

Neurochemical measurements in the zebrafish brain

Lauren Jones¹, James McCutcheon¹, Andrew Young¹, William Norton^{1*}

¹Neuroscience, Psychology and Behaviour, University of Leicester, United Kingdom

Submitted to Journal:
Frontiers in Behavioral Neuroscience

Article type:
Original Research Article

Manuscript ID:
148886

Received on:
22 Apr 2015

Revised on:
18 Aug 2015

Frontiers website link:
www.frontiersin.org

In review

Neurochemical measurements in the zebrafish brain

Lauren J Jones, James E McCutcheon, Andrew MJ Young* and William HJ Norton*

Department of Neuroscience, Psychology and Behaviour, University of Leicester, Leicester, LE1 7RH, UK

*Correspondence:

Dr Andrew Young

Department of Neuroscience, Psychology and Behaviour

University of Leicester,

University Rd,

Leicester, LE1 7RH, UK

amjy1@le.ac.uk

and

Dr William Norton

Department of Neuroscience, Psychology and Behaviour

University of Leicester,

University Rd,

Leicester, LE1 7RH, UK

whjn1@le.ac.uk

7330 words and 10 figures

Abstract

The zebrafish is an ideal model organism for behavioural genetics and neuroscience. The high conservation of genes and neurotransmitter pathways between zebrafish and other vertebrates permits the translation of research between species. Zebrafish behaviour can be studied at both larval and adult stages and recent research has begun to establish zebrafish models for human disease. Fast scan cyclic voltammetry (FSCV) is an electrochemical technique that permits the detection of neurotransmitter release and reuptake. In this study we have used in vitro FSCV to measure the release of analytes in the adult zebrafish telencephalon. We compare different stimulation methods and present a characterisation of neurochemical changes in the wild-type zebrafish brain. This study represents the first FSCV recordings in zebrafish, thus paving the way for neurochemical analysis of the fish brain.

Keywords: Zebrafish, Fast scan cyclic voltammetry, Adult brain, Dopamine, 5-HT, Histamine, pH, Neurochemistry

In review

Introduction

A central goal of neuroscience is to understand how the brain processes stimuli in order to tailor an appropriate behavioural response. Initially, each behaviour was thought to be driven by a dedicated neural circuit in the brain (Zupanc and Lamprecht, 2000). However, recent research suggests that discrete behaviours can be produced by the interaction of diffuse neural networks with overlapping functions (Bargmann, 2012). Thus, dramatically different behaviours can be driven by the same neurons acting in parallel circuits. Rather than being hard-wired entities, neural circuits exhibit plasticity due to short-term neuromodulatory activity and longer-term structural reorganisation at the synaptic level (Zupanc and Lamprecht, 2000; Bargmann, 2012). Therefore, a combination of approaches combining information from a range of scientific disciplines is needed to identify the network components that drive behaviour.

The zebrafish is a powerful model organism for developmental biology and neuroscience. Zebrafish are also an ideal species to investigate the neural circuits that drive behaviour since their relative transparency until larval stages permits the visualisation and manipulation of neurons within the intact brain (Feierstein et al.; Fetcho and Liu, 1998; Arrenberg and Driever, 2013; Bonan and Norton, 2015). Neural circuits can be mapped using calcium imaging, bioluminescence or electrophysiology to monitor neural activity in freely behaving fish (Higashijima et al., 2003; Naumann et al., 2010). Techniques such as genetic ablation or optogenetics can then be used to functionally connect circuits to behaviour (Nagel et al., 2003; Curado et al., 2007; Zhang et al., 2007; Del Bene and Wyart, 2012). An alternative approach, based upon forward genetics, is to identify mutant lines that exhibit interesting behavioural phenotypes. The expression profile of the mutated loci can be used as a starting point to examine the alterations to brain structure and function that underpin aberrant behaviour (Webb et al., 2009; Norton et al., 2011; Ziv et al., 2013).

Previous studies have used quantitative PCR, immunohistochemistry and electrophysiology to investigate the function of the zebrafish brain. In our laboratory we complement these approaches by using fast-scan cyclic voltammetry (FSCV) to quantify the release- and reuptake of neurotransmitters at the synapse on a sub-second time-scale (Stamford, 1990; Heien et al., 2004). A voltage waveform is applied to a carbon fibre microelectrode causing oxidation and reduction of electroactive compounds at the surface of the electrode (Stamford, 1990; John and Jones, 2007a). These oxidation and reduction reactions lead to changes in electrical current which are proportional to the concentration of the compound being measured. This data can be visualised as a colour plot where current is encoded using a false colour scheme and plotted against both applied electrical potential (E_{app}) and time (Heien et al., 2004). Typically, in vitro neurotransmitter release is evoked by using either electrical stimulation or bath application of a high concentration of potassium. Different analytes, including many neurotransmitters, can be identified on the basis of their voltammograms (a plot of applied voltage against current) using attributes such as the position, shape and relative amplitude of the oxidation and reduction peaks (Stamford, 1990; Heien et al., 2004). Further specificity can be achieved by placing the recording electrode into brain areas containing a single neurotransmitter or by applying specific drugs that modify neurotransmitter reuptake (Dankoski and Wightman, 2013). Finally, if stimulation evokes the release of several analytes, the overlapping voltammograms can be separated by principal component analysis (Heien et al., 2004).

In this study we present a method for FSCV recordings in sagittal slices of the adult zebrafish brain and characterise the release of analytes in the telencephalon. This research provides a basis for examination of zebrafish mutants that display intriguing behavioural phenotypes by uncovering alterations to the dynamics of neurotransmitter release in the brain.

Materials and Methods

Fish Stocks

All experiments were performed on adults of the AB/AB wild-type strain. Standard fish keeping protocols and conditions were followed (Westerfield, 1995). Fish were anaesthetised in MS333 and culled by decapitation (a Schedule 1 procedure under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012). All protocols are covered by appropriate personal licences: PIL 60/13671 (WHJN) and PIL I98F58CD7 (LJJ).

FSCV equipment

The setup for FSCV was custom built and consists of a tissue bath, stimulating electrode, recording and reference electrodes connected to a computer and amplifier (Fig. 1A). Carbon fibre microelectrodes (tip size 7x120 μm) were used as recording electrodes and an Ag/AgCl electrode as a reference. The recording and reference electrodes were connected to a potentiostat and headstage circuit (ChemClamp, Dagan Instruments, USA) and a computer running TarHeel (Chapel Hill, University of North Carolina) voltammetry software. The waveform (Fig. 1A) was applied at 10 Hz.

Electrode manufacture

Glass capillary electrodes were used for all our FSCV experiments. Electrodes were manufactured as described in Fortin et al., 2015. A single carbon fibre was aspirated into a borosilicate glass capillary (World Precision Instruments, 100 mm length, 1/0.58 mm OD/ID). The glass was pulled to a fine tip using a vertical needle puller (PE-21, Narishige) and the exposed carbon fibre was cut to a length of 100 μm using a scalpel. A wire coated in silver conductive paint (Coating Silver Print II, GC Electronics) was inserted into the capillary, secured with a gold pin (Newark) and heat shrink-wrapped to the capillary (FP-301, 3M). In each case, electrodes were tested to ensure a suitable background (non-Faradaic) current profile by applying the voltage waveform (see below) at 60 Hz. If the current signal was adequate the electrode was cycled at 60 Hz for a minimum of 15 minutes to reduce background drift.

Voltage input waveforms

The voltage input waveform was scanned at a rate of 400 V/second in the following pattern: 0 V \rightarrow +1.2 V \rightarrow -0.6 V \rightarrow 0 V (John and Jones, 2007a). With this waveform, dopamine oxidises at \sim +0.6 V and shows one reduction peak at \sim -0.2 V; 5-HT oxidises at \sim +0.6 V and shows two reduction peaks at \sim 0 V and \sim -0.5 V; and histamine shows two reduction peaks on the forward scans of the waveform at \sim +0.25 V and \sim -0.4 V and an oxidation peak on the reverse scan at \sim +1.05 V.

Flow cell experiments

Flow cell experiments were performed in a custom-built Y-shaped chamber (University of Illinois at Chicago Biology Workshop (Sinkala et al., 2012)). The cell permits a carbon-fibre electrode to be exposed to known concentrations of neurotransmitter solutions. All analytes were dissolved in artificial fish cerebrospinal fluid (aCSF) and delivered by gravity perfusion at a rate of 1 ml per minute. aCSF (pH 7.4) contained (in mM) 131 NaCl, 2 KCL, 1.25 KH_2PO_4 , 2 MgSO_4 , 20 NaHCO_3 , 2.5 CaCl, and 10 glucose (Vargas et al., 2011). For some experiments, we prepared aCSF containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) adjusted to pH 7.4. The voltage waveform was applied to electrodes at 60 Hz for 15 minutes prior to the start of each experiment to precondition the electrode (Fortin et al., 2015). Known concentrations of dopamine, histamine, 5-HT, and pH-adjusted aCSF were perfused for 5 seconds and changes in current were recorded.

Preparation of tissue for FSCV

Following decapitation, the brain was manually dissected from the skull in a Petri dish containing ice-cold aCSF. Brains were sliced sagittally and transferred to a tube containing additional ice-cold aCSF. Sections were mounted in an organ bath and perfused with oxygenated aCSF (constantly bubbled with 95% O_2 and 5% CO_2 and warmed to 32°C by a Peltier heater) at a rate of 1.5 ml per minute. Tissue sections were allowed to equilibrate in warmed aCSF for 10-15 minutes before recording began. The

flow rate was regulated by a gravity flow system containing an intravenous dial flow-regulator (World Precision Instruments) and waste aCSF was aspirated using a Dymax 5 suction pump (Charles Austen pumps and RS Components). A micromanipulator (R.C-2R adjustable clamp, Narishige) was used to insert the electrodes into the telencephalon at a depth of approximately 150 μm (Fig. 1B).

Stimulation of neurotransmitter release

Neurotransmitter release was evoked by either bath application of a high concentration of potassium or electrical stimulation. Potassium-evoked release was performed by perfusing tissue with high K^+ (100 mM, replacing an equimolar amount of NaCl) aCSF for 1 minute once a stable 30-second baseline recording had been obtained. Electrically-evoked release was performed with a bipolar stimulating electrode placed close to the carbon fibre recording electrode within the dorsal telencephalon. Current pulses were generated by the acquisition software and applied via a stimulus isolator (Iso-Flex; AMP Instruments). The tissue was allowed to recover for a minimum of either 5 minutes (electrical stimulation) or 30 minutes (for high K^+ stimulation) between stimulations. In some experiments we added drugs targeting neurotransmitter systems to the aCSF: 10 μM GBR 12909 (selective DA reuptake inhibitor; Sigma Aldrich D052); or 10 μM cocaine hydrochloride (DA, NA and 5-HT reuptake inhibitor; Sigma Aldrich C5776). GBR 12909 was first dissolved in DMSO with gentle warming before being directly added to the aCSF. Cocaine was made into a stock solution in water before being added to aCSF. Drugs were not perfused onto tissue until at least 3 stable baseline recordings had been obtained.

Fast-scan cyclic voltammetry procedure

Voltage waveforms were applied to electrodes using TarHeel software and the resulting changes to current were recorded and analysed. Carbon fibre microelectrodes generate a characteristic background signal that can be subtracted to yield the Faradaic current caused by oxidation and reduction of compounds (Fig. 1C (Baur et al., 1988; John and Jones, 2007a)). Neurotransmitters were identified upon the basis of their cyclic voltammograms (noting the position and height of oxidation and reduction peaks) and colour plots permitted the visualisation of release dynamics over time.

Statistical analyses

The percentage of variance in experimental data (voltammograms recorded in the telencephalon) accounted for by template data (voltammograms generated in a flow cell) was assessed using the CV match programme in TarHeel (Robinson et al., 2003). Analyses were only performed on experimental data obtained using the same electrode as that used to collect template data. We reported the values as r^2 , which was deemed to be significant when exceeding a threshold of $r^2 = 0.75$ as described in Heien et al., 2003. Principal component analysis was performed in TarHeel (Heien et al., 2004; Heien et al., 2005; Keithley and Wightman, 2011). An in vitro training set composed of cyclic voltammograms for dopamine, 5-HT, histamine and both acidic and basic pH shifts and combinations of these four factors were used for the final analysis (Heien et al., 2004; Heien et al., 2005). At least 5 voltammograms for each species (neurotransmitter / pH change) or combination were included. Training sets were deemed to fit the data appropriately if the maximum value shown on the Q plot did not exceed the threshold Qa value (686681). Current vs. time data recorded following application of neurotransmitter reuptake inhibitors to the tissue were extracted from TarHeel software and imported into Clampfit 10.2 (part of pCLAMP 10.2 software package; Molecular Devices). Baseline correction was applied to all recordings to account for increasing / decreasing baselines and electrode drift thus ensuring a stable flat baseline for analysis. Clampfit was also used for peak analysis, providing values for peak amplitude and multiple time parameters reflective of reuptake. This included half width, (the time taken for the peak to reach- and return to half peak amplitude), T half (time taken to decay to half peak amplitude from peak), tau decay (a time constant representing decay of current), and peak area ($\text{nA}\cdot\text{s}$) all of which have been deemed appropriate measures of neurotransmitter reuptake (Yorgason et al., 2011). Statistical analyses were conducted using GraphPad Prism 6 for Windows. One-way repeated measures ANOVA tests were performed for all measures, with time as the repeated measures variable. Control values were obtained by averaging values from 3 control stimulations prior to drug

perfusion. When statistical significance was indicated ($p < 0.05$), post-hoc analyses were conducted using Dunnett's multiple comparisons test, comparing each time-point to control and adjusting p values accordingly. Non-parametric Friedman tests were used when data were not normally distributed. When statistical significance was indicated, post-hoc analyses were conducted using Dunn's multiple comparisons test adjusting p values accordingly.

Results

Characterisation of neurotransmitter profiles in a flow cell

As a first step towards characterising analyte release in zebrafish we collected template FSCV data for dopamine, 5-HT and histamine, neurotransmitter systems that send extensive projections to the telencephalon. We used a flow cell (Sinkala et al., 2012), a microfluidic device that permits electrodes to be exposed to standard neurotransmitter solutions, to collect representative colour plots and cyclic voltammograms. We exposed carbon fibre electrodes to a known concentration of each neurotransmitter or pH shift (using the voltage waveform shown in Fig. 3B). Application of 1 μ M dopamine produced an increase in current at $\sim +0.6$ V and a reduction peak at ~ -0.25 V (Fig. 2A and 2B). A 0.5 μ M 5-HT solution also produced an increase in current $\sim +0.6$ V but reduction peaks occurred at ~ 0 V and ~ -0.5 V (Fig. 2C and 2D). Application of 40 μ M histamine produced a very different response, with an increase in current on the reverse scan at $\sim +1.0$ V and two reduction peaks on the forward scan at $\sim +0.25$ V and ~ -0.4 V (Fig. 2E and Fig. 2F). We also investigated the effect of altering pH on the oxidation potentials of voltammograms. An acidic change of -0.25 pH units (i.e. pH 7.15) produced an oxidation peak at $\sim +0.5$ V and reduction peaks at both $\sim +1.1$ V and ~ -0.3 V (Fig. 2G and 2H). An acidic change of -1.0 pH units (i.e. pH 6.4) produced a similar voltammogram with a sharp oxidation peak at $\sim +0.5$ V and reduction peaks at both $\sim +1.1$ V and ~ -0.3 V (Fig. 2I and 2J). A basic pH change of +1.0 units (i.e. pH 8.4) produced a large oxidation peak on the reverse scan at $\sim +1.1$ V and reduction peaks on the forward scans at $\sim +0.4$ V and ~ -0.5 V, similar to the neurotransmitter histamine (Fig. 2K and 2L). Together, these experiments demonstrate the characteristic shapes of cyclic voltammograms that are produced by exposing electrodes to neurotransmitter solutions and changes in pH.

Fast scan cyclic voltammetry in the adult zebrafish brain

We next investigated whether fast scan cyclic voltammetry (FSCV) could be used to measure the release of analytes in sagittal sections of the adult zebrafish brain. The zebrafish dorsal telencephalon receives extensive 5-HT-positive projections from the raphe- and pretectal nuclei (Lillesaar et al., 2009). We therefore applied a voltage waveform optimised for measurements of 5-HT (John and Jones, 2007a) and depolarised neurons and terminals with aCSF containing a high concentration of K^+ (100 mM K^+ ; hereafter high K^+ aCSF). Bath application of high K^+ aCSF led to changes in current at several points in the voltage waveform. A cyclic voltammogram extracted at ~ 10 seconds after stimulation displays characteristics that could reflect the oxidation of dopamine and/or 5-HT, including a prominent peak in current on the forward scan at $\sim +0.6$ V (Fig. 3E). A rapid increase in oxidative current is observed at the point in the waveform that corresponds to the peak of this signal ($\sim +0.6$ V; Fig. 3C). However, this current vs. time plot also exhibits a striking dip in current which is most likely due to the decrease in current at around $\sim +0.2$ V masking the oxidation peak at $\sim +0.6$ V.

The cyclic voltammogram extracted 30 seconds after application of high K^+ aCSF displays a different set of characteristics that are suggestive of an additional compound being oxidised on the reverse scan of the waveform (Fig. 3F). These features include a dip in current at $\sim +0.6$ V, a large increase in current at around $\sim +1.0$ V and two decreases in current at $\sim +0.2$ V and ~ -0.4 V suggesting that reduction occurs on the forward scan of the waveform (Fig 3D and 3F). Examination of the current vs. time plot at +1.0 V shows a large increase in current that returns towards baseline upon washout of the high K^+ aCSF (Fig 3D). The cyclic voltammogram of the second analyte exhibited characteristics of the voltammogram for the neurotransmitter histamine (Fig. 2F) which has distinct characteristics

including a reduction peak on the forward scan and an oxidation peak on the reverse scan (in contrast to the oxidation and reduction profile of dopamine (Fig. 2B) and 5-HT (Fig. 2D) (Pihel et al., 1995; Hashemi et al., 2011; Chang et al., 2012). Interestingly, both current vs. time plots reveal a long time-course of release with analytes failing to return fully to pre-stimulation baseline levels. We next compared FSCV data obtained from multiple independent experiments (n=12 sagittal sections from 8 fish for data extracted at ~10 seconds, and n=12 sagittal sections from 8 fish for data extracted at ~30 seconds). The shape of the voltammograms that we obtained was highly reproducible, both within- and across experiments (Fig. 3G and 3H). Furthermore, average voltammograms compiled from these experiments looked similar to representative recordings in the telencephalon, with oxidation- and reduction peaks occurring at the same potentials (compare Fig. 3I to 3E and 3J to 3F). This suggests that we can compare FSCV data across different animals or mutant lines.

Electrically-evoked neurotransmitter release in the zebrafish telencephalon

Bath application of high K^+ aCSF produced a current vs. time plot with a prolonged release profile that did not return to pre-stimulation baseline levels. In order to clarify whether this was an artefact caused by electrode drift during the long time necessary for complete washout to occur, we used electrical stimulation to evoke the release of analytes (Fig. 4A). Electrical stimulation of local terminals using optimal parameters (20 pulses with a pulse width of 4 ms, 60 Hz, 500 μ A) resulted in an increase in current on the forward part of the waveform (Fig. 4C and 4F) that rapidly returned to baseline (n=8 stimulations from a single sagittal section in Fig. 4C and 4B). It also produced a cyclic voltammogram with a shape similar to that obtained using high K^+ aCSF suggesting that both types of stimulation evoke the release of a similar mixture of analytes (Fig. 4B and 4E). We tested this possibility using the CV match algorithm in TarHeel. Comparison of an example electrical stimulation with an example K^+ stimulation gave an r^2 value of 0.876, indicating that both types of stimulation evoke similar neurochemical changes in the tissue. However, the oxidation peak at ~+0.6 V and reduction peak at ~-0.2 V were much more prominent when using electrical stimulation than in voltammograms obtained using high K^+ aCSF. The lowest intensity stimulation that we could use to trigger analyte release in the telencephalon was 20 pulsed with a pulse width of 4 ms, 60 Hz, 300 μ A. This produced a voltammogram with an oxidation peak at ~+0.6 V and a smaller reduction peak at ~-0.2 V (Fig. 4H and 4I). In contrast to this, high intensity stimulation (1 mA, 60 Hz, 60 pulses, pulse width 4 ms) produced a cyclic voltammogram with a similar shape to that extracted ~30 s after K^+ stimulation (Fig. 4J and 4K) with a small oxidation peak occurring on the reverse scan at ~1.1 V and a large reduction peak at ~-0.4 V). Furthermore, the change in current ~+0.6 V showed a large decrease similar to the dip in current observed following K^+ stimulation (Fig. 4L). The prolonged time-course of alterations in current suggested that an artefact such as a change in pH had occurred. This indicates that stimulation parameters are an important consideration when attempting to obtain reproducible measurements of neurotransmitter release that are not masked by pH shifts or electrode drift.

Analysis of the effects of pH changes on current following stimulation of the telencephalon

We investigated whether a shift in pH could be contributing to the changes in the current that we measured by adding HEPES buffer to the aCSF. Stimulation using high K^+ HEPES-buffered aCSF altered the release profile of analytes occurring at the oxidation and reduction potentials for both dopamine and histamine. The large dip normally present at ~10 seconds after application of high K^+ aCSF was reduced (compare Fig. 5A and 5C with 5B and 5D). Furthermore, addition of HEPES caused the current to return to baseline following stimulation (Fig. 5D) suggesting a less prominent shift in background signal. The resulting voltammogram, taken ~10 seconds after stimulation, (Fig. 5E) no longer showed a large reduction in signal at around +0.2 V which seemed to mask the oxidation peak at +0.6 V in previous experiments (compare to Fig. 3A and 3C). A second cyclic voltammogram taken ~30 seconds after stimulation showed a small increase in current observed at ~+0.7 V (Fig. 5F).

Combinations of neurotransmitter solutions in the flow cell

The colour- and voltage plots that we obtained from recordings in the zebrafish telencephalon appeared to be influenced by the release of more than one analyte. We applied combinations of dopamine, 5-HT and histamine and pH changes to the electrode in the flow cell and measured changes in current. A combination of 1 μM dopamine and 40 μM histamine produced a cyclic voltammogram with a small oxidation peak at $\sim +0.6$ V, a larger oxidation peak at around $+1.0$ V and three reduction peaks at $\sim +0.25$ V, ~ -0.2 V and ~ -0.4 V (Fig. 6A). A combination of 2 μM dopamine and 20 μM histamine produced a very large oxidation peak at $\sim +0.6$ V, a second oxidation peak on the reverse scan at $\sim +1.0$ V and reduction peaks at $\sim +0.2$ V, ~ -0.2 V and ~ -0.4 V (Fig. 6B). Likewise, combining 5-HT and histamine produced a voltammogram with a similar oxidation profile but different reduction profile. A mixture of 0.25 μM 5-HT and 40 μM histamine led to oxidation peaks at $\sim +0.6$ V and $\sim +1.0$ V and reduction peaks at $\sim +0.3$ V, ~ 0 V and ~ -0.4 V (Fig. 6C). We also examined the current changes produced by mixing all three neurotransmitters. A combination of 0.25 μM 5-HT, 1 μM dopamine and 20 μM histamine produced a current plot with oxidation peaks at $\sim +0.6$ V and $\sim +1.0$ V and four reduction peaks at $\sim +0.2$ V, ~ 0 V, ~ -0.2 V and ~ -0.4 V (Fig. 6D). Together, these data indicate that it should be possible to separate signals composed of these three neurotransmitters, since each individual peak is neither inflated nor altered by the presence of a second compound, apart from the overlapping oxidation peak at $\sim +0.6$ V for dopamine and 5-HT. However, 5-HT can still be identified by its unique reduction peaks, permitting the visual dissociation of these two transmitters. We explored the possibility that pH changes could be influencing the signals that we recorded in the telencephalon by altering the pH of dopamine, histamine and 5-HT mixtures in a flow cell. A combination of -0.5 pH units, 1 μM dopamine and 40 μM histamine (Fig. 6E) produced a cyclic voltammogram similar to the voltammogram obtained ~ 10 seconds after high K^+ aCSF stimulation (Fig. 2D). Altering the pH of histamine alone (80 μM histamine and -0.25 pH units; Fig. 6F) provided a good in-vitro representation of the voltammogram for the second analyte obtained with high K^+ HEPES-buffered aCSF (Fig. 6E,F). Despite not being completely identical, it showed changes in current at $\sim +0.2$ V, $\sim +1.0$ V, and ~ -0.4 V. A basic change in pH (+1.0 units) coupled to 1 μM dopamine and 40 μM histamine produced a voltammogram with a broad reduction peak around $+0.4$ V (Fig. 6G) but no large reduction peak at ~ -0.4 V (Fig. 5E and 5F) suggesting that the pH change was not likely to be basic. Furthermore, addition of 5-HT to the flow cell mixture also produced a voltammogram with a very different shape (1 μM 5-HT, 80 μM histamine and -0.5 pH units) suggesting that 5-HT was unlikely to have contributed to the analytes measured in the telencephalon (Fig. 6H).

CV match analysis of in vitro stimulation data

In order to compare the similarity of experimental recordings in tissue to template flow cell data we used the CV match function in TarHeel. We calculated r^2 values between experimental cyclic voltammograms from the telencephalon obtained using optimal stimulation parameters and multiple template cyclic voltammograms (Robinson and Wightman, 2007). Comparison of voltammograms produced by electrical stimulation (4 experiments from 2 sagittal sections taken from 2 fish) to a mixture of 2 μM dopamine, 80 μM histamine and an acidic pH shift of -0.25 units produced r^2 values that exceeded the accepted threshold of 0.75 in 3 out of 4 stimulations analysed (for example, Fig. 7A and 7C, $r^2 = 0.846$; 7A and 7E, $r^2 = 0.833$; for further values see Table 1). In one case, a template of 4 μM dopamine, 160 μM histamine and an acidic pH shift of -0.25 units produced the highest r^2 value of 0.878 (see table 1). High K^+ aCSF stimulation of the telencephalon produced a voltammogram at ~ 10 s following stimulation, which we also compared to multiple template cyclic voltammograms (2 experiments from 2 fish) that were significantly similar to a combination of 2 μM dopamine and 20 μM histamine (Fig. 7B and 7D, $r^2 = 0.857$; for further values see Table 1). However it was not possible to produce a template voltammogram in the flow cell that gave a significant match to a voltammogram extracted at ~ 30 s following high K^+ aCSF stimulation. In summary, the analytes released by electrical stimulation of the zebrafish forebrain are significantly similar to a combination of dopamine, histamine and an acidic pH change of -0.25 units, whereas the analytes released ~ 10

seconds after high K^+ stimulation are indicated to be more similar to a combination of dopamine and histamine.

Principal component analysis of electrical stimulation data

We examined our data using principal component analysis to provide an estimate of actual neurotransmitter concentration in the brain and to determine the relative contribution of each neurotransmitter to the changes in current that we measured (Heien et al., 2005; Keithley and Wightman, 2011). We constructed training sets of voltammograms that included responses to both single neurotransmitters and mixtures of neurotransmitters in the flow cell. Full details of these training sets are provided in Table 1. We used data from electrical stimulation experiments (Fig. 8A) for this analysis because recordings using high K^+ aCSF did not fit the statistical model well, perhaps due to the prolonged time-course of the changes that can cause the baseline to drift. We obtained the best fit for our electrical stimulation data (i.e. the lowest residual values) when using a training set that included dopamine, 5-HT, histamine and both acidic- and basic pH shifts. The resulting concentration vs. time plots suggest that dopamine (Fig. 8C), 5-HT (Fig. 8D) and histamine (Fig. 8E) are all likely to be present following electrical stimulation. Importantly, the resulting Qt plot did not pass the threshold of 686681 at any point (Fig. 8G) suggesting that our training set fits the in vitro data well. The increase of dopamine is ~ 100 nM, 5-HT ~ 8.0 nM and the increase of histamine is ~ 8.0 μ M. In addition, it appears that there is also an acidic pH shift of ~ 0.05 units (Fig. 8F). To confirm that our PCA was accurate in its representation of type- and concentration- of analytes, we examined a combination of 0.25 μ M 5HT, 1 μ M dopamine, 40 μ M histamine and an acidic pH shift of +1.0 unit obtained in the flow cell. This provided a highly accurate prediction of the concentration of each species (Fig. 8H), suggesting that the training set was indeed appropriate for the main analysis.

Pharmacological inhibition of dopamine reuptake

The identity of analytes released during FSCV can be further confirmed by pharmacological validation (Dankoski and Wightman, 2013). The results of the CV match and PCA analyses suggest that dopamine is likely to be a major contributor to the changes in current that we measured. We further investigated this prediction by manipulating dopamine pharmacologically. We treated sagittal slices of the adult zebrafish brain with either cocaine, a non-selective monoamine reuptake inhibitor that has been shown to increase dopamine reuptake within the rodent nucleus accumbens, caudate putamen and substantia nigra (Jones et al., 1995a; Jones et al., 1995b; Davidson et al., 2000; John and Jones, 2007a; John and Jones, 2007b; España et al., 2008; Yorgason et al., 2011) or the selective long-acting dopamine reuptake inhibitor GBR 12909 (España et al., 2008; Esposti et al., 2013). Treatment with 10 μ M cocaine produced an increase in current at $\sim +0.6$ V that appeared to be prolonged (Fig. 9B-D) compared to controls (Fig. 9A). Current vs. time plots showed peaks that become broader over time (Fig. 9E-H) indicating a slowing down of reuptake kinetics. Comparison of cyclic voltammograms from these experiments further demonstrated that oxidation- and reduction peaks became more prominent at $\sim +0.65$ V and ~ -0.25 V respectively (Fig. 9I-L) following cocaine application, with a shape that was more similar to the dopamine voltammogram obtained in the flow cell (Fig. 2B). Current vs. time plots further illustrated this, as peaks became broader over time (Fig. 9I-L) indicating a slowing down of reuptake kinetics. Moreover, there was a slight increase in the amplitude of peaks (Fig. 9M,N), suggesting that cocaine may also affect dopamine release in the zebrafish telencephalon. We used one-way repeated measures ANOVA tests followed by Dunnett's multiple comparisons tests with p -value adjustment to compare the average value of three control stimulations with 4 time-points following cocaine perfusion ($n=6$ fish in each case). Non parametric tests were used when the data were not normally distributed. There was a significant effect of cocaine on peak height (nA; $F(4,20) = 4.91$, $p < 0.01$). Post hoc Dunnett's tests revealed that peak height was significantly larger than control at 10 minutes ($p < 0.005$), 20 minutes ($p < 0.005$) and 30 minutes ($p < 0.05$) after cocaine perfusion, but not after 40 minutes ($p > 0.05$). Cocaine also had a significant effect on half width (s; $F(4,20) = 23.14$, $p < 0.0001$). Half width was significantly increased 10 minutes ($p < 0.01$), 20 minutes ($p < 0.0001$), 30 minutes ($p < 0.0001$) and 40 minutes ($p < 0.0001$) after perfusion. There was also a significant effect on T Half (s; $F(4,20) = 23.23$, $p < 0.0001$). T Half was significantly increased at 10 minutes ($p = 0.005$), 20 minutes, ($p < 0.0001$), 30 minutes ($p < 0.0001$) and 40

minutes ($p < 0.0001$). Cocaine also significantly influenced peak area (nA*s; $F(4,20) = 19.17$, $p < 0.0001$). Dunnett's tests revealed peak area was significantly larger 10 minutes, ($p < 0.005$), 20 minutes ($p < 0.0001$), 30 minutes ($p < 0.0001$) and 40 minutes ($p < 0.0001$) following cocaine perfusion. Tau decay (s) was also significantly altered ($\chi^2(5) = 18.53$, $p = 0.001$ Friedman test). Post hoc tests showed that tau decay was significantly larger 20 minutes ($p = 0.001$), 30 minutes ($p < 0.05$) and 40 minutes ($p < 0.005$) following cocaine application. Treatment with 10 μ M of the more selective dopamine reuptake inhibitor GBR 12909 led to an increase in the amplitude and time course of current at $\sim +0.6$ V following electrical stimulation (Fig. 10 A-D). The related current vs. time plots show that peaks become larger and somewhat broader over time indicating an increase in release and possibly a slowing of reuptake kinetics as well (Fig. 10E-H). Comparison of cyclic voltammograms from these experiments provided further evidence for an increase in the amplitude of release, as the peak around the oxidation potential for dopamine ($\sim +0.65$ V) became considerably larger (Fig. 10I-L). The effects of 10 μ M GBR 12909 application were analysed using non parametric tests to account for significant deviation from normality. Friedman tests followed by post hoc Dunn's multiple comparisons with p -value adjustment were used to compare the average value of three control stimulations with 4 time-points post GBR 12909 perfusion ($n=6$ fish in each case). GBR 12909 had a significant effect on peak height (nA; $\chi^2(5) = 16.93$, $p < 0.005$). Peak height was significantly increased after 30 minutes, ($p < 0.05$) and 40 minutes ($p < 0.0001$) drug application. GBR 12909 did not significantly alter half width (s; $p = 0.0504$), however there was a significant effect on T half (s; $\chi^2(5) = 10.4$, $p < 0.05$). Dunn's tests revealed that T half was significantly increased after 20 minutes, ($p < 0.05$), 30 minutes ($p < 0.05$) and 40 minutes ($p < 0.05$). There was also a significant effect of GBR 12909 on peak area (nA*s; $\chi^2(5) = 14.67$, $p < 0.01$). Peak area was significant increased after 20 minutes, ($p < 0.05$), 30 minutes, ($p < 0.05$), and 40 minutes, ($p < 0.005$) GBR 12909 perfusion. GBR 12909 did not significantly alter tau decay ($p > 0.05$). Taken together, the combination of statistical analysis and pharmacological studies demonstrates that stimulation of the telencephalon evokes the release of dopamine, with possible release of histamine and a concomitant acidic change in pH as well.

Discussion

In this study we have established a protocol to record the release of analytes in the zebrafish telencephalon by FSCV. We evoked neurotransmitter release by either bath application of high K^+ aCSF or by electrical stimulation of local terminals. Neurotransmitters were detected and identified upon the basis of their oxidation and reduction profiles at the surface of a carbon fibre electrode. Using this method we obtained voltammograms that are significantly similar to the simultaneous measurement of dopamine and histamine coupled to a change in pH. We have also provided pharmacological validation that we have measured the release of dopamine. To the best of our knowledge, this study represents the first FSCV recordings in zebrafish, thus paving the way for neurochemical analysis of the fish brain.

Comparison of slice preparation and method of stimulation

There are several advantages to using an in vitro slice preparation rather than recording from whole brains. Firstly, we were able to accurately place electrodes in a specific area, something that would be difficult to achieve in intact brains in the absence of a stereotaxic atlas. Our setup also gave us fine-control of environmental parameters (including temperature and pH) and obviated the need to use an anaesthetic which could potentially alter the dynamics of neurotransmitter release (John and Jones, 2007a). We also compared stimulation of neurotransmitter release by either perfusion of high K^+ aCSF or electrical stimulation. The results that we obtained with both methods were comparable once we had identified the best parameters for electrical stimulation (20 pulses with a pulse width of 4 ms, 60 Hz, 500 μ A). The data that we obtained using low intensity stimulation was not very reproducible, and the oxidation peak was less prominent than in experiments using optimal parameters. In contrast to this, high intensity electrical- or K^+ aCSF stimulation triggered a prolonged release profile that did not return to baseline. Electrical stimulation is ideal for examining local neurotransmitter release and

could in theory be used to map neural circuits in the brain. However, we will have severed a large number of axon tracts when sectioning the brain meaning that the action of some endogenous control mechanisms (such inhibitory neurotransmitters) may have been disrupted (Dankoski and Wightman, 2013). The results from our slice preparation thus need to be interpreted with caution.

Identity of neurotransmitters recorded in the telencephalon

A major challenge of fast scan cyclic voltammetry is to characterise the analytes that are released following stimulation. Wightman and colleagues have suggested five criteria that can be used to identify endogenously released substances (Dankoski and Wightman, 2013): good correlation between voltammograms obtained during an experiment and standard data (e.g. when exposing electrodes to neurotransmitter solutions in a flow cell); independent verification of the presence of the neurotransmitter; precise anatomical positioning of the electrode in the region of interest; correct physiological release properties for the transmitter being measured; and pharmacological validation of each compound (Dankoski and Wightman, 2013). We have taken these criteria into account in this study. We compared our FSCV recordings in brain slices to template cyclic voltammograms generated in a flow cell using the CV match software (Fig. 2, 6 and 7). 5-HT, dopamine and histamine have already been shown to be present in the zebrafish forebrain by high pressure liquid chromatography and radioactive immunoassay (Norton et al., 2011; Buske and Gerlai, 2012). Furthermore, the position of our recording electrode was chosen based upon studies of the projection patterns of dopamine, 5-HT- and histamine neurons (Kaslin and Panula, 2001; Lillesaar et al., 2009). The voltammograms that we obtained from recordings in the telencephalon appear to represent a combination of more than one neurotransmitter. We do not appear to be measuring 5-HT since the voltammograms in Fig. 3D do not show the characteristic reduction peak at 0 V (see Fig. 3D). Our telencephalic recordings are most similar to an acidic (pH 6.95) solution of ~2 μ M dopamine and 80 μ M histamine recorded in the flow cell (Fig. 7). We further validated this result by using the selective dopamine reuptake inhibitor GBR 12909 (Esposti et al., 2013). Application of GBR 12909 led to a significant alteration in peak amplitude, area and reuptake. We could not use the Michaelis-Menten kinetics to analyse this data, since the rate of dopamine reuptake in zebrafish (i.e. the rate of DOPAC formation in tissue slices) has not been calculated (Near et al., 1988) and it is unlikely that the stimulated release saturated reuptake, a prerequisite for Michaelis-Menten modelling. However, peak amplitude, area and reuptake parameters have already been used to examine FSCV data following pharmacological manipulation (Yorgason et al., 2011) demonstrating the validity of this approach.

Characteristics of neurotransmitter release in the zebrafish telencephalon

The initial current vs. time plots that we recorded following high K^+ aCSF stimulation of the telencephalon had two striking characteristics: a long release profile that lasted approximately 5 minutes (Fig. 3C, 3E); and the presence of a second analyte that did not return to baseline (Fig. 3F). Increasing the intensity of electrical stimulation also led to a longer time-course of release (Fig. 4L). This result was surprising, since measurements of neurotransmitter release in the rodent brain typically only last for a few seconds (Hashemi et al., 2011). However, it could also indicate that a pH change has altered the background signal and thus inflated our measurements of current (Jones et al., 1994; Takmakov et al., 2010). Acidifying or alkalinising pH shifts can occur in conjunction with neurotransmitter release and are an indicator of neural activity (Chesler, 2003; Venton et al., 2003; Takmakov et al., 2010). Therefore, care is needed to avoid confusing changes in pH and the release of neurotransmitters such as dopamine (Venton et al., 2003). In contrast to this, electrical stimulation with optimal parameters triggered a release with a shorter time-course that rapidly returned to baseline, making electrical stimulation with optimal parameters much more suitable for FSCV recordings in zebrafish. We obtained similar cyclic voltammograms by either electrically stimulating the adult zebrafish telencephalon or exposing an electrode in the flow cell to dopamine and histamine in the presence of an acidic pH shift (-0.5 pH units) (Fig. 4E and 6E). This acidification fits within the normal physiological limit of pH changes (Chesler, 2003) and suggests that some of our current measurements in the adult zebrafish brain may have been influenced by alterations in pH concomitant with neurotransmitter release. Interestingly, the low concentration of 5-HT predicted by principal component analysis (PCA) suggests that this neurotransmitter may not contribute to the in

vitro signal that we recorded; the trace level of 5-HT detected here could be an overestimation, caused by the overlapping oxidation potentials of 5-HT and dopamine, or the slight shift in oxidation potentials that can occur in tissue vs. flow cell recordings (Keithley and Wightman, 2011). Further experiments would be required to investigate this issue.

Validation of FSCV data

We used CV match and PCA (Heien et al., 2005; Keithley and Wightman, 2011) to assess how well our training set fitted the data and to separate the constituent parts of the voltammograms elicited following stimulation of the telencephalon. The CV match programme calculated r^2 values that exceeded the statistical threshold of 0.75 in each case (Fig. 7), permitting us to conclude that it was highly likely we were measuring the release of dopamine, histamine and a change of -0.25 pH units. The CV match algorithm returns very conservative r^2 estimates (Heien et al., 2003) meaning that we can be confident in the accuracy of this result. In contrast to this, we could not produce an electrochemical trace in the flow cell that surpassed the r^2 threshold of 0.75 when compared to the change occurring ~30 seconds after high K^+ aCSF stimulation (Fig. 2F and 7F), perhaps due a distortion of the signal by high K^+ levels and a change in pH (Threlfell and Cragg, 2007). We therefore cannot conclude anything about the analytes which may contribute to this change in current. We used PCA to partially account for our in vitro data by uploading a training set containing dopamine, 5-HT and histamine and both acidic- and basic pH changes (Fig. 8). The quality of the PCA may be improved by using a biologically relevant training set rather than in vitro flow cell data. However, this was not achievable since initially we did not know which analytes we were detecting in the zebrafish brain. Future analyses could be improved by using green fluorescent protein (GFP) labelling to unambiguously identify neurons – for example, the ETvmat2:eGFP line labels all monoaminergic neurons in the brain (Wen et al., 2008).

Neurochemical profile of the zebrafish brain

Stimulation of the zebrafish telencephalon appeared to trigger release of more than one neurotransmitter. FSCV has already been used to demonstrate the simultaneous release of 5-HT and histamine in the rat substantia nigra pars reticulata (Hashemi et al., 2011). Furthermore, the histamine H3 heteroreceptor can modulate the activity of many types of neurons (including dopamine and 5-HT neurons) thus permitting cross-talk between neurotransmitter systems (Haas et al., 2008). Zebrafish dopaminergic and histaminergic neurons project extensively throughout the brain (Eriksson et al., 1998; Kaslin and Panula, 2001) making it perhaps unsurprising that histamine and dopamine could be co-released in the telencephalon. The primary target of ascending histamine projections is the rostral medial dorsal telencephalon with some fibres innervating the caudal medial dorsal telencephalon (Eriksson et al., 1998). Therefore, the anatomical localisation of histamine-positive fibres suggests that release of histamine could contribute to the change in current that we measured. Histamine plays a role in aggression, sleep, anxiety, locomotion and long-term memory in zebrafish (Peitsaro et al., 2000; Peitsaro et al., 2003; Renier et al., 2007; Norton et al., 2011). The absence or scarcity of this neurotransmitter in the periphery (Eriksson et al., 1998) raises the possibility that histamine may have a more important role in the central nervous system of zebrafish than other vertebrates. Further work comparing the behavioural function of histamine in fish and other model organisms will be required to clarify this issue. Importantly, the protocol that we have established here can be used to examine the zebrafish dopaminergic system, including comparisons of dopamine reuptake in different brain areas and looking at the effect of pharmacological manipulations on neurotransmitter release. In addition, it will provide the basis for further characterisation of zebrafish mutant lines that display alterations in behaviour. FSCV thus represents another useful tool for in-depth characterisation of the zebrafish brain.

Author contributions

LJ, JM, AY and WN designed the experiments. LJ conducted the experiments. WN wrote the first version of the manuscript. LJ, JM, AY and WN improved the manuscript and approved the final version. We have no conflicts of interest to declare.

Acknowledgements

We are grateful to Carl Breaker for maintaining our aquarium facilities and fish care. We also thank all members of the Norton lab for helpful discussions about this data. Sandra Beleza and Jonathan McDermid helped improve an early version of this manuscript. Vincenzo Marra, Mike Jay and Bethany Denton kindly provided help and advice during in vitro experiments and statistical analysis. Research in our laboratory is funded by the BBSRC MIBTP programme (LJJ), the European Union's seventh framework programme Aggressotype grant agreement no. 602805 and the Marie Skłodowska-Curie Foundation international training network "MiND" grant agreement no. 643051.

Figure legends

Figure 1. Fast scan cyclic voltammetry (FSCV) setup and position of the stimulating- and recording electrodes.

(A) Diagram showing components of the FSCV setup and the position of the recording electrode in the adult zebrafish brain. The applied voltage waveform (top graph) and a representative cyclic voltammogram for dopamine (lower graph) are also shown, with the forward scan in black and the reverse scan in red. (B) Schematic representation showing a lateral and dorsal view of the adult zebrafish brain. The black asterisk marks the position of the recording electrode in the telencephalon. (C) Diagram showing characteristic non-Faradaic background signal that is subtracted to generate the background-subtracted voltammograms shown throughout the paper. Abbreviations: Cb, cerebellum; Hy, hypothalamus; OB, olfactory bulb; T, telencephalon; TeO, optic tectum.

Figure 2. Comparison of cyclic voltammograms generated by exposing electrodes to dopamine, 5-HT, histamine and pH changes in a flow cell.

Colour plots (A,C,E,G,I and K) and cyclic voltammograms (B,D,F,H,J and L; black lines represent forward scan and red lines reverse scan) taken at the time point indicated by the dashed white lines. (A,B) 1 μ M dopamine solution. (C,D) 0.5 μ M 5-HT solution. (E,F) 40 μ M histamine solution. (G,H) -0.25 units acidic pH change (pH 7.4 \rightarrow pH 7.15). (I,J) -1.0 units acidic pH change (pH 7.4 \rightarrow pH 6.4). (K,L) +1.0 units basic pH change (pH 7.4 \rightarrow pH 7.4 \rightarrow pH 8.4). Scale bar in A,C,E,G,I and K represents 5 seconds.

Figure 3. Comparison of analytes evoked by high K⁺ aCSF stimulation of the zebrafish telencephalon.

(A) Colour plot showing changes in current following stimulation with high K⁺ aCSF. (B) Applied waveform ranging from 0 V \rightarrow +1.2 V \rightarrow -0.6 V \rightarrow 0 V (the holding potential). The forward scan of the waveform is coloured black and the reverse scan is red. (C) Current vs. time plot showing the profile of current changes at $E_{app} \sim +0.6$ V, the point in the waveform indicated by the black circle in (E). (D) Current vs. time plot showing the profile of current changes at $E_{app} \sim +1.0$ V on the reverse scan of the waveform, the point indicated by the circle in (F). (E) Representative cyclic voltammogram taken at the time point indicated by the thick dashed white line in (A). (F) Representative cyclic voltammogram taken at the time point indicated by the thin dashed white line in (A). (G) Cyclic voltammograms from 24 separate stimulations taken at the time point indicated by the thick dashed white line in (A). (H) Cyclic voltammograms from 20 independent experiments taken at the time point indicated by the thin dashed white line in (A). (I) Average cyclic voltammogram

derived from data presented in (G). (J) Average cyclic voltammogram derived from data presented in (H).

Figure 4. Comparison of electrically-stimulated release of analytes in the telencephalon.

(A) Schematic representation showing lateral view of zebrafish telencephalon. The red crosses show the position of tips of the stimulating electrode and the black cross shows the position of the recording electrode used in these experiments. (B,C) Voltammograms and current vs. time plots from 8 repeated stimulations in one slice using optimal parameters (20 pulses (pulse width of 4 ms) and a voltage of 500 μ A, 60 Hz). (D-F) Colour plot, voltammogram and current vs. time plot from a single representative experiment using optimal stimulation. (G-I) Colour plot, voltammogram and current vs. time plot from a single representative experiment using low intensity stimulation parameters (20 pulses (pulse width of 4 ms), and a voltage of 300 μ A, 60 Hz). (J-L) colour plot and voltammogram and current vs. time plots from a single representative experiment using high intensity stimulation parameters (60 pulses (pulse width of 4 ms), voltage of 1 mA, 60 Hz). High intensity stimulation led to the release of analytes at two different points in the voltammogram (black and red lines in L). (D,G,J) Dashed lines show the position at which the voltammograms were extracted and black and red circles depict points at which current vs. time plots were taken.

Figure 5. Comparison of stimulation with high K^+ aCSF with- and without HEPES buffer

(A) Colour plot showing changes in current evoked by stimulation with high K^+ aCSF without HEPES. The same colour plot is shown in Fig. 2A. (B) Colour plot showing changes in current evoked by stimulation with high K^+ aCSF containing HEPES buffer. Thick dashed white line shows the time point and potential at which the first analyte was maximal. Thin dashed white line shows the time point at which the second analyte was observed. Current vs. time plots for responses obtained in high K^+ aCSF without HEPES (C) (same traces as shown in Fig. 2C and 2E for comparison) compared to aCSF containing HEPES buffer (D). Blue lines show dopamine-like current and black lines show histamine-like current. (E) Voltammogram extracted at time point indicated by thick dashed white line in (B). Circle corresponds to blue line in (D). (F) Voltammogram extracted at time point indicated by thin white line in (B). Circle corresponds to black line in (D).

Figure 6. Comparison of cyclic voltammograms generated by exposing electrodes to mixtures of dopamine, 5-HT and histamine and pH changes in a flow cell.

(A) 1 μ M dopamine and 40 μ M histamine solutions. (B) 2 μ M dopamine and 20 μ M histamine solutions. (C) 0.25 μ M 5-HT and 40 μ M histamine solutions. (D) 0.25 μ M 5-HT, 1 μ M dopamine and 20 μ M histamine solutions. (E) Voltammogram resulting from exposing an electrode in a flow cell to 1 μ M dopamine and 40 μ M histamine in the presence of an acidic pH shift (-0.5 pH units, pH 7.4 \rightarrow pH 6.9). (F) Voltammogram resulting from exposing an electrode in a flow cell to 80 μ M histamine in the presence of an acidic pH shift (-0.25 pH units; pH 7.4 \rightarrow pH 7.15). (G) Voltammogram resulting from exposing an electrode in a flow cell to 1 μ M dopamine and 40 μ M histamine in the presence of a basic pH shift (+1.0 pH units, pH 7.4 \rightarrow pH 8.4). (H) Voltammogram resulting from exposing an electrode in a flow cell to 1 μ M 5-HT and 80 μ M histamine in the presence of an acidic pH shift (-0.5 pH units, pH 7.4 \rightarrow pH 6.9). Black lines represent forward scan and red lines reverse scan.

Figure 7. Statistical analysis of stimulated release

(A) Template voltammogram resulting from exposing an electrode in a flow cell to 2 μ M dopamine and 80 μ M histamine in the presence of an acidic pH shift (-0.25 pH units, pH 7.4 \rightarrow pH 7.15). (B) Template voltammogram resulting from exposing an electrode in a flow cell to 2 μ M dopamine and 20 μ M histamine. (C-F) Cyclic voltammograms obtained by either electrical- (C,E) or high K^+ aCSF stimulation (D,F) of the telencephalon. The r^2 value for (A) and (C) is 0.846; the r^2 value for (A) and (E) is 0.833; the r^2 value for (B) and (D) is 0.857 and the r^2 value for (B) and (F) is 0.416.

Figure 8. Principal component analysis

Principal component analysis (PCA) of data obtained from stimulation of zebrafish telencephalon. (A) Colour plot showing current changes resulting from electrical stimulation. (B) Residual colour plot showing changes not accounted for by the PCA model. (C) Time vs. concentration plot for dopamine obtained from PCA. (D) Time vs. concentration plot for 5-HT obtained from PCA. (E) Time vs. concentration plot for histamine obtained from PCA. (F) Time vs. pH units plot obtained from PCA. (G) Qt plot showing that data does not exceed the Q threshold of 686681. (H) Application of the model to a cyclic voltammogram representing a combination of 1 μ M dopamine, 0.25 μ M 5HT, 40 μ M histamine and a pH shift of +1.0 unit measured in a flow cell.

Figure 9. Dopamine reuptake inhibition with cocaine modifies analyte release profile in the telencephalon

(A-D) Colour plots showing the time course (x-axis) of changes in current as a function of the applied waveform (y-axis) after a control stimulation (A) and 10 (B), 20 (C) and 40 (D) minutes after 10 μ M cocaine administration. (E-H) Current vs. time plots showing the time course of changes in current after a control stimulation (E) and 10 (F), 20 (G) and 40 (H) minutes after 10 μ M cocaine administration. (I-L) Cyclic voltammograms extracted immediately following onset of release with oxidation and reduction peaks becoming larger at $\sim +0.65$ V and ~ -0.25 V over time; control stimulation (I) and 10 (J), 20 (K) and 40 (L) minutes after 10 μ M cocaine administration. (M-P) Individual data points showing peak height (M), peak area (N), half width (O) and T half (P) for control stimulations (C1, C2, C3) or at the time points indicated.

Figure 10. Dopamine reuptake inhibition with GBR 12909 modifies analyte release profile in the telencephalon

(A-D) Colour plots showing the time course (x-axis) of changes in current as a function of the applied waveform (y-axis) after a control stimulation (A) and 10 (B), 20 (C) and 40 (D) minutes after 10 μ M GBR 12909 administration. (E-H) Current vs. time plots showing the time course of changes in current after a control stimulation (E) and 10 (F), 20 (G) and 40 (H) minutes after 10 μ M GBR 12909 administration. (I-L) Cyclic voltammograms extracted immediately following onset of release, with oxidation and reduction peaks becoming larger at $\sim +0.65$ V and ~ -0.25 V over time; control stimulation (I) and 10 (J), 20 (K) and 40 (L) minutes after 10 μ M GBR 12909 administration. Current vs. time plots showing the time course of changes in current after a control stimulation (I) and 10 (J), 20 (K) and 40 (L) minutes after 10 μ M GBR 12909 administration. (M-P) Individual data points showing peak height (M), peak area (N), half width (O) and T half (P) for control stimulations (C1, C2, C3) or at the time points indicated.

References

- Arrenberg, A.B., and Driever, W. (2013). Integrating anatomy and function for zebrafish circuit analysis. *Frontiers in Neural Circuits* 7, 74. doi: 10.3389/fncir.2013.00074.
- Bargmann, C.I. (2012). Beyond the connectome: How neuromodulators shape neural circuits. *BioEssays* 34, 458-465. doi: 10.1002/bies.201100185.
- Baur, J.E., Kristensen, E.W., May, L.J., Wiedemann, D.J., and Wightman, R.M. (1988). Fast-scan voltammetry of biogenic amines. *Analytical Chemistry* 60, 1268-1272. doi: 10.1021/ac00164a006.
- Bonan, C.D., and Norton, W.H.J. (2015). The utility of zebrafish as a model for behavioural genetics. *Current Opinion in Behavioral Sciences* 2, 34-38. doi: <http://dx.doi.org/10.1016/j.cobeha.2014.07.003>.
- Buske, C., and Gerlai, R. (2012). Maturation of shoaling behavior is accompanied by changes in the dopaminergic and serotonergic systems in zebrafish. *Developmental Psychobiology* 54, 28-35. doi: 10.1002/dev.20571.

- Chang, S.-Y., Jay, T., Muñoz, J., Kim, I., and Lee, K.H. (2012). Wireless fast-scan cyclic voltammetry measurement of histamine using WINCS -- a proof-of-principle study. *The Analyst* 137, 2158-2165. doi: 10.1039/c2an16038b.
- Chesler, M. (2003). Regulation and Modulation of pH in the Brain. *Physiol Rev.* 83, 1183-1221. doi: 10.1152/physrev.00010.2003.
- Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E., and Stainier, D.Y.R. (2007). Conditional targeted cell ablation in zebrafish: A new tool for regeneration studies. *Developmental Dynamics* 236, 1025-1035. doi: 10.1002/dvdy.21100.
- Dankoski, E.C., and Wightman, R.M. (2013). Monitoring serotonin signaling on a subsecond time scale. *Frontiers in integrative neuroscience* 7, 44. doi: 10.3389/fnint.2013.00044.
- Davidson, R.J., Putnam, K.M., and Larson, C.L. (2000). Dysfunction in the neural circuitry of emotion regulation - A possible prelude to violence. *Science* 289, 591-594.
- Del Bene, F., and Wyart, C. (2012). Optogenetics: A new enlightenment age for zebrafish neurobiology. *Developmental Neurobiology* 72, 404-414. doi: 10.1002/dneu.20914.
- Eriksson, K.S., Peitsaro, N., Karlstedt, K., Kaslin, J., and Panula, P. (1998). Development of the histaminergic neurons and expression of histidine decarboxylase mRNA in the zebrafish brain in the absence of all peripheral histaminergic systems. *European Journal of Neuroscience* 10, 3799-3812. doi: 10.1046/j.1460-9568.1998.00394.x.
- España, R.A., Roberts, D.C.S., and Jones, S.R. (2008). Short-acting cocaine and long-acting GBR-12909 both elicit rapid dopamine uptake inhibition following intravenous delivery. *Neuroscience* 155, 250-257. doi: 10.1016/j.neuroscience.2008.05.022.
- Esposti, F., Johnston, J., Rosa, Juliana m., Leung, K.-M., and Lagnado, L. (2013). Olfactory Stimulation Selectively Modulates the OFF Pathway in the Retina of Zebrafish. *Neuron* 79, 97-110. doi: 10.1016/j.neuron.2013.05.001.
- Feierstein, C.E., Portugues, R., and Orger, M.B. Seeing the whole picture: A comprehensive imaging approach to functional mapping of circuits in behaving zebrafish. *Neuroscience* [Online]. Available: <http://www.sciencedirect.com/science/article/pii/S0306452214010148>.
- Fetcho, J.R., and Liu, K.S. (1998). Zebrafish as a Model System for Studying Neuronal Circuits and Behavior. *Annals of the New York Academy of Sciences* 860, 333-345. doi: 10.1111/j.1749-6632.1998.tb09060.x.
- Fortin, S.M., Cone, J.J., Ng-Evans, S., Mccutcheon, J.E., and Roitman, M.F. (2015). Sampling phasic dopamine signaling with fast-scan cyclic voltammetry in awake, behaving rats. *Curr Protoc Neurosci* 70, 7.25.21-27.25.20. doi: 10.1002/0471142301.ns0725s70.
- Haas, H.L., Sergeeva, O.A., and Selbach, O. (2008). Histamine in the Nervous System. *Physiological Reviews* 88, 1183-1241. doi: 10.1152/physrev.00043.2007.
- Hashemi, P., Dankoski, E.C., Wood, K.M., Ambrose, R.E., and Wightman, R.M. (2011). In vivo electrochemical evidence for simultaneous 5-HT and histamine release in the rat substantia nigra pars reticulata following medial forebrain bundle stimulation. *Journal of Neurochemistry* 118, 749-759. doi: 10.1111/j.1471-4159.2011.07352.x.
- Heien, M.L., Phillips, P.E., Stuber, G.D., Seipel, A.T., and Wightman, R.M. (2003). Overoxidation of carbon-fiber microelectrodes enhances dopamine adsorption and increases sensitivity. *Analyst* 128, 1413-1419. doi: 10.1039/b307024g.
- Heien, M.L.a.V., Johnson, M.A., and Wightman, R.M. (2004). Resolving Neurotransmitters Detected by Fast-Scan Cyclic Voltammetry. *Analytical Chemistry* 76, 5697-5704. doi: 10.1021/ac0491509.
- Heien, M.L.a.V., Khan, A.S., Ariansen, J.L., Cheer, J.F., Phillips, P.E.M., Wassum, K.M., and Wightman, R.M. (2005). Real-time measurement of dopamine fluctuations after cocaine in the brain of behaving rats. *Proceedings of the National Academy of Sciences of the United States of America* 102, 10023-10028. doi: 10.1073/pnas.0504657102.
- Higashijima, S.-I., Masino, M.A., Mandel, G., and Fetcho, J.R. (2003). Imaging Neuronal Activity During Zebrafish Behavior With a Genetically Encoded Calcium Indicator. *Journal of Neurophysiology* 90, 3986-3997. doi: 10.1152/jn.00576.2003.
- John, C.E., and Jones, S.R. (2007a). "Fast Scan Cyclic Voltammetry of Dopamine and Serotonin in Mouse Brain Slices," in *Electrochemical Methods for Neuroscience*, eds. A.C. Michael & L.M. Borland. (Boca Raton (FL): CRC Press).

- John, C.E., and Jones, S.R. (2007b). Voltammetric characterization of the effect of monoamine uptake inhibitors and releasers on dopamine and serotonin uptake in mouse caudate-putamen and substantia nigra slices. *Neuropharmacology* 52, 1596-1605.
- Jones, S.R., Garriss, P.A., Kilts, C.D., and Wightman, R.M. (1995a). Comparison of Dopamine Uptake in the Basolateral Amygdaloid Nucleus, Caudate-Putamen, and Nucleus Accumbens of the Rat. *Journal of Neurochemistry* 64, 2581-2589. doi: 10.1046/j.1471-4159.1995.64062581.x.
- Jones, S.R., Garriss, P.A., and Wightman, R.M. (1995b). Different effects of cocaine and nomifensine on dopamine uptake in the caudate-putamen and nucleus accumbens. *Journal of Pharmacology and Experimental Therapeutics* 274, 396-403.
- Jones, S.R., Mickelson, G.E., Collins, L.B., Kawagoe, K.T., and Mark Wightman, R. (1994). Interference by pH and Ca²⁺ ions during measurements of catecholamine release in slices of rat amygdala with fast-scan cyclic voltammetry. *Journal of Neuroscience Methods* 52, 1-10. doi: [http://dx.doi.org/10.1016/0165-0270\(94\)90048-5](http://dx.doi.org/10.1016/0165-0270(94)90048-5).
- Kaslin, J., and Panula, P. (2001). Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *The Journal of Comparative Neurology* 440, 342-377. doi: 10.1002/cne.1390.
- Keithley, R.B., and Wightman, R.M. (2011). Assessing Principal Component Regression Prediction of Neurochemicals Detected with Fast-Scan Cyclic Voltammetry. *ACS Chemical Neuroscience* 2, 514-525. doi: 10.1021/cn200035u.
- Lillesaar, C., Stigloher, C., Tannhäuser, B., Wullmann, M.F., and Bally-Cuif, L. (2009). Axonal projections originating from raphe serotonergic neurons in the developing and adult zebrafish, *Danio rerio*, using transgenics to visualize raphe-specific pet1 expression. *The Journal of Comparative Neurology* 512, 158-182. doi: 10.1002/cne.21887.
- Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proceedings of the National Academy of Sciences of the United States of America* 100, 13940-13945. doi: 10.1073/pnas.1936192100.
- Naumann, E.A., Kampff, A.R., Prober, D.A., Schier, A.F., and Engert, F. (2010). Monitoring neural activity with bioluminescence during natural behavior. *Nat Neurosci* 13, 513-520. doi: http://www.nature.com/neuro/journal/v13/n4/supinfo/nn.2518_S1.html.
- Near, J.A., Bigelow, J.C., and Wightman, R.M. (1988). Comparison of uptake of dopamine in rat striatal chopped tissue and synaptosomes. *Journal of Pharmacology and Experimental Therapeutics* 245, 921-927.
- Norton, W.H.J., Stumpfenhorst, K., Faus-Kessler, T., Folchert, A., Rohner, N., Harris, M.P., Callebert, J., and Bally-Cuif, L. (2011). Modulation of Fgfr1a Signaling in Zebrafish Reveals a Genetic Basis for the Aggression–Boldness Syndrome. *The Journal of Neuroscience* 31, 13796-13807. doi: 10.1523/jneurosci.2892-11.2011.
- Peitsaro, N., Anichtchik, O.V., and Panula, P. (2000). Identification of a Histamine H3-like Receptor in the Zebrafish (*Danio rerio*) Brain. *Journal of Neurochemistry* 75, 718-724. doi: 10.1046/j.1471-4159.2000.0750718.x.
- Peitsaro, N., Kaslin, J., Anichtchik, O.V., and Panula, P. (2003). Modulation of the histaminergic system and behaviour by α -fluoromethylhistidine in zebrafish. *Journal of Neurochemistry* 86, 432-441. doi: 10.1046/j.1471-4159.2003.01850.x.
- Pihel, K., Hsieh, S., Jorgenson, J.W., and Wightman, R.M. (1995). Electrochemical detection of histamine and 5-hydroxytryptamine at isolated mast cells. *Analytical Chemistry* 67, 4514-4521. doi: 10.1021/ac00120a014.
- Renier, C., Faraco, J.H., Bourgin, P., Motley, T., Bonaventure, P., Rosa, F., and Mignot, E. (2007). Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. *Pharmacogenet Genomics* 17, 237-253. doi: 10.1097/FPC.0b013e3280119d62.
- Robinson, D.L., Venton, B.J., Heien, M.L., and Wightman, R.M. (2003). Detecting subsecond dopamine release with fast-scan cyclic voltammetry in vivo. *Clin Chem* 49, 1763-1773.
- Robinson, D.L., and Wightman, R.M. (2007). "Rapid Dopamine Release in Freely Moving Rats.," in *Electrochemical Methods for Neuroscience.*, eds. Michael A.C. & B. L.M. (Boca Raton (FL): CRC Press).

- Sinkala, E., Mccutcheon, J.E., Schuck, M., Schmidt, E., Roitman, M.F., and Eddington, D.T. (2012). Electrode Calibration with a Microfluidic Flow Cell for Fast-scan Cyclic Voltammetry. *Lab on a chip* 12, 2403-2408. doi: 10.1039/c2lc40168a.
- Stamford, J.A. (1990). Fast cyclic voltammetry: Measuring transmitter release in 'real time'. *Journal of Neuroscience Methods* 34, 67-72. doi: [http://dx.doi.org/10.1016/0165-0270\(90\)90043-F](http://dx.doi.org/10.1016/0165-0270(90)90043-F).
- Takmakov, P., Zachek, M.K., Keithley, R.B., Bucher, E.S., Mccarty, G.S., and Wightman, R.M. (2010). Characterization of Local pH Changes in Brain Using Fast-Scan Cyclic Voltammetry with Carbon Microelectrodes. *Analytical Chemistry* 82, 9892-9900. doi: 10.1021/ac102399n.
- Threlfell, S., and Cragg, S.J. (2007). "Using Fast-Scan Cyclic Voltammetry to Investigate Somatodendritic Dopamine Release," in *Electrochemical Methods for Neuroscience*, eds. A.C. Michael & L.M. Borland. (Boca Raton (FL): CRC Press
- Taylor & Francis Group, LLC).
- Vargas, R., Jóhannesdóttir, I.P., Sigurgeirsson, B., Þorsteinsson, H., and Karlsson, K.Æ. (2011). The zebrafish brain in research and teaching: a simple in vivo and in vitro model for the study of spontaneous neural activity. *Adv Physiol Educ* 35, 188-196. doi: 10.1152/advan.00099.2010.
- Venton, B.J., Michael, D.J., and Wightman, R.M. (2003). Correlation of local changes in extracellular oxygen and pH that accompany dopaminergic terminal activity in the rat caudate-putamen. *Journal of Neurochemistry* 84, 373-381. doi: 10.1046/j.1471-4159.2003.01527.x.
- Webb, K.J., Norton, W.H.J., Trümbach, D., Meijer, A.H., Ninkovic, J., Topp, S., Heck, D., Marr, C., Wurst, W., Theis, F.J., Spaink, H.P., and Bally-Cuif, L. (2009). Zebrafish reward mutants reveal novel transcripts mediating the behavioral effects of amphetamine. *Genome Biology* 10, R81-R81. doi: 10.1186/gb-2009-10-7-r81.
- Wen, L., Wei, W., Gu, W., Huang, P., Ren, X., Zhang, Z., Zhu, Z., Lin, S., and Zhang, B. (2008). Visualization of monoaminergic neurons and neurotoxicity of MPTP in live transgenic zebrafish. *Developmental Biology* 314, 84-92. doi: <http://dx.doi.org/10.1016/j.ydbio.2007.11.012>.
- Westerfield, M. (1995). *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 3rd Edition. Eugene, OR: University of Oregon Press.
- Yorgason, J.T., España, R.A., and Jones, S.R. (2011). Demon voltammetry and analysis software: Analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *Journal of neuroscience methods* 202, 158-164. doi: 10.1016/j.jneumeth.2011.03.001.
- Zhang, F., Wang, L.-P., Brauner, M., Liewald, J.F., Kay, K., Watzke, N., Wood, P.G., Bamberg, E., Nagel, G., Gottschalk, A., and Deisseroth, K. (2007). Multimodal fast optical interrogation of neural circuitry. *Nature* 446, 633-639. doi: http://www.nature.com/nature/journal/v446/n7136/suppinfo/nature05744_S1.html.
- Ziv, L., Muto, A., Schoonheim, P.J., Meijsing, S.H., Strasser, D., Ingraham, H.A., Schaaf, M.J.M., Yamamoto, K.R., and Baier, H. (2013). An affective disorder in zebrafish with mutation of the glucocorticoid receptor. *Molecular psychiatry* 18, 681-691. doi: 10.1038/mp.2012.64.
- Zupanc, G.K.H., and Lamprecht, J. (2000). Towards a Cellular Understanding of Motivation: Structural Reorganization and Biochemical Switching as Key Mechanisms of Behavioral Plasticity. *Ethology* 106, 467-477. doi: 10.1046/j.1439-0310.2000.00546.x.

Table 1

Output for CV Match linear regression analysis comparing template cyclic voltammograms obtained using a flow cell with cyclic voltammograms extracted from experimental data obtained using either optimal electrical stimulation parameters (n=4 stimulations from 2 sagittal sections taken from 2 fish) or K⁺ stimulation (n=2 stimulations from 2 fish). Bold type indicates a significant r^2 value, and bold red type indicates the highest r^2 value for the example stimulation. Legend: DA, dopamine; HA, histamine; Estim, electrical stimulation; K⁺, high K⁺ aCSF stimulation.

DA (μ M)	HA (μ M)	5-HT (μ M)	Acid pH (pH units)	Basic pH (pH units)	ESTi m1 (r^2)	Esti m2 (r^2)	Esti m3 (r^2)	Esti m4 (r^2)	K ⁺ 10s 1 (r^2)	K ⁺ 30s 1 (r^2)	K ⁺ 10s 2 (r^2)	K ⁺ 30s 2 (r^2)
0.5	0	0	0	0	0.053	0.213	0.321	0.121	0.551	0.123	0.542	0.081
1	0	0	0	0	0.639	0.657	0.602	0.638	0.78	0.316	0.836	0.408
2	0	0	0	0	0.61	0.624	0.56	0.512	0.742	0.325	0.81	0.369
0	10	0	0	0	0.167	0.164	0.366	0.377	0.75	0.373	0.465	0.361
0	20	0	0	0	0.061	0.2	0.155	0.222	0.691	0.306	0.417	0.313
0	40	0	0	0	0.199	0.284	0.053	0.179	0.702	0.468	0.559	0.52
0	0	0.1	0	0	0.661	0.678	0.641	0.73	0.758	0.414	0.74	0.612
0	0	0.5	0	0	0.625	0.611	0.671	0.664	0.694	0.375	0.659	0.402
0	0	0.25	0	0	0.529	0.456	0.474	0.493	0.548	0.319	0.482	0.248
0	0	0	0	0.5	0.468	0.436	0.703	0.679	0.478	0.197	0.418	0.139
0	0	0	0	1	0.669	0.579	0.814	0.843	0.353	0.154	0.55	0.211
0	0	0	0	2	0.599	0.604	0.834	0.871	0.235	0.288	0.533	0.205
0	0	0	0.5	0	0.348	0.254	0.641	0.652	0.633	0.139	0.198	0.071
0	0	0	0.25	0	0.368	0.297	0.624	0.688	0.556	0.166	0.245	0.045
0	0	0	1	0	0.291	0.311	0.59	0.588	0.624	0.194	0.275	0.145
0	0	0	3.5	0	0.385	0.485	0.576	0.509	0.465	0.51	0.528	0.432
1	40	0	0	0	0.561	0.611	0.352	0.47	0.833	0.45	0.76	0.524
2	20	0	0	0	0.814	0.787	0.771	0.651	0.857	0.416	0.866	0.441
0	40	0.25	0	0	0.71	0.67	0.588	0.65	0.762	0.425	0.674	0.463
1	20	0.25	0	0	0.776	0.746	0.784	0.733	0.853	0.426	0.814	0.431
1	0	0	0	0.5	0.31	0.101	0.379	0.399	0.475	0.374	0.267	0.011
4	0	0	0	1	0.427	0.328	0.209	0.202	0.327	0.508	0.508	0.337
2	0	0	0.5	0	0.833	0.755	0.832	0.868	0.734	0.282	0.752	0.285
4	0	0	0.25	0	0.841	0.802	0.818	0.873	0.781	0.318	0.819	0.341
1	0	0	1	0	0.667	0.588	0.724	0.801	0.588	0.19	0.626	0.151
0	40	0	0	0.5	0.19	0.254	0.402	0.48	0.651	0.24	0.325	0.216
0	40	0	0	2	0.403	0.447	0.641	0.725	0.426	0.169	0.344	0.317
0	160	0	0.5	0	0.59	0.466	0.676	0.678	0.37	0.273	0.487	0.252
0	160	0	0.25	0	0.473	0.511	0.581	0.593	0.228	0.14	0.504	0.376
0	40	0	1	0	0.548	0.435	0.705	0.713	0.245	0.148	0.454	0.092
0	0	1	0	1	0.552	0.519	0.541	0.581	0.61	0.314	0.549	0.258
0	0	0.5	0	2	0.514	0.504	0.742	0.746	0.208	0.27	0.42	0.224
0	0	0.5	2	0	0.385	0.419	0.566	0.645	0.426	0.191	0.423	0.126
2	80	0	0	0.5	0.54	0.523	0.272	0.372	0.759	0.442	0.658	0.53

1	40	0	0	1	0.312	0.206	0.427	0.488	0.552	0.156	0.241	0.078
1	40	0	0.5	0	0.795	0.755	0.75	0.804	0.826	0.435	0.808	0.483
2	160	0	0.5	0	0.845	0.739	0.84	0.853	0.675	0.414	0.75	0.355
2	80	0	0.25	0	0.846	0.833	0.863	0.877	0.777	0.421	0.824	0.426
4	160	0	0.25	0	0.845	0.806	0.863	0.878	0.752	0.441	0.801	0.421
1	80	0	1	0	0.752	0.676	0.799	0.831	0.178	0.248	0.702	0.243
0	160	1	0.5	0	0.75	0.727	0.836	0.813	0.762	0.481	0.712	0.461
1	40	0.5	0	1	0.197	0.05	0.315	0.361	0.609	0.25	0.251	0.062

842

843

844

845

846

In review

Figure 1.TIF

Figure 1

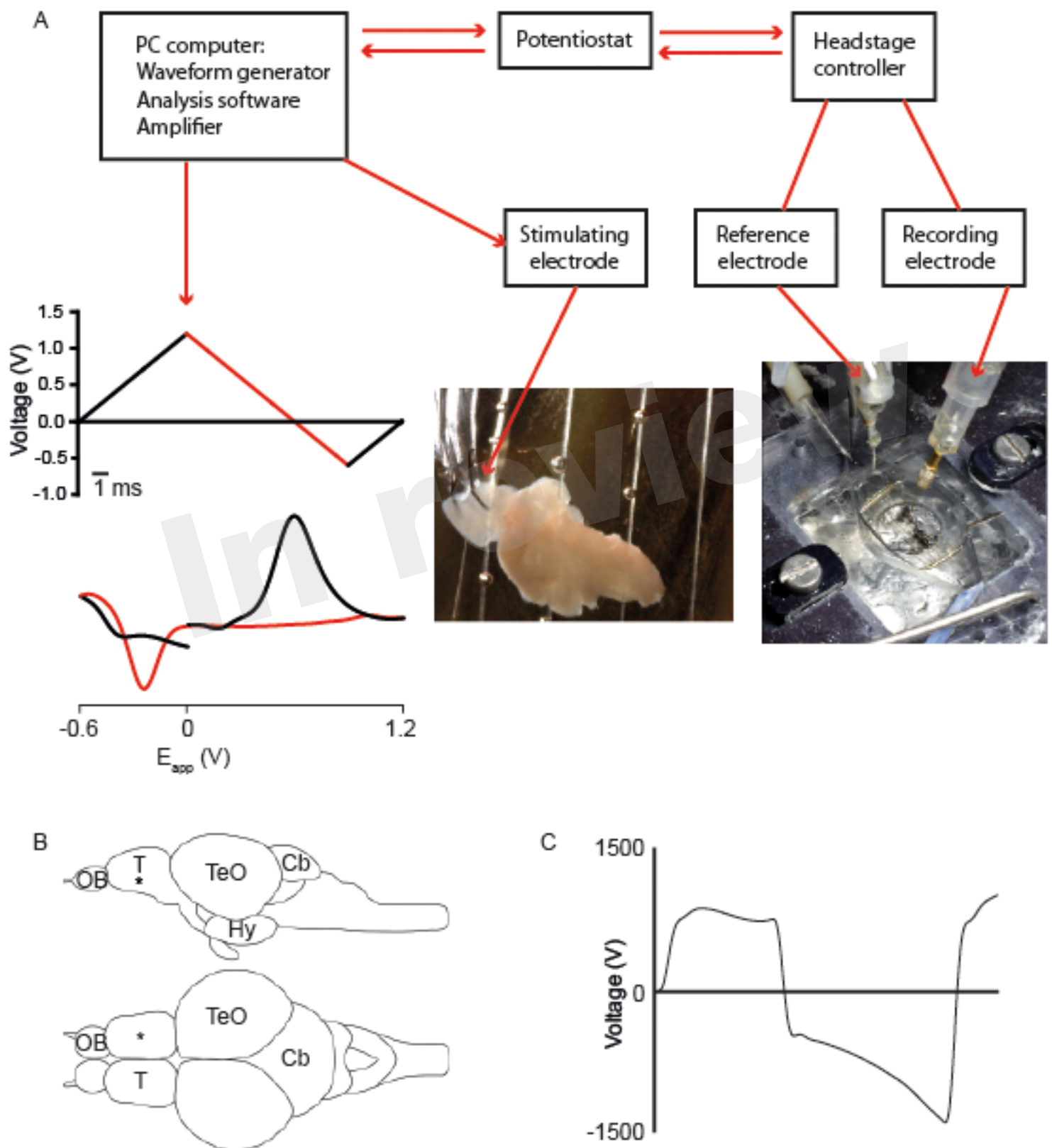


Figure 10.TIF

Figure 10

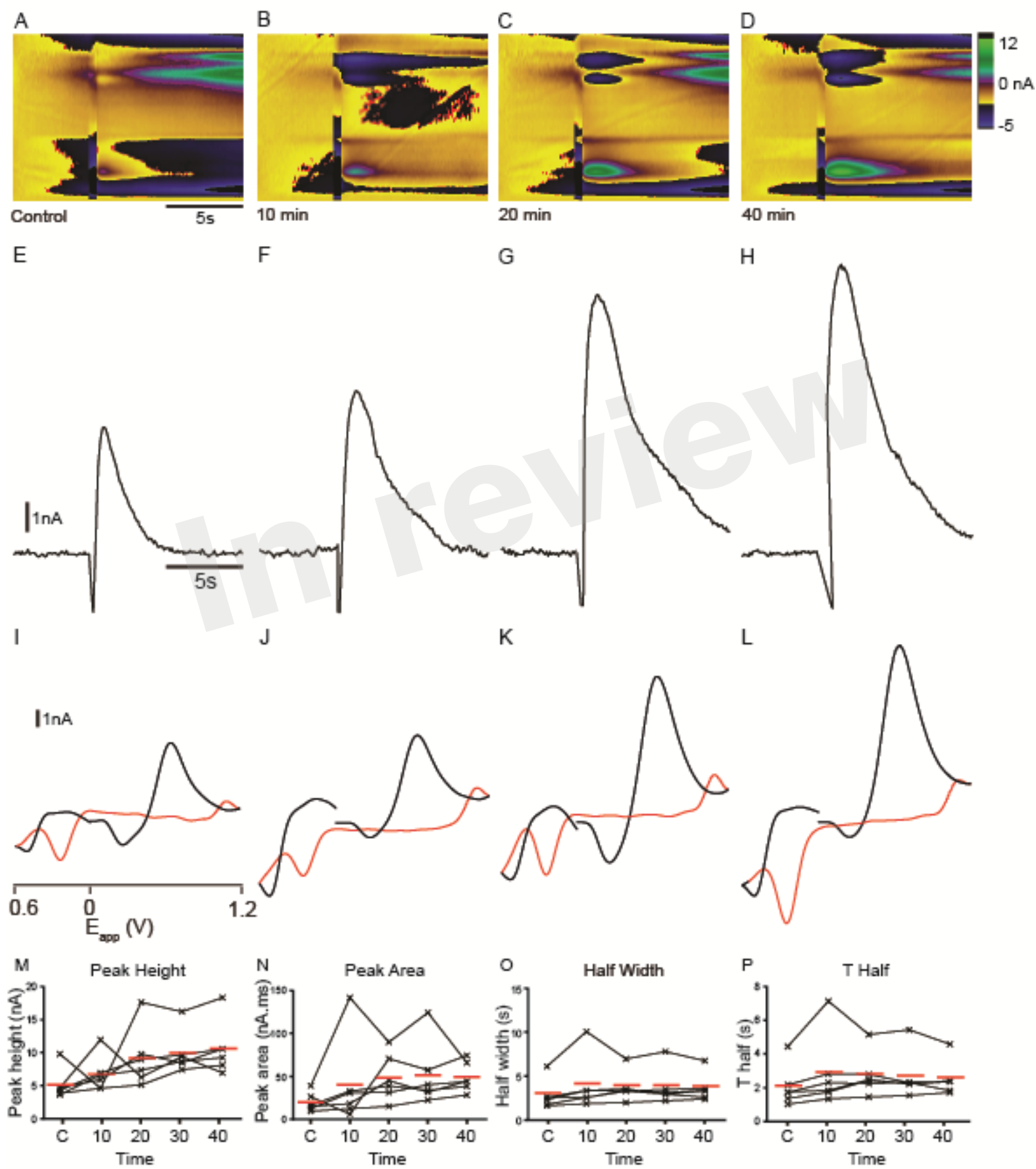


Figure 2

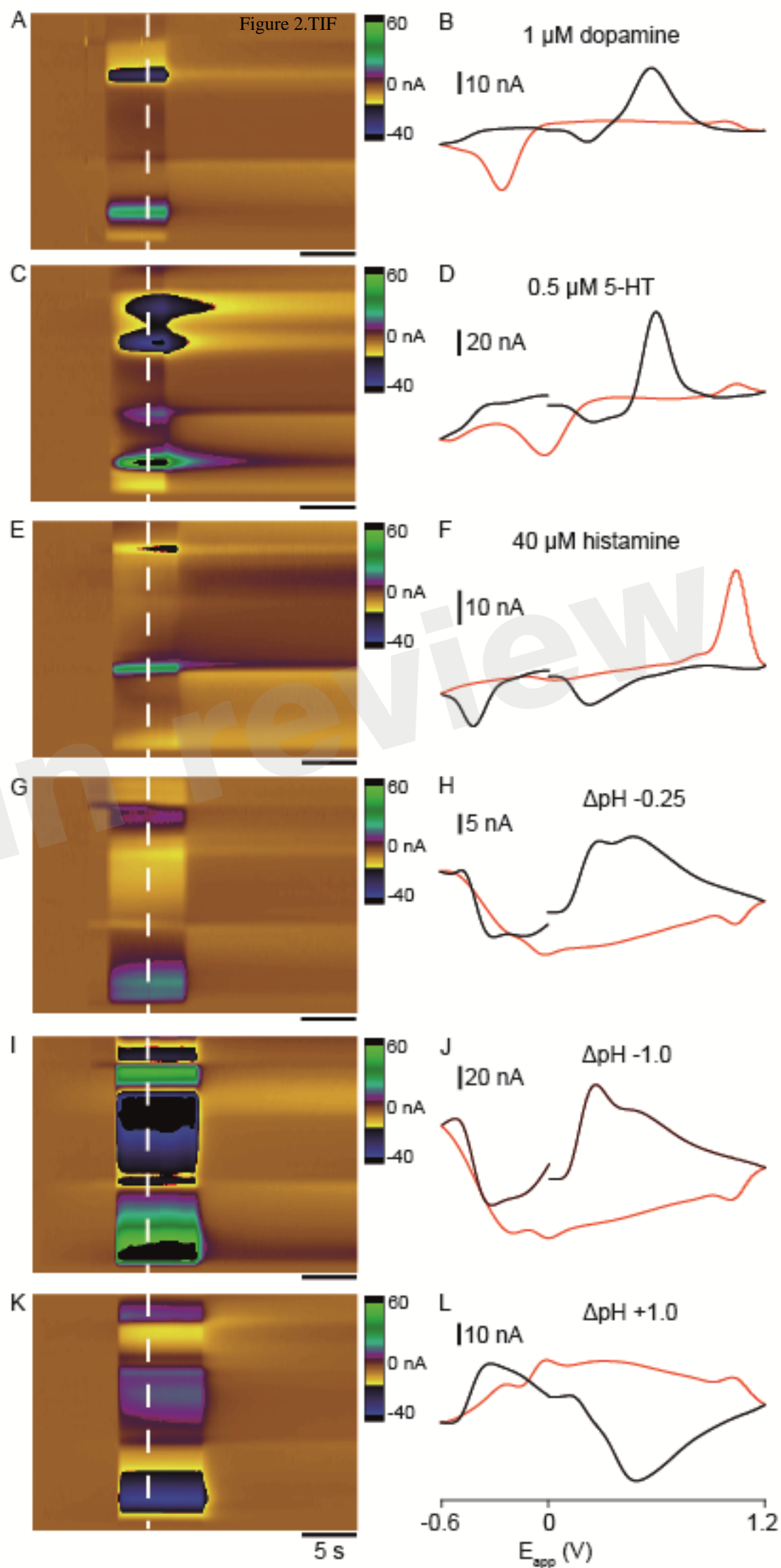


Figure 3

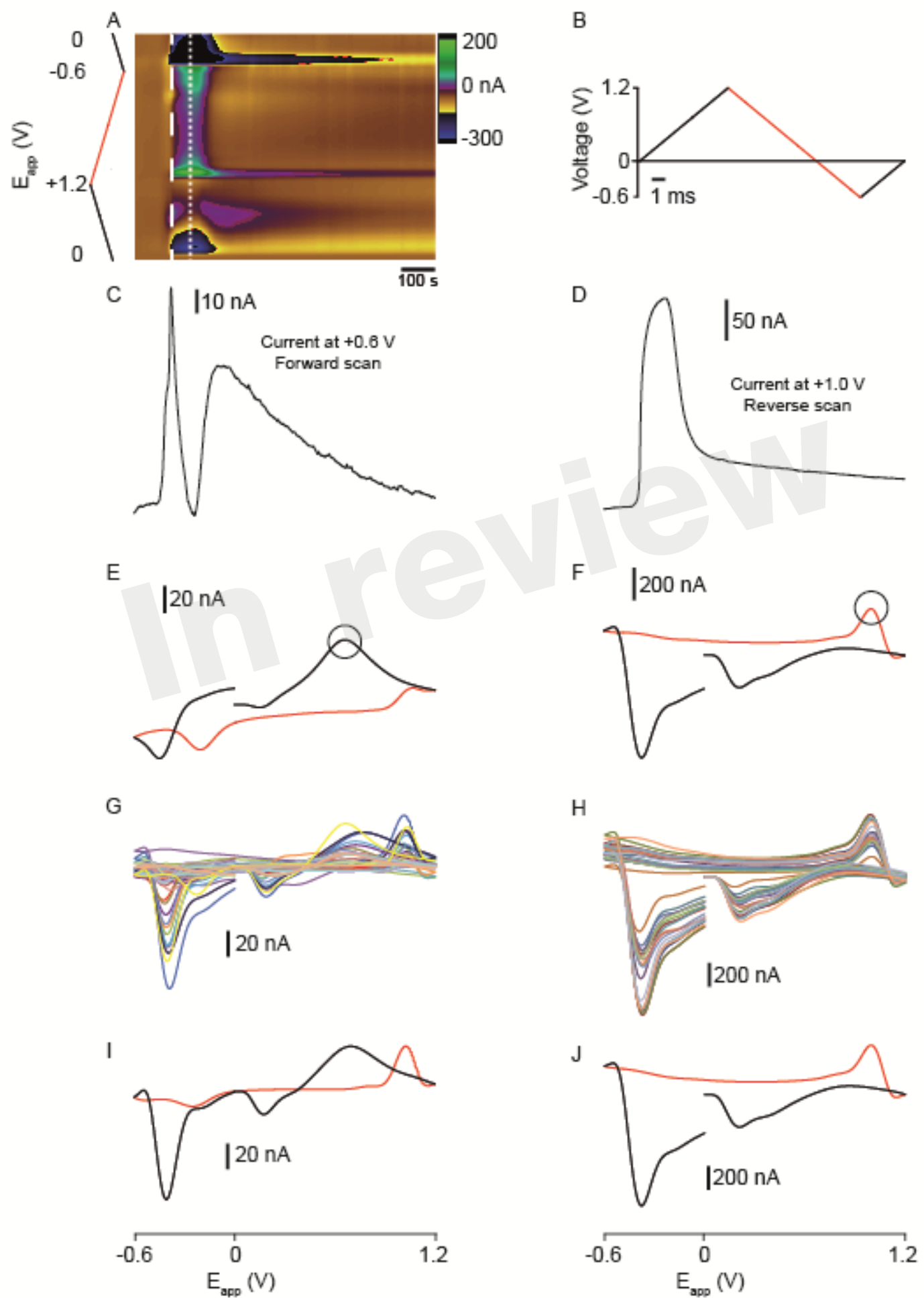


Figure 4

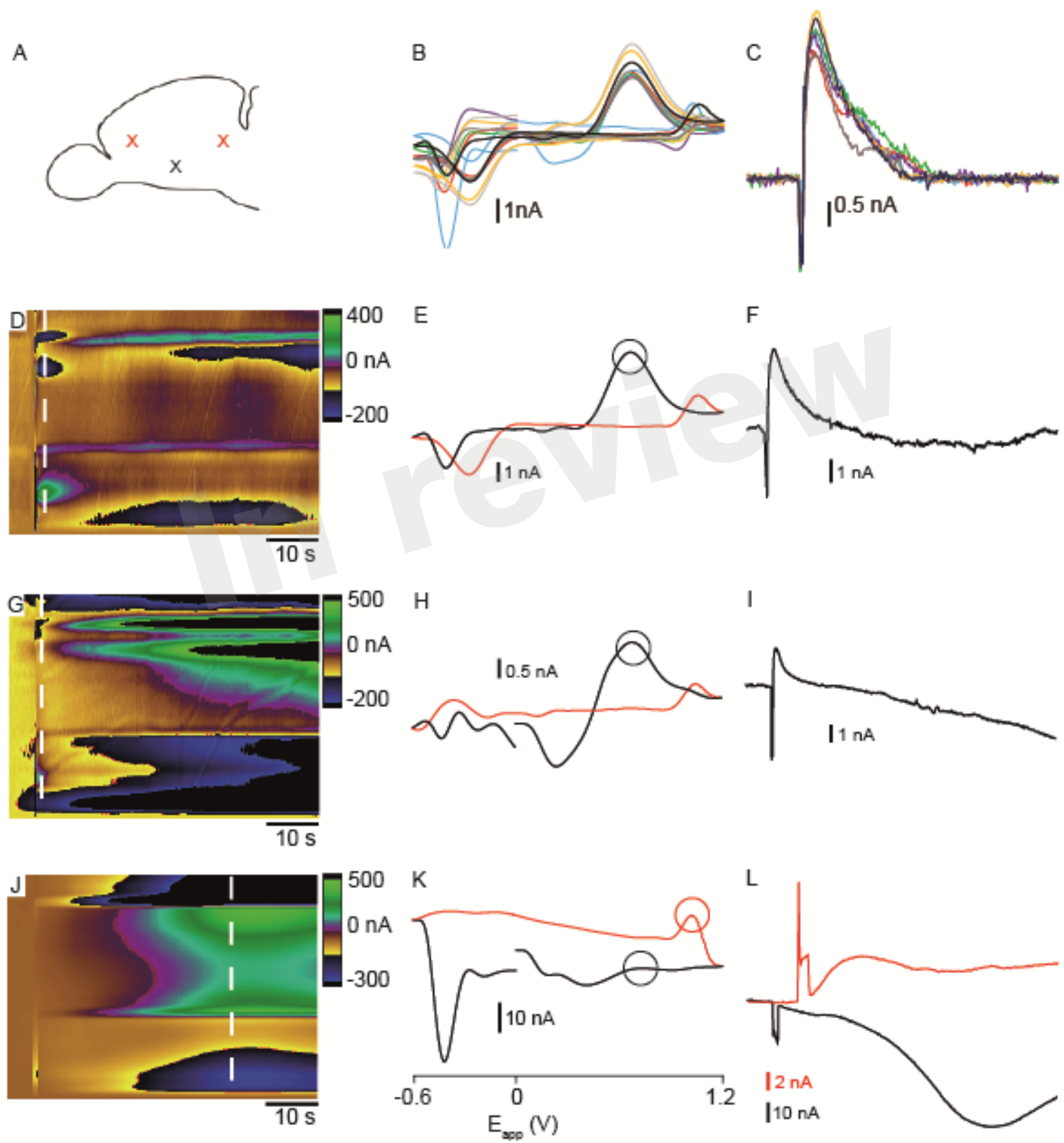


Figure 5

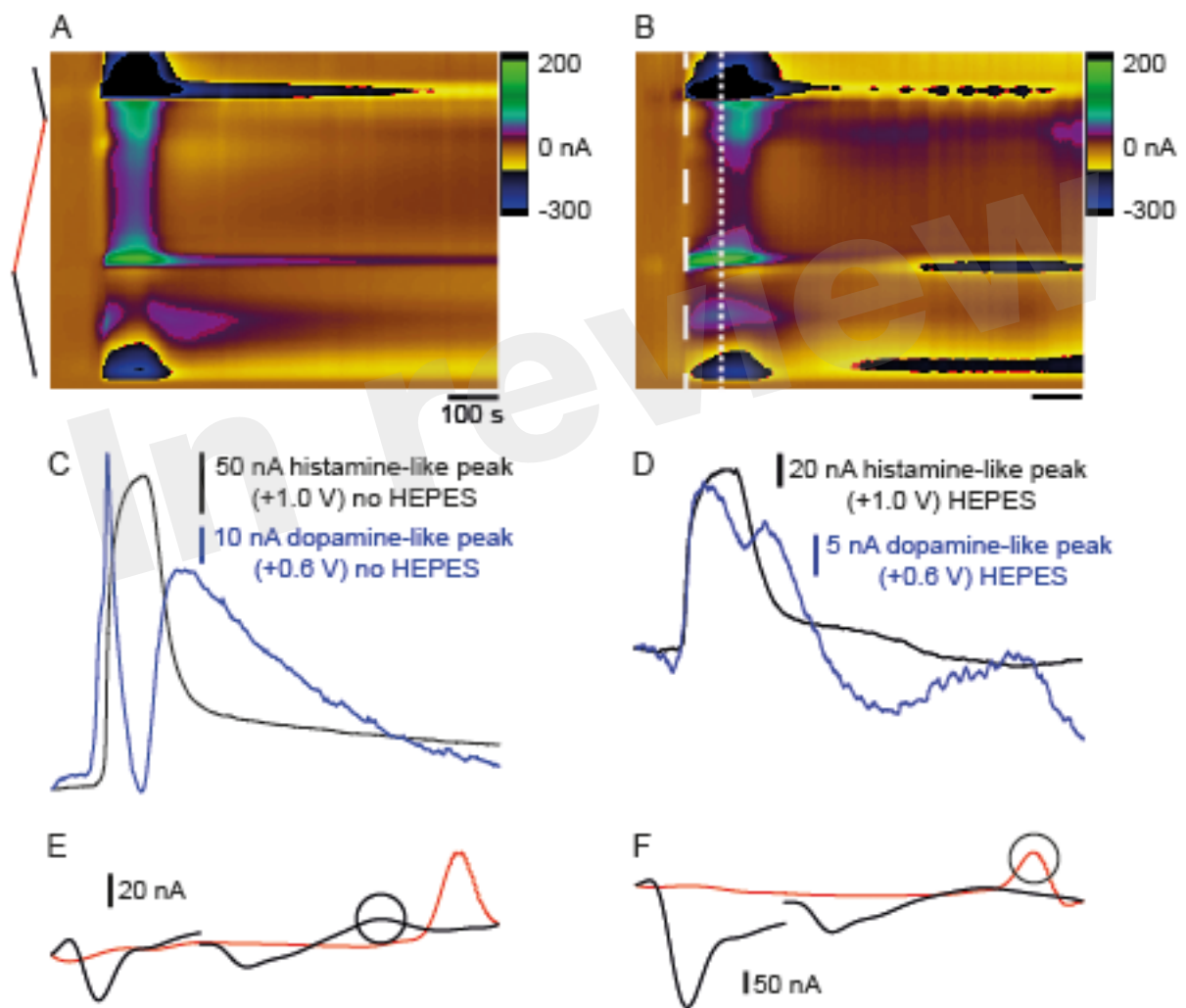


Figure 6

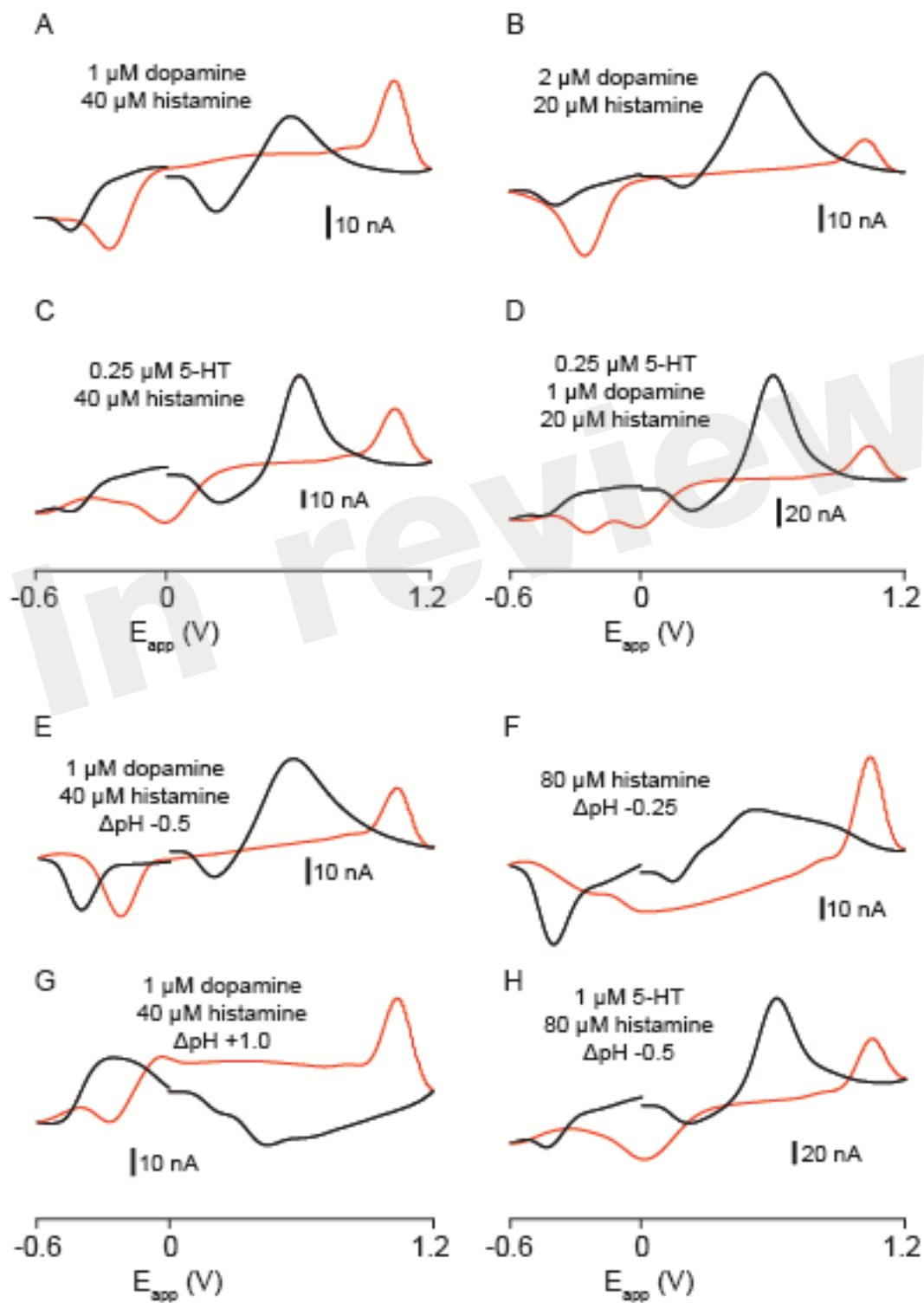


Figure 7

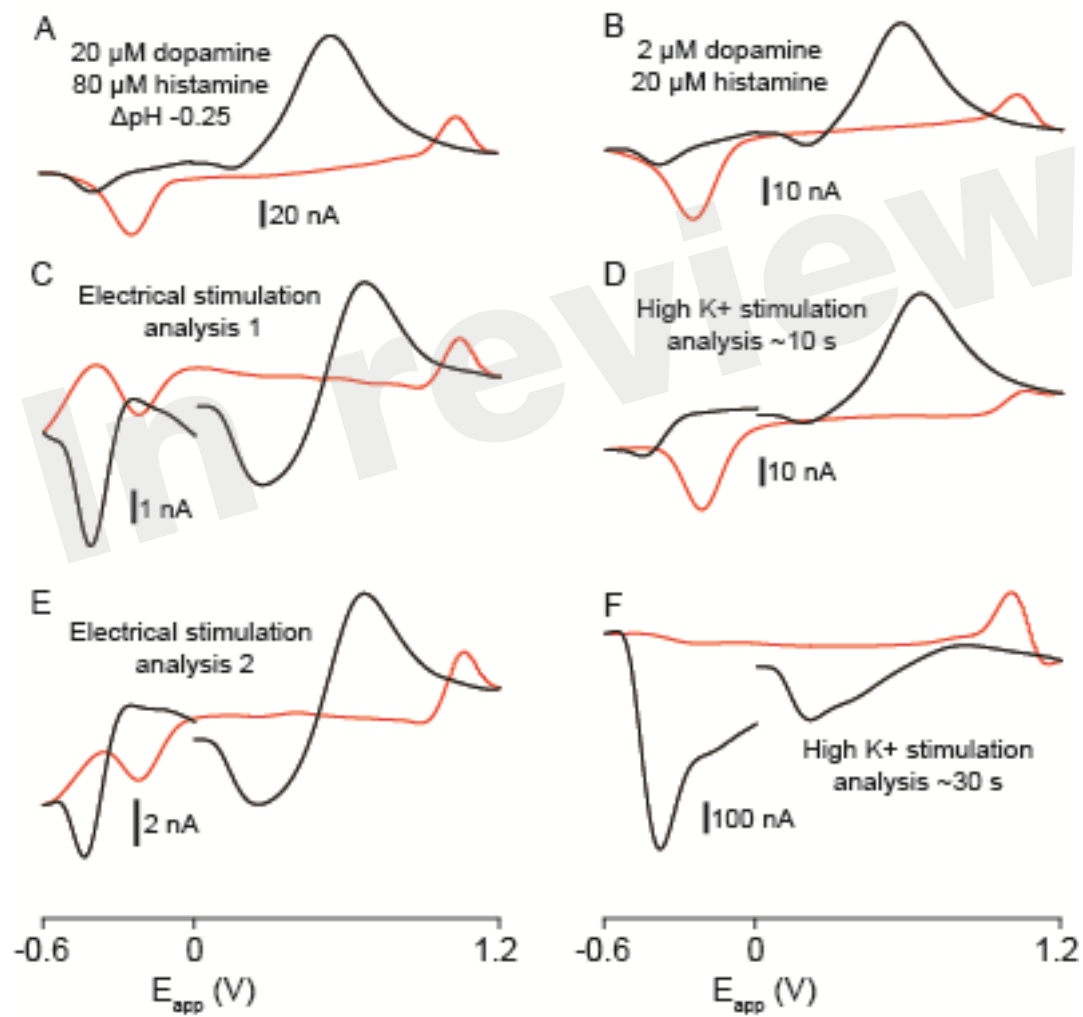


Figure 8

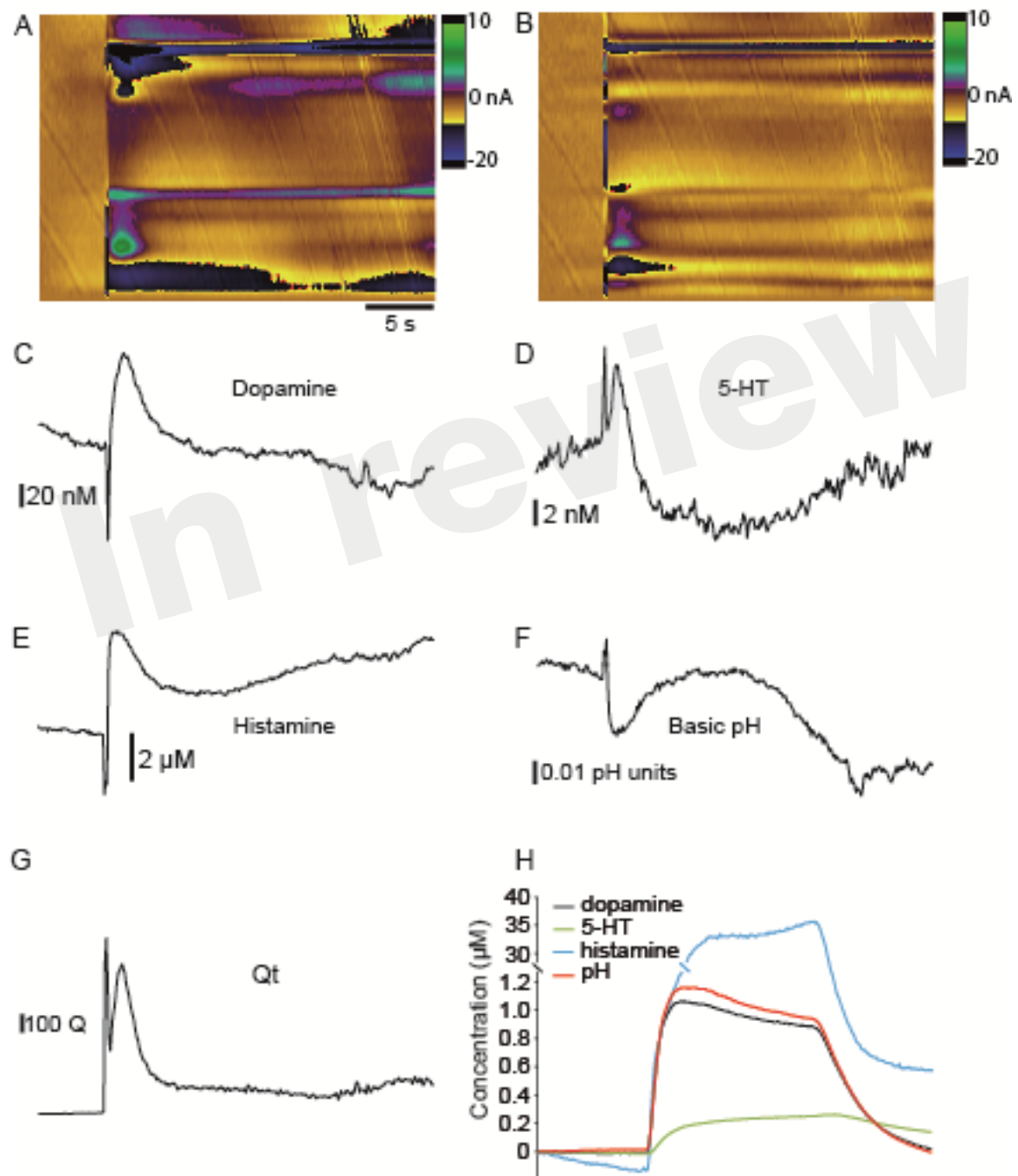


Figure 9

