammagram of evoked dopamine. (B) SEM of a short CNP like the one used *in vivo*. (C) Color plot showing stimulated dopamine in a *Drosophila* larval ventral nerve cord. Red light was applied from 5 to 10 seconds. (D) Extracellular concentration of dopamine over time as red light stimulates release (red line).

Table 1

Average electrochemical parameters for 1 μM dopamine detection for CNPEs and CFMEs at 1.0 V and 1.3 V waveforms.

	$i_{p,a}$ (nA)	Background current (nA)	ΔEP (V)
CFME 1.0V[a]	11 ± 2	330 ± 70	0.69 ± 0.02
CNPE 1.0V[b]	7.4 ± 2	490 ± 110	0.50 ± 0.02[d]
CFME 1.3V[a]	19 ± 2	410 ± 80	0.66 ± 0.01
CNPE 1.3V[c]	14 ± 3	570 ± 160	0.52 ± 0.01[d]

$i_{p,a}$ is oxidative peak current; ΔEP is the difference between the oxidative and reductive potentials.

[a] n=6.

[b] n=8.

[c] n=11.

[d] Data for CNPEs are significantly different than for CFMEs at the same waveform (p<0.0001).