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TITLE:

A Plate-Based Assay for The Measurement of Endogenous Monoamine Release in Acute Brain Slices

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KEYWORDS:

monoamine release, acute brain slices, monoamines, dopamine, norepinephrine, serotonin, neurotransmission

SUMMARY:

This method introduces a simple technique for the detection of endogenous monoamine release using acute brain slices. The setup uses a 48-well plate containing a tissue holder for monoamine release. Released monoamine is analyzed by HPLC coupled with electrochemical detection. Additionally, this technique provides a screening method for drug discovery.

ABSTRACT:

Monoamine neurotransmitters are associated with numerous neurologic and psychiatric ailments. Animal models of such conditions have shown alterations in monoamine neurotransmitter release and uptake dynamics. Technically complex methods such as electrophysiology, Fast Scan Cyclic Voltammetry (FSCV), imaging, in vivo microdialysis, optogenetics, or use of radioactivity are required to study monoamine function. The method presented here is an optimized two-step approach for detecting monoamine release in acute brain slices using a 48-well plate containing tissue holders for examining monoamine release, and high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) for

monoamine release measurement. Briefly, rat brain sections containing regions of interest, including prefrontal cortex, hippocampus, and dorsal striatum were obtained using a tissue slicer or vibratome. These regions of interest were dissected from the whole brain and incubated in an oxygenated physiological buffer. Viability was examined throughout the experimental time course, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The acutely dissected brain regions were incubated in varying drug conditions that are known to induce monoamine release through the transporter (amphetamine) or through the activation of exocytotic vesicular release (KCl). After incubation, the released products in the supernatant were collected and analyzed through an HPLC-ECD system. Here, basal monoamine release is detected by HPLC from acute brain slices. This data supports previous in vivo and in vitro results showing that AMPH and KCl induce monoamine release. This method is particularly useful for studying mechanisms associated with monoamine transporter-dependent release and provides an opportunity to screen compounds affecting monoamine release in a rapid and low-cost manner.

INTRODUCTION:

A plethora of neurological and psychiatric diseases are associated with dysregulation or insufficient maintenance of monoamine neurotransmitter (dopamine [DA], serotonin [5-HT], norepinephrine [NE]) homeostasis[1–3]. These conditions include, but are not limited to, depression[1,2], schizophrenia[2], anxiety[2], addiction[4], menopause[5–7], pain[8], and Parkinson's disease[3]. For instance, several rat models of menopause have shown that the dysregulation or reduction of monoamines within the hippocampus, prefrontal cortex, and striatum may be associated with both depression and cognitive decline, which is seen in women experiencing menopause. The dysregulation of monoamines in these models have been extensively examined using HPLC-ECD, although the studies did not discriminate between measured neurotransmitter content versus neurotransmitter release[5–7]. Monoamines are classically released into the extracellular space through Ca^{2+}-dependent vesicular release[9], and are recycled back through their respective plasma membrane re-uptake system (dopamine transporter, DAT; serotonin transporter, SERT; norepinephrine transporter, NET)[10,11]. Conversely, data suggests that these transporters are able to release or efflux monoamines, since drugs of abuse such as amphetamine (AMPH) and 3,4-Methylenedioxymethamphetamine (MDMA) are known to release DA and 5-HT, respectively through their transporter systems[12–17]. Thus, a proper mechanistic understanding of monoamine release dynamics is crucial for developing specific and targeted pharmacotherapies.

A wide range of techniques have been employed to study monoamine release such as Fast Scan Cyclic Voltammetry (FSCV)[18], in vivo microdialysis[13], imaging[19], preincubation with radiolabeled monoamines[20], optogenetics, and more recently, genetically encoded fluorescent sensors and photometry[21,22]. FSCV and in vivo microdialysis are the primary techniques used for studying monoamine release. FSCV is used to study the stimulated exocytotic release of, primarily, DA in acute brain slices and in vivo[23]. Because FSCV uses electrodes to stimulate or evoke release, the primary source of neurotransmitter release is Ca^{2+}-dependent vesicular release[18,24–31]. In vivo microdialysis coupled with HPLC measures changes in extracellular neurotransmitter levels using a probe placed in a brain area of interest[13,32]. Similar to FSCV, a major limitation to in vivo

microdialysis is the difficulty in determining the source of neurotransmitter release: Ca^{2+} dependent vesicular release or transporter dependent. Noteworthy, both methods allow for the direct measurement of monoamine release. Through the recent advancement of optogenetics, research demonstrates detection of 5-HT and DA release in a short timespan with exquisite cell-type specificity[21,22]. However, these strategies require complex and costly techniques and equipment, and indirectly measure monoamine release, specifically through monoamine binding to receptors. Further, radiolabeled monoamines are also used for studying monoamine dynamics. Radiolabeled monoamines may be preloaded into various model systems such as heterologous cells overexpressing each monoamine transporter[20,33–40], primary neurons[20], synaptosomes[33,39,41,42], and acute brain slices[43,44]. However, radioactivity poses potential harm to the experimenter, and the tritium-labeled analytes may not faithfully recapitulate endogenous monoamine dynamics[45,46]. Superfusion systems combined with off-line detection methods such as HPLC-ECD have allowed for the detection of monoamines from multiple tissue sources. Here, this protocol provides as an optimized and low-cost, simple, and precise method using acute brain slices to directly measure endogenous basal and stimulated monoamine release.

Acute brain slices allow for testing mechanistic hypotheses, primarily as they preserve the in vivo anatomical microenvironment, and maintain intact synapses[47–52]. In a few studies, acute brain slices or chopped brain tissue have been used in conjunction with a superfusion technique using KCl to stimulate Ca^{2+} mediated release[53–56]. Superfusion systems have been critical to advance the field's understanding of neurotransmitter release mechanisms, including monoamines. However, these systems are relatively expensive, and the number of chambers available for tissue analysis ranges from 4–12. In comparison, the method presented here is inexpensive, allows the measurement of 48 tissue samples, and may be refined to use up to 96 tissue samples. Each well within the 48-well plate contains tissue holders that use filters to separate the released product from the tissue, and released monoamines are then collected and analyzed by HPLC-ECD. Importantly, this method allows for the simultaneous measurement of 5-HT, DA, and NE release from different brain areas such as the prefrontal cortex, the hippocampus, and the dorsal striatum after treatment with pharmacological agents that modulate monoamine release. Thus, the experimenter can answer multiple questions using an inexpensive multi-well system that increases the number of samples tested and thereby reducing the number of animals used.

PROTOCOL:

All experiments, including animal handling and tissue collection, were carried out in accordance with the University of Florida and the City College of New York Institutional Animal Care and Use Committee (IACUC), following the approved protocol 201508873 (UF) and 1071 (CCNY). For reagents and buffer please refer to the **Supplementary File**.

1. Prepare acute rat brain slices

NOTE: In this experiment adult male rats (250–350 g) were used. However, this set up is functional for different developmental points, female rats, and other species. If using a smaller animal, such as mice, the experimenter may adjust to optimize the protocol by using a different number of brain slices or punches per condition. Dissection buffer will be referred to as Buffer 1;

efflux buffer will be referred to as Buffer 2.

1.1. Prepare Buffer 1 as mentioned in the **Supplementary File**. Saturate Buffer 1 with oxygen by bubbling with 95%/5% (O_2/CO_2) for 20 min on ice. Remove 50 mL of Buffer 1, and chill on ice in a small beaker or a Petri dish. This buffer is used to hold the acutely harvested whole brain.

1.1.2. Anesthetize one or two adult rats (250–350 g) with 1%–2% isoflurane, decapitate them using a guillotine, and rapidly remove their brains. Immediately place the brain in ice-cold oxygenated Buffer 1 in the container from step 1.1.

NOTE: Ensure isoflurane and guillotine are used safely. Open isoflurane under a fume hood.

1.1.3. Using a vibratome or compresstome, cut 300 µm coronal brain sections from each region of interest (**Figure 1**). Bubbling Buffer 1 must be present while sections are being made. Using a stainless-steel spatula, carefully and immediately transfer brain slices into a new Petri dish filled with ice-cold oxygenated Buffer 1 (**Figure 2**).

1.1.4. Further dissect brain slices (e.g., punches, cut out) by carefully moving the slices to glass slides (**Figure 1G**) using rat brain atlas[57]. For instance, identify the dorsal striatum based off its dark, striated structure, and identify the hippocampus based off its proximity to the cortex and unique spiral structure. The right and left hemispheres may be separated to use as control and experimental slices (**Figures 2G–H**). Here, the dorsal striatum was further dissected into 2 mm punches (**Figure 1G**).

1.1.5. Using a plastic transfer pipette with the tip cut off, transfer slices or brain punches into small containers immersed in oxygenated ice-cold Buffer 1 with oxygen bubbling. These containers may be stainless steel mesh, or small Petri dishes filled with buffer (**Figure 1H**).

2. Ex-vivo endogenous monoamine release from brain slices or punches

NOTE: The device used for this section consists of a 48-well plate and a tissue holder made of six microcentrifuge filter units without the inset-filters connected to a carbogen line (**Figure 2**). To make the holder, use a sturdy plastic rod (e.g., from a cell scrapper) and super glue the microcentrifuge filter units without the inset-filters to it. Let it dry for 1–2 days. Time required for the endogenous monoamine release experiment and concentrations of amphetamine, fluoxetine, and cocaine are based on the current literature and previous protocols[13,20,58].

2.1. Tissue activation

2.1.1. Transfer the brain tissue from step 1.1.5 to each well of the efflux chamber and allow recovery for 30–50 min at 37 °C in 0.5–1 mL of oxygenated Buffer 2, with constant, gentle bubbling (**Figure 2B1**).

2.1.2. During this incubation, dilute the drugs to the desired concentration for the experiment.

All the drugs must be dissolved in Buffer 2, and concentrations are based off the current literature.

2.2. First incubation

2.2.1. Move the tissue holder with brain tissue to wells containing 500 µL of oxygenated Buffer 2 and incubate for 20 min at 37 °C. Ensure that minimal to no buffer is transported over by tapping the holder on the edge of the well until no excess buffer is in the holder.

2.2.2. In experiments with pharmacological agents such as monoamine transporter inhibitors, incubate the tissue samples with the drugs diluted in oxygenated Buffer 2 (e.g., 10 µM Fluoxetine, 40 µM cocaine; see **Figure 2B2**). The final volume in each well will be 500 µL.

2.3. Second incubation

2.3.1. Move the holder with the tissue to a new set of wells containing 500 µL total Buffer 2 with or without the desired concentration of each drug. Ensure there is no excess buffer leftover. Each well represents an n = 1 for experimental conditions. Each experimental condition is performed in triplicate.

2.3.2. One well includes an oxygenated Buffer 2, the next 10–30 µM AMPH, and the final well includes 10–30 µM AMPH plus monoamine transporter inhibitors. Each drug is dissolved in oxygenated Buffer 2.

2.3.3. Incubate the tissue for 20 min at 37 °C with 500 µL of the drug condition.

NOTE: Additional wells may include an oxygenated high K+ Buffer 2 with or without the monoamine transporter inhibitors. Dissolve each drug in the oxygenated Buffer 2 (500 µL).

2.3.4. During this second incubation of 20 min, collect the solution from the wells from the first incubation in step 2.2.1 and transfer to microcentrifuge tubes containing 50 µL of 1 N perchloric acid or phosphoric acid (dependent on the type of HPLC, final concentration 0.1 N). The final volume of the sample will be 550 µL. Maintain microcentrifuge tubes on ice, and label the tubes #1.

2.3.5. After the second incubation of 20 min, move the tissue holder with brain sections or punches to an empty well and maintain the plate on ice. Transfer the supernatant to microcentrifuge tubes containing 50 µL of 1 N perchloric acid or phosphoric acid. The final volume of the sample will be 550 µL. Maintain microcentrifuge tubes on ice, and label the tubes #2.

2.3.6. Add 1 mL of ice-cold Buffer 1 to each well containing tissue. Collect all of the tissue using small tweezers, and transfer to clean microcentrifuge tubes.

2.3.7. Maintain tubes with brain tissue at -80 °C. Discard the 1 mL of Buffer 1 (**Figure 2B4**).

2.3.8. Filter solutions obtained from each incubation using microcentrifuge filter tubes (0.22 µm) at 2,500 x *g* for 2min. Use the filtrate to determine monoamine content using HPLC with electrochemical detection (**Figure 2B5**).

3. Tissue viability

3.1. MTT assay

NOTE: A significant concern regarding this experimental setup is tissue viability as the tissue may be used for up to several hours[59]. An MTT assay[60,61] is used to determine tissue viability by the end of the experimentation. This assay is based on the conversion of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by viable cells with adequate metabolism.

3.1.1. Post experiment maintain a separate group of tissue samples and separate them into two groups.

3.1.2. Incubate one group for 20 min at 37 °C in Triton X-100 (1%) dissolved in Buffer 2 as a control. Triton X-100 treatment results in cell death. Maintain the second group in Buffer 2, and do not incubate in Triton X-100 (tissue viability control).

3.1.3. Add MTT (stock solution 5 mg/mL in PBS, pH 7.4) to both groups in the oxygenated Buffer 2 to a final concentration of 0.5 mg/mL.

3.1.4. Incubate the tissue samples for 20 min at 37 °C, wash with PBS, and transfer into microcentrifuge tubes containing 250 µL of a mixture of SDS (10%, w/v), DMF (25%, v/v), and water to dissolve the formazan crystals.

3.1.5. Incubate the samples for 24 h.

3.1.6. Centrifuge the tubes at 10,000 x *g* for 10 min and measure the absorbance of the supernatant (200 µL) at 562 nm and 690 nm using a microplate reader. Tissue viability is calculated as follows: $(A_{562}–A_{690})$/tissue weight.

4. HPLC analysis of monoamines

4.1. Quantify monoamine release from each experimental condition using HPLC-ECD according to previous protocols[13,44], using a reverse phase column.

4.1.1. Prepare the mobile phase required for detection. This consists of 100 mM phosphoric acid, 100 mM citric acid, 0.1 mM EDTA-Na_2, 600 mg/L octanesulfonic acid, 8% v/v acetonitrile (final pH 6.0). The composition of the mobile phase is dependent on type of HPLC and column used.

4.1.2. Set the potential of the electrochemical detector (2 mm glassy carbon electrode,) to 0.46 V, and set the flow rate to 0.05 mL/min.

4.1.3. Load 5 µL of each sample, including neurotransmitter standards into the HPLC for autoinjection and detection. The amount of each sample added depends on the type of HPLC used.

4.1.4. Once the HPLC has completed the run, use the given HPLC analysis software to acquire and analyze the chromatograph data.

4.1.5. Analyze monoamine content using a standard curve composed of each monoamine (Dopamine: DA, Norepinephrine: NE, and Serotonin: 5-HT; **Figure 2C**). Use the resulting chromatograms to obtain the area under the curve (AUC) based on the manufacturer's guidelines.

5. Preparing tissue lysates for protein quantification

5.1. Protein assay

5.1.1. Add ice-cold lysis buffer plus protease inhibitors (0.1 g/1 mL) to each microcentrifuge tube containing brain sections/punches and homogenize using a pestle homogenizer. The microcentrifuge tubes must be maintained on ice while homogenizing to prevent protein degradation.

5.1.2. Incubate tissue homogenates for 1 h at 4 °C with light rotation.

5.1.3. Centrifuge tissue homogenates at 16,000 x *g* for 15 min at 4 °C and recover the supernatant.

5.1.4. Determine protein concentration in the supernatants, with bovine serum albumin (BSA) as a standard.

5.1.5. Normalize the monoamine content in each brain sample to the total content of protein (µg) measured in 250 µL of brain tissue lysed. Use the below formula to determine the nmol monoamine/g protein. df = dilution factor.

$$nmol\ monoamine\ per\ gram\ of\ protein = \frac{nM\ Monoamine * 5\mu L * 110\ (df)}{\mu g\ protein}$$

6. Statistical analysis

6.1. Analyze monoamine release (nmol/g) using one-way ANOVA followed by Sidak's multiple comparisons test for post-hoc comparisons.

6.1.2. Analyze tissue viability using an Unpaired Student's *t*-test for independent groups (Control vs. 1% Triton X-100).

6.1.3. For all statistical analyses, set the alpha level to ≤ 0.05.

REPRESENTATIVE RESULTS:

This technique describes the use of brain slices to measure the release of endogenous monoamines using HPLC with electrochemical detection based in a 48-well plate with an internal tissue holder. Experimental set up is depicted in **Figure 1** and **Figure 2**. Initially, to ensure tissue viability by the end of the experimentation, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay was performed. After functional experiments, the acute brain slices are metabolically active and stay viable as compared with those that are incubated with 1% Triton X-100, a condition of cell death (**Figure 3**).

Acute, 20 min, treatment of hippocampal and prefrontal cortex brain sections with AMPH induces a significant increase in the extracellular levels of each monoamine (**Figure 4A,B**). AMPH (30 µM) increased the level of extracellular 5-HT from hippocampal slices and prefrontal cortex slices by 220-fold and 64-fold, extracellular NE by 19-fold and 8-fold, and extracellular DA by 8-fold and 7-fold, respectively. Similar experiments were performed in the presence of fluoxetine (10 µM), a selective-serotonin reuptake inhibitor. Inhibition of SERT with fluoxetine prevents the increase of extracellular 5-HT induced by AMPH in both the hippocampus and the prefrontal cortex. In contrast, fluoxetine does not impact the effect of AMPH on extracellular DA or NE in the same brain areas, consistent with its selectivity for SERT (**Figure 4A,B**). All experimental conditions were performed in triplicate.

The release of monoamines from 2 mm dorsal striatum punches were next measured. Acute, 20 min, treatment of dorsal striatum punches with AMPH (10 µM) induces a 35-fold increase in the extracellular levels of DA (**Figure 4C**) over basal levels. DA detection was focused in the dorsal striatum due to the lower basal levels of 5-HT and NE previously reported in this region[62,63]. Thus, AMPH induces a minor dose-dependent increase in extracellular 5-HT as compared to extracellular DA levels induced by AMPH (data not shown). Incubation of dorsal striatum punches with cocaine (40 µM), a monoamine transporter blocker, resulted in a significant inhibition of AMPH-induced extracellular DA when compared to punches incubated solely with AMPH (**Figure 4C**). This data further supports previous findings indicating that AMPH induced DA efflux through DAT[16].

Finally, to demonstrate exocytotic release of monoamines, brain sections were incubated with KCl (40 mM). Increasing the concentration of extracellular KCl to evoke membrane depolarization via incubation with 40 mM of KCl is sufficient to induce the exocytotic release of monoamines when compared against control conditions (**Figure 5**). Neither fluoxetine nor cocaine blocks the increase in the extracellular levels of monoamines induced by KCl membrane depolarization.

FIGURE AND TABLE LEGENDS:

Figure 1: Acute rat brain slice and sectioning preparation. (**A**) A rat brain is placed in a brain matrix. A superior cut denotes the top of the optic chiasm; the inferior cut is 3 mm posterior of the base of the hypothalamus. Cuts were made to remove the hippocampus and striatum, and to ensure a horizontal base to glue the specimen to the compresstome or vibratome securely prior to slicing. (**B–D**) Super glue was spread around the base of the stage, brains were glued on, immediately covered with agarose, and the agarose was solidified using the frozen clamp in (**D**). (**E–F**) A compresstome was used to make 300 µm slices for the rat brain, and slices were placed in oxygenated buffer until use. (**G**) Sections were placed on a slide and 2 mm punches of the dorsal striatum were made. (**G**) Punches of the dorsal striatum (top), cut-outs of the hippocampus (middle), and cut-outs of the cortex (bottom) are maintained at 4 °C in oxygenated dissection buffer before initiating functional experiments.

Figure 2: Experimental set up for efflux experiment. (**A**) The efflux chamber consists of a 48-well tissue culture plate and a tissue holder tray connected to the carbogen line. (**B**) A diagram showing the experimental design for the endogenous monoamine efflux experiment in which the tissue activation (**B1**), pre-incubation with/without monoamine transporter inhibitors (**B2**), efflux experiment (**B3**), and the final sample processing are presented (**B4–B5**). (**C**) The left-hand panel depicts an experimenter loading the perfusate into the HPLC in preparation for auto-injection. The right-hand panel shows a representative chromatogram denoting the monoamine standard peaks. The area under the curve (AUC) is measured for each monoamine standard and brain samples. After calibration, the AUC measured for each brain sample is converted to nM concentration.

Figure 3: **Acute brain slices were viable by the end of the experimentation.** An MTT assay was performed to determine tissue viability and compared to Triton X-100 1%, which induces cell death. The result of the MTT assay showed that by the end of 6 h, tissue samples were still viable. Results are expressed as the mean ± SEM (N = 6). **** $P < 0.0001$, unpaired *t* test.

Figure 4: Amphetamine induces monoamine release in acute brain slices from the hippocampus, prefrontal cortex, and punches of the dorsal striatum. (**A–B**) Hippocampal and prefrontal cortex slices incubated in 30 µM AMPH results in a significant increase in 5-HT, DA, and NE release. Fluoxetine significantly inhibits 5-HT release, but has no impact on DA or NE release in these regions. (**C**) 2 mm dorsal striatum punches were incubated in cocaine (40 µM) or AMPH (30 µM). AMPH stimulated DA release and pretreatment with cocaine led to a significant reduction of AMPH induced DA release. All measurements are in nmol/g of protein. Statistics represent a one-way ANOVA followed by Sidak's multiple comparison test. Results are expressed as the mean ± SEM (N = 6). Statistics represent a one-way ANOVA with Sidak's multiple comparison test (** $p = 0.01$, *** $p = 0.001$, **** $p = 0.0001$).

Figure 5: High extracellular K^+ results in monoamine release through membrane depolarization. (**A–B**) KCl (40 mM) induces membrane depolarization and the release of all three monoamines in the HPC and PFC. In both brain areas, pretreatment with fluoxetine (10 µM) does not affect the effect of KCl on extracellular monoamine release. (**C**) KCl (40 mM) induces membrane

depolarization and the release of DA in the dorsal striatum, and pretreatment with cocaine (40 µM) does not affect the effect of KCl on DA release. Statistics represent a one-way ANOVA followed by Sidak's multiple comparison test. Results are expressed as the mean ± SEM (N = 6). Statistics represent a one-way ANOVA with Sidak's multiple comparison test (***p < 0.001, ****p < 0.0001).

Supplementary File: Recipes for buffers and solutions.

DISCUSSION:

Monoamine release measurements have been performed for years in a number of systems such as heterologous cells, neuronal cultures, brain synaptosomes, ex vivo acute brain slices, and whole animals[13,20,41,42,58,64–68]. Such preparations have allowed the field of neuroscience to explore basic neurotransmitter release mechanisms that may lead to the discovery of novel pharmacological agents for neurologic and psychiatric disorders where monoamines play a role. Despite the wide use of such methods, there are certain limitations regarding the source and/or the amount of endogenous monoamine release, particularly in radioactive procedures. In addition, ex vivo acute brain slice preparations have been widely used in conjunction with electrophysiological, pharmacological, genetic, molecular, immunocytochemical and other approaches[18,24,25,47,50,51,59,69,70], as they preserve the tissue architecture, and retain both neuronal activity and synaptic connections. Thus, brain slices offer exceptional advantages when compared with other in vitro models such as heterologous systems, primary cultured neurons, and synaptosomes. Largely, their advantage is that these systems can reproduce many aspects of the in vivo environment.

Electrophysiological, optogenetic, fluorescent sensors, and voltametric approaches offer exquisite temporal and spatial resolution to examine mechanisms associated with monoamine release, particularly DA. However, the basic premise for the use of these approaches is that the electrical or light-induced stimulation of neurons induces the classic, and well-documented calcium-dependent exocytotic vesicular release of neurotransmitters[18,21,22,24,27,30]. One of the more discernible limitations of these approaches is that monoamines released via alternative mechanisms (i.e., non-vesicular release) are not detected by these techniques. Radiolabeled neurotransmitter molecules have also been used to study monoamine release, but this approach has significant limitations. Cells or tissue samples are loaded with non-physiological concentrations of labeled neurotransmitters that do not faithfully recapitulate the native environment[20,42,46]. Interestingly, a few studies document the use of brain slices in superfusion systems to examine endogenous monoamine release[53,54,56]. However, these studies use radioactive neurotransmitters, and those that examine the endogenous neurotransmitter focus only on K^+ and non-physiological conditions to induce neurotransmitter release.

The currently presented method can be used to examine transporter-mediated monoamine release from native tissue. This allows the experimenter to overcome the limitations of tritiated neurotransmitters. In addition, this approach provides a simple setup to measure endogenous monoamine release more accurately through a direct detection of monoamines rather than the indirect measurement when using fluorescent sensors or radiolabeled monoamine. It is well

established that amphetamine acts as a monoamine transporter release agent in the prefrontal cortex[71], dorsal striatum[56,72], and hippocampal[39] brain regions. These findings were confirmed using this 48-well plate system. Additionally, this method may prove to be a supplement to currently used methods which measure total monoamine content using HPLC-ECD but have not examined monoamine release[5–7]. This method provides a novel aerator designed to measure the endogenous release of monoamines from acute brain slices using HPLC coupled with electrochemical detection.

While using this method, it is critical that the brain tissues are kept cold in oxygenated buffer during the experiment to prevent deterioration. Additionally, it is crucial that the tissues used are activated in a buffer containing pargyline to prevent monoamine degradation. Further, the experimenter may have to troubleshoot multiple aspects of this method. First, depending on the developmental timepoint or species of the animal, one may need to create smaller or larger sections or use more or less sections, slices, or punches per condition. Second, depending on the brain region of interest, there will be varying amounts of each neurotransmitter. Third, while it is critical to ensure consistent bubbling of oxygen when noted, the experimenter must be careful to not provide excess oxygenation as this may lead to the accidental removal of tissue from the well. Finally, as there are various types of HPLC devices and different separation columns, the experimenter will have to determine, based on the literature, which device or column would work best for their experiment.

Although this method provides the experimenter with the ability to quickly and precisely obtain ex-vivo data about the release of endogenous monoamines, there are limitations that must be kept in mind. As this is an ex vivo approach, networks and connections are severed, thus the slices or punches are not representative of an intact system. Another important limitation of this approach is the lack of temporal, and spatial resolution as monoamine release is measured in a time scale of minutes, and from a population of release sites. Future refinement of the approach might allow an optimization of time and space resolution. Additional experiments will also examine the mechanisms associated with release events. Having demonstrated the validity of the current method, future experiments will require to dissect the molecular events leading to monoamine release. Additional experiments will include Ca^{2+}-free efflux buffer, and selective inhibitors of vesicular release as additional controls. As the regional distribution of the monoamines and their transporters are three independent events, future experiments must incorporate more extensive pharmacology and a time-course study, as different drug conditions may require shorter or longer incubation times. For instance, based on regional distribution or type of tissue used, further experiments may use more specific pharmacological blockers for NET, SERT, or DAT such as desipramine, fluoxetine, and GBR12909, respectively. Further, although the tissue remained viable throughout the experiment, the experimenter is unable to rule out the possibility that monoamine transporter function might have been affected during the duration of the entire process. The equipment required for this method is low-cost, however, there is a need to have access to an expensive HPLC-ECD. This may be mitigated by core facilities, as many currently have access to HPLC-ECDs for communal use. Despite such limitations, the current method provides a fundamental procedure, which may be further manipulated to investigate monoamine release.

In general, this method provides a simple, high throughput, and low-cost two-step procedure to evaluate the simultaneous release of monoamines from adult rodent neurons using ex vivo acute brain slices from different brain regions. Ideally, this method may be combined with in vivo protocols, and it provides preliminary data thus allowing the experimenter to decrease the number of animals required in in vivo models, as recommended in the "three Rs" (Replacement, Reduction, and Refinement) of animal welfare. Thus, it is feasible to implement this ex vivo platform for the screening of potentially therapeutic molecules with the goal of discovering novel pharmacological agents for the treatment of conditions associated with deregulations in monoamine homeostasis.

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DISCLOSURES:

The authors have no disclosures.

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694

Figure 1

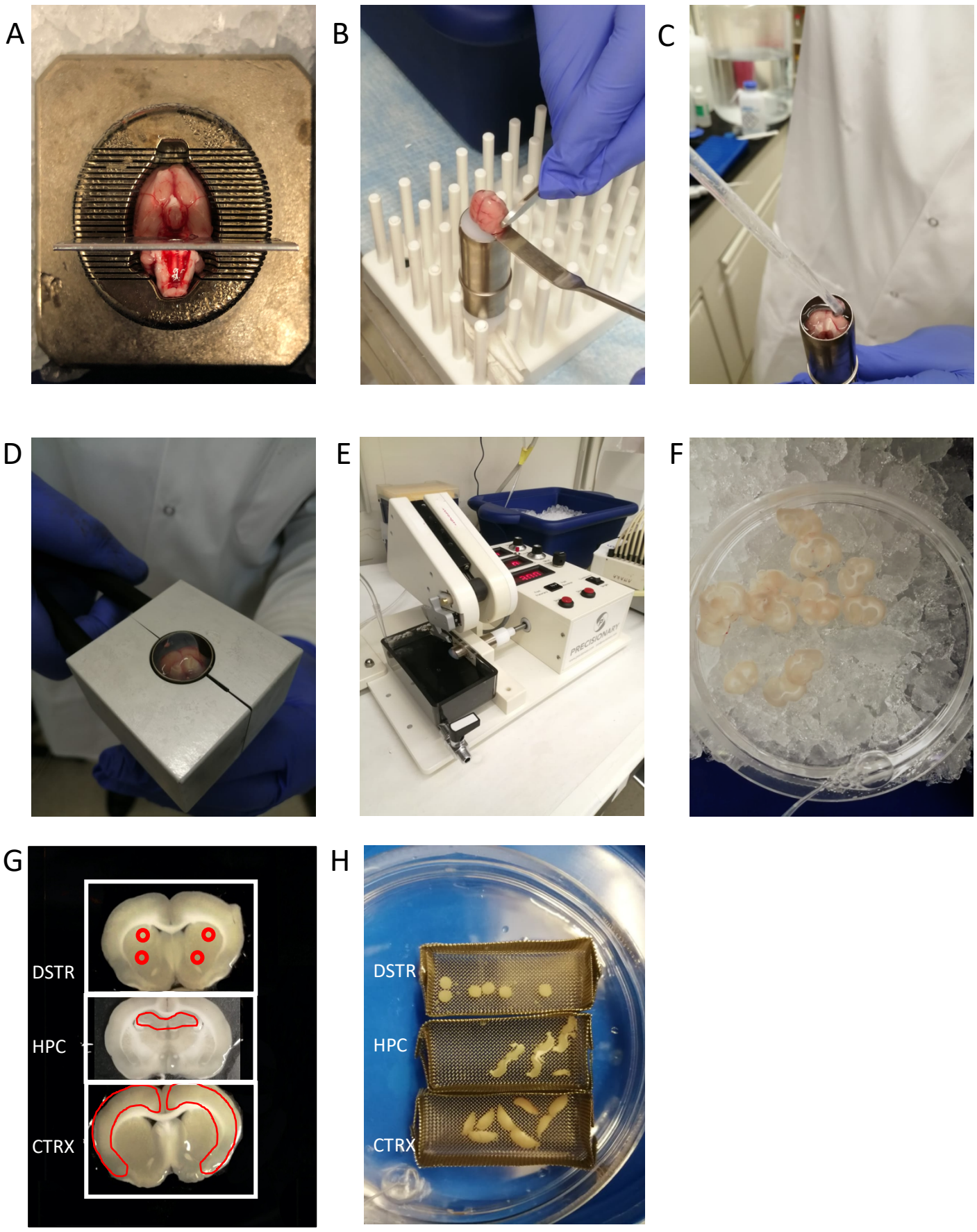
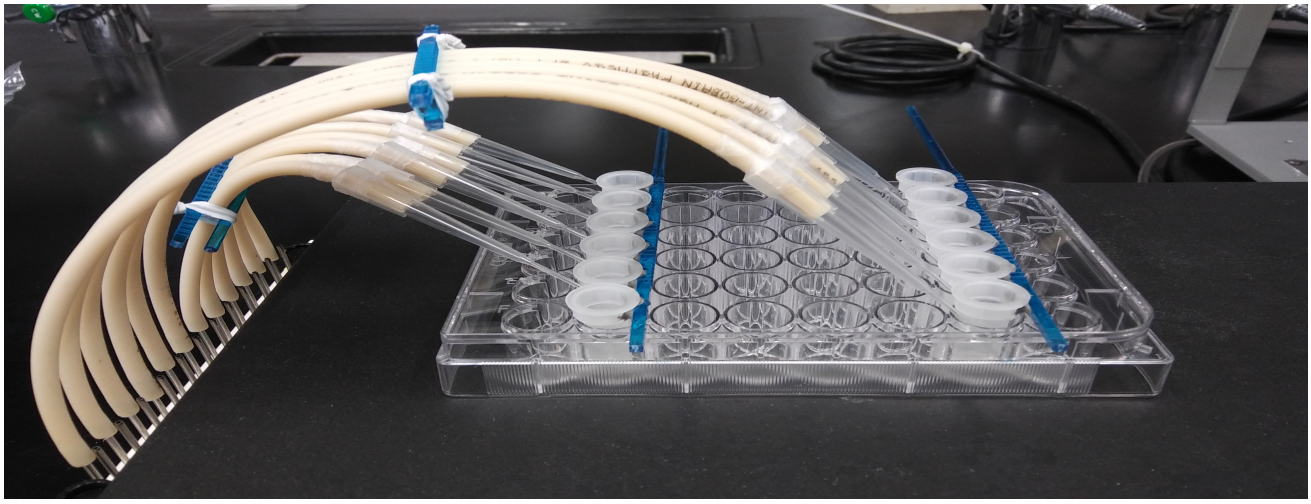
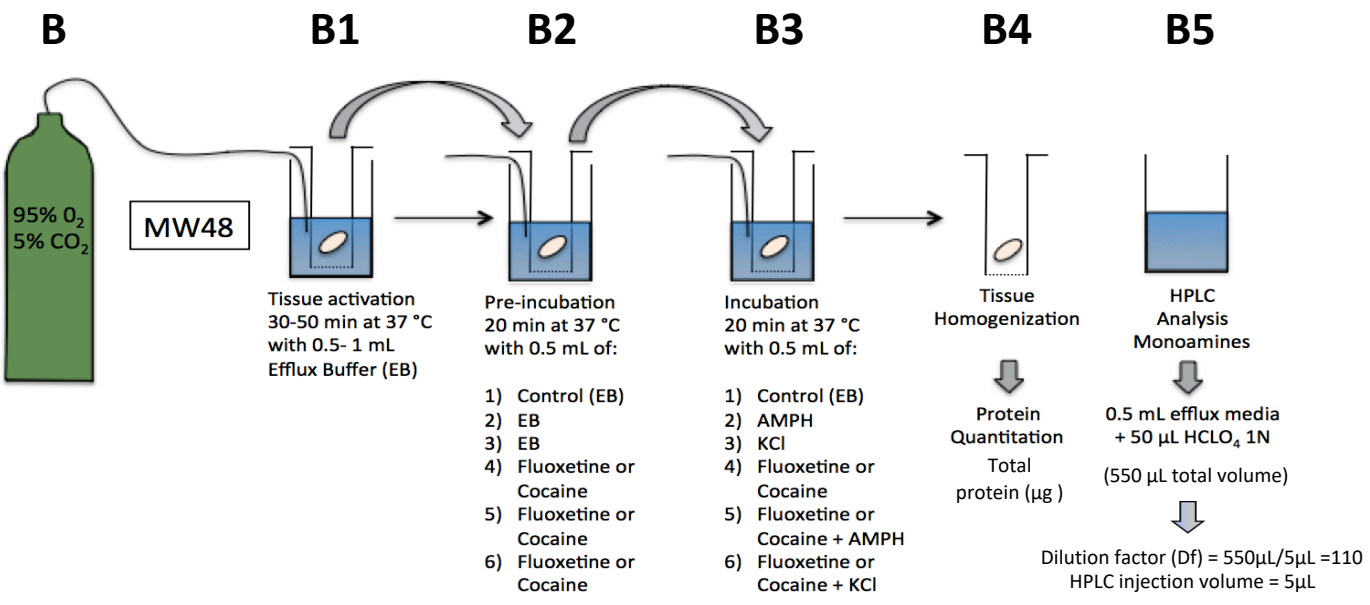


Figure 2

A



B



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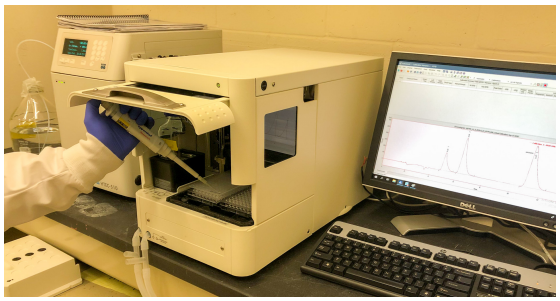


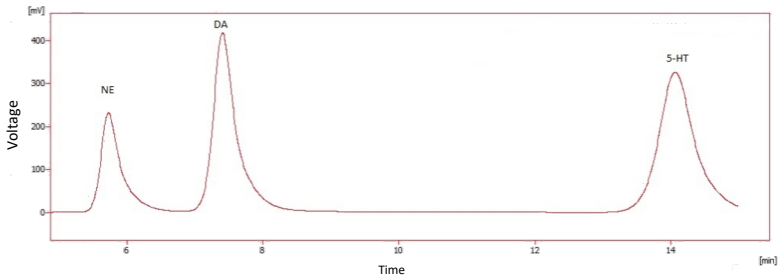
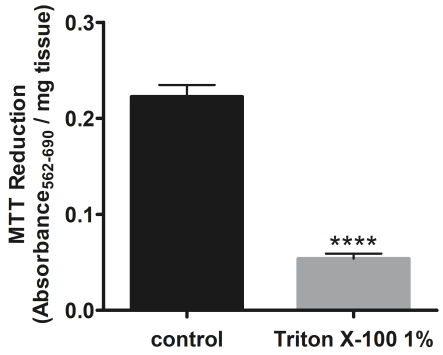
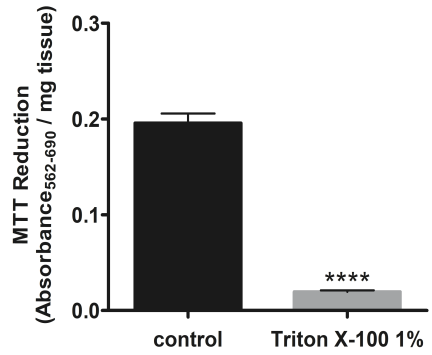


Figure 3

A. Hippocampus Slices



B. Prefrontal Cortex Slices



C. Dorsal Striatum Punches

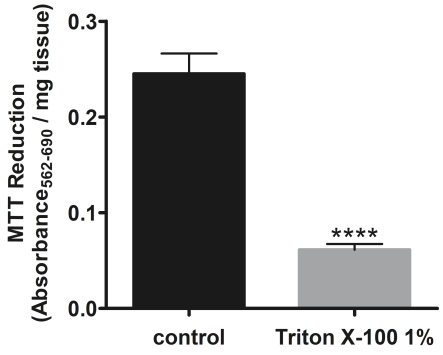
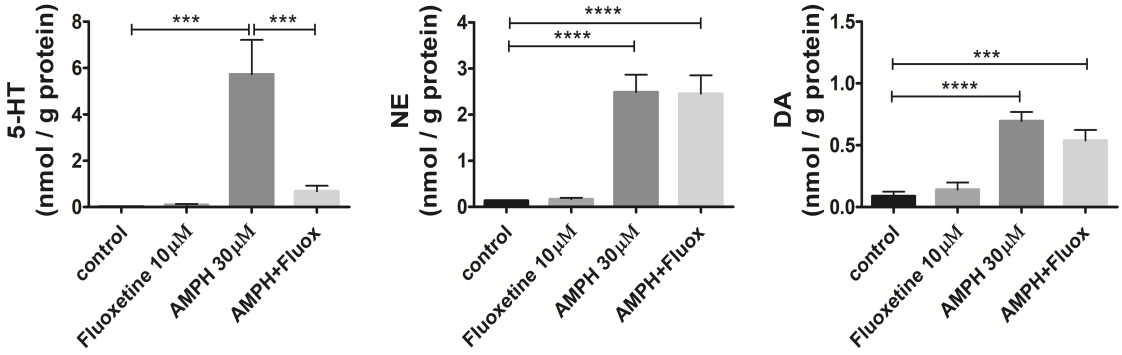
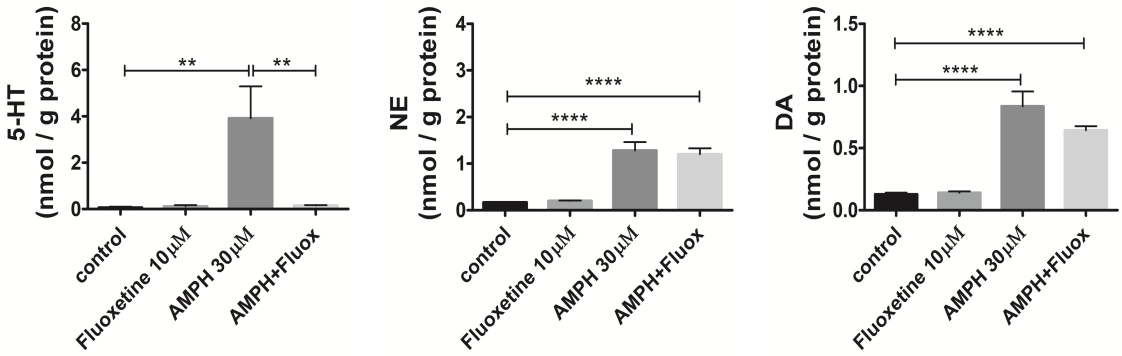


Figure 4

A. Hippocampal Slices



B. Prefrontal Cortex Slices



C. Dorsal Striatum Punches

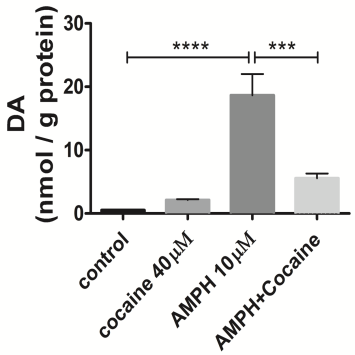
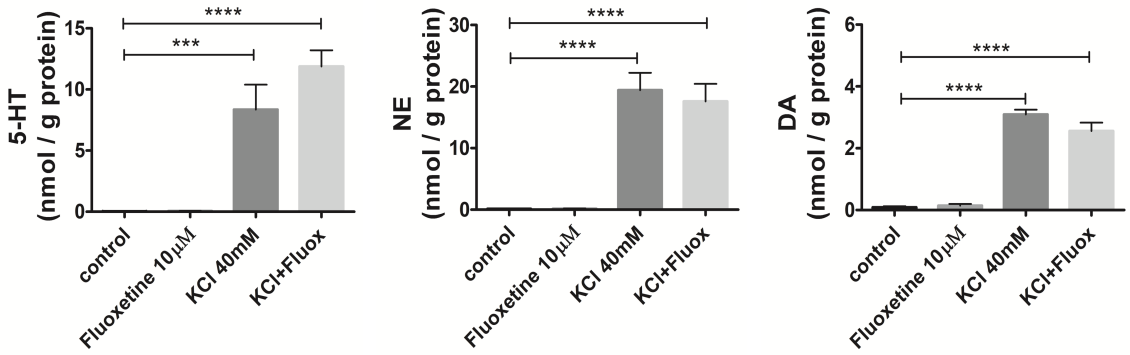
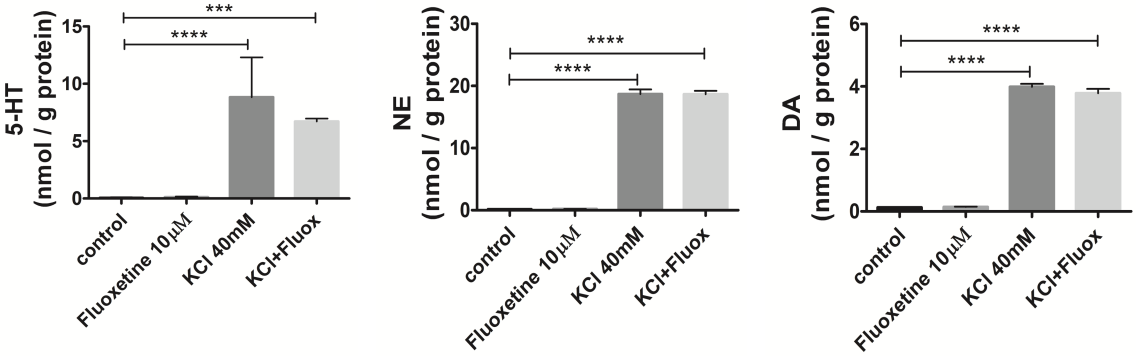


Figure 5

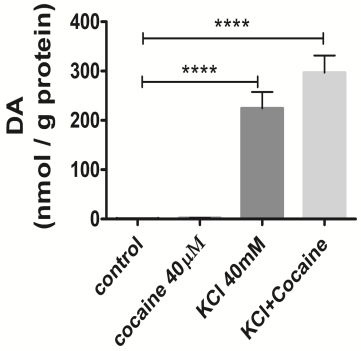
A. Hippocampal Slices



B. Prefrontal Cortex Slices



C. Dorsal Striatum Punches



Name of Material/Equipment	Company	Catalog Number	Comments/Description
48 Well plate	NA	NA	Assay
Acetonitrile	Fischer Scientific	A998-1	Mobile Phase
			Modified Artifical Cerebrospinal Fluid OR Efflux
Calcium Chloride Ahydrous	Sigma Aldrich	C1016	Buffer
Clarity Software	Anetc		
Citric Acid	Sigma Aldrich		Mobile Phase
D-(+)-Glucose	Sigma	1002608421	Dissection Buffer
DMF	Sigma Aldrich	D4551	MTT Assay
EDTA-Na2	Sigma Aldrich		Mobile Phase
GraphPad Software	Graphpad Software, Inc		Statistical Analysis
Glycerol	Sigma Aldrich	G5516	Lysis buffer
HEPES	Sigma Aldrich	H3375	Lysis buffer
HPLC, Decade Amperometric	Anetc		HPLC, LC-EC system
HPLC	Amuza		HPLC HTEC-510.
L-Asrobic Acid	Sigma Aldrich	A5960	Dissection Buffer
Magnesium Sulfate	Sigma	7487-88-9	KH Buffer
Microcentrifuge Filter Units UltraFree	Millipore	C7554	Assay - 6 to fit in 48 well plate
MTT	Thermo Fisher	M6494	MTT Assay
Nanosep	VWR	29300-606	Assay; protein assay
Octanesulfonic acid	Sigma Aldrich	V800010	Mobile Phase
			Modified Artifical Cerebrospinal Fluid OR Efflux
Pargyline Clorohydrate	Sigma Aldrich	P8013	Buffer
Phosphoric Acid	Sigma Aldrich		Mobile Phase
Potassium Chloride	Sigma	12636	KH Buffer
Potassium Phosphate Monobasic	Sigma	1001655559	KH Buffer
Precisonary VF-21-0Z	Precissonary		Compresstome

Protease Inhibitor Cocktail	Sigma Aldrich	P2714	Lysis buffer.
Sodium Bicarbonate	Sigma	S5761	Dissection Buffer
Sodium Bicarbonate	Sigma Aldrich	S5761	Dissection Buffer
Sodium Chloride	Sigma	S3014	KH Buffer
Sodium Dodecyl Sulfate	Sigma Aldrich	L3771	Lysis buffer
Triton X-100	Sigma Aldrich	T8787	MTT Assay / Lysis buffer

The City College of New York
CUNY School of Medicine
Department of Molecular, Cellular & Biomedical Sciences

We would like to thank the JOVE Editorial members as well as the manuscript reviewers for their insightful comments. We have revised the paper to include all suggestions. Below is a detail response to all the comments:

Editorial comments:
Changes to be made by the Author(s):

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.**
    a. The manuscript has been thoroughly proofread and all spelling and/or grammar issues have been corrected.
2. **Please use the headings mentioned in the instructions for authors and JoVE's style guide: summary instead of short abstract; abstract instead of long abstract.**
    a. The headings have been adjusted to JoVE's style guide.
3. **Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Please rewrite the entire protocol in the form of numbered steps (1, 1.1, 1.1.1, etc) with text that cannot be written in the imperative tense added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.**
    a. The protocol section has been adjusted to read in the imperative tense, and numbering has been resolved.
4. **JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Nanosep®; Decade amperometric HPLC; Antec LC-EC system, Antec, Leyden BV, the Netherlands; Acquity (Waters Co., Milford, MA); SenCell, Antec; Clarity software, Precisionary VF-31-0Z etc**
    a. All commercial language has been removed from the manuscript and referenced in the Table of Materials and Reagents.
5. **Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**
    a. More specific details have been added to the protocol steps to ensure viewers can easily replicate the protocol.
6. **After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.**
    a. One line space has been added after each protocol step, and relevant sections of the protocol have been highlighted.

7. **As we are a methods journal, please also add to the Discussion the following in detail with citations:**
    a. Critical steps, modifications, troubleshooting, and limitations have all been added to the Discussion section of the manuscript.
        i. Critical steps within the protocol
        ii. Any modifications and troubleshooting of the technique
        iii. Any limitations of the technique
8. **Please remember to add spaces between numbers and units/opening parentheses in figures.**
    a. Spaces have been added between number sand units/opening parentheses in the figures.
9. **Please sort the Materials Table alphabetically by the name of the material.**
    a. The Materials Table has been put into alphabetical order.

Reviewers' comments:
Reviewer #1:
The current manuscript by Pino et al reported that they optimized an approach for detecting monoamine release in acute brain slices through the use of HPLC-ECD. It is claimed that the method demonstrates basal monoamine release is detected through HPLC from acute brain slices, and their data support previous in vivo and in vitro results showing that AMPH and KCl induce monoamine release.
The study presents some interesting methodology and data and is considered relevant to the field. However, there are a few important issues that require to be carefully addressed before consideration for publication.

a. The introduction section as well as the discussion section requires further improvement. There are a few important relevant information related to the study topic that are neither sufficiently presented in the introduction, nor adequately discussed in the discussion section.

The Authors thank Reviewer #1 for their thoughtful and constructive comments regarding the manuscript, and for bringing this exciting literature to our attention. We have added this information and the references to the manuscript.

It is stated that "To overcome the limitations of traditionally used techniques, this technique represents an optimized a low-cost, simple, and precise method…". Since it is an "optimized" method, it is reasonable and necessary for the readers to know what the current method is optimized from? Compared to the old method, what improvements have been made and what are the specific, comparative advantages of new method in terms of cost, simplicity, precision etc. There should be a dedicated session/table to address the issue.

This technique is optimized from the use of radiolabeled monoamines, and superfusion assays. The superfusion system is costly, and only allows the experimenter to use ~6 wells at a time whilst this method allows the experimenter to theoretically use 48 wells at once in a much simpler device. This information was further explained in the text.

Is it the case that the HPLC-ECD analytical method is unmodified, but the prior sample collecting and processing was modified? This part could be confusing and requires some efforts to make it straight.

The HPLC-ECD analytical method is unmodified and has been addressed in the text.

c. **Provide some details of how to locate and dissect the specific brain regions, as such information are missing. Rationale for the concentrations selected for agents such as AMPH, fluoxetine should be provided.**

More information has been added regarding how to locate and dissect specific brain regions, and the citation for the brain atlas used was added. Concentrations of the selected agents are based off of previous literature and our own dose response curves.

d. MTT assay is used to test the tissue viability. Note that even if the tissue is viable/cell is undead, as demonstrated by MTT, it doesn't mean the functions of transport and release of neurotransmitter is unaltered. The magnitude of change in ex vivo relative to in vivo is not well known. The data should be interpreted with great caution and in context. Were the magnitudes of the neurotransmitter releases observed in current study in the ballpark compared to previous reported data?

We agree with Reviewer #1 and have addressed this in the discussion. The magnitude of neurotransmitter release observed in the current study is similar to those found in previous literature, and confirms said findings.

e. What are the limitations of the current method? It would be better if these should be added in the end of Discussion section.

This has been added to the discussion section.

f. Statistics: Information regarding statistical analysis software (type and version) should be added.

Commercial language is not permissible in the JoVE manuscript, but the statistical software Graphpad has been added to the materials table.

g. Were the assays conducted in duplicate, triplicate or..?
Assays were conducted in triplicate, and this information has been added to the text.

h. Figures: marking significance like *p <0.05 , p <0.01, *** p < 0.001, **** p < 0.0001 appears unnecessary and too detailed , which sometimes make the data presentation rather crowded. It is not a critical issue though.**
We agree that this is not a critical issue. P-values are only written out in the figure legend and not within the figure itself.

Reviewer #2:
Manuscript Summary:
This manuscript describes a low-cost fast throughput assay for detecting monoamine release from brain slices. The manuscript is easy to follow and the procedures are well-described. The results from the assay are straightforward and as expected from the different brain areas. The statistical design is also clear. There are also controls for slice and cell viability.

Major Concerns:
The main concern with the manuscript is the assertion that this protocol can clearly delineate Ca2+-dependent release of monoamines from transporter efflux. However,

additional controls need to be added to the protocol. The protocol should include a 0Ca2+ buffer and the addition of tetrodotoxin (TTX) to eliminate action potential activity within the slices during incubation. The high K+ condition for 20 min is a "hammer" on cells within slices and the additional controls should provide additional support for the interpretation.

The authors thank Reviewer #2 for their thoughtful and detailed response to the current manuscript. We agree that the protocol should have included a buffer sans Ca^{2+} as a control. However, the purpose of this paper is to provide researchers with a new technique to answer such pressing questions and fully dissect out between vesicular (KCl) versus transporter mediated (Amphetamine) release. We have made necessary adjustments in the text indicating that we cannot fully dissect out these differences, however, this allows for future experiments to determine a release mechanism with a given agent.

Minor Concerns: NA

Reviewer #3:
Manuscript Summary: This is an easy to follow and very useful protocol for estimating monoamine transporter mediated monoamine release in an ex vivo preparation

Minor Concerns: The examples of experiments are useful but could be enhanced by adding a couple of things. It would be good to see a time course of the release experiment. All experiments appear to be carried out at 20 minutes and a demonstration of a more complete time course would increase our understanding of its linear range and what the assay can be used for. For the various types of tissue a cleaner pharmacology using more specific drugs would also be useful. Fluoxetine is appropriate for teasing apart SERT mediated uptake but cocaine is not specific for any of the three transporters so including a NET (e.g. Desipramine) and DAT (e.g. GBR12909) specific blocker would be useful. Finally, it would also be useful to include more information on how to actually perform the HPLC analysis with more detail and maybe even a figure.

The authors thank Reviewer #3 for their thoughtful and detailed comments on the manuscript. Whilst we agree that it would be interesting to perform more detailed or cleaner pharmacology, the goal of this paper was to provide researchers with a new technique to dissect out specific mechanisms of action with a given agent. This information has been added to the text for a bigger-picture understanding of the technique.

Finally, it would also be useful to include more information on how to actually perform the HPLC analysis with more detail and maybe even a figure.
We have expanded the protocol section with information about the HPLC procedure and added a figure depicting both, the HPLC system and a chromatogram as a result of the HPLC detection (see new Figure 2).

Sincerely,

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Required solutions:

Dissection buffer – Ensure that this is prepared fresh on the day of experimentation

Ice-cold Krebs-Henseleit (KH) buffer supplemented with: 116 mM NaCl, 3 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.4 mM ascorbic acid, 11 mM D-glucose, pH 7.4 Prepare the dissection buffer as a 10X stock solution. On the day of experimentation, prepare 1X working solutions from the 10X stock, supplementing with sodium bicarbonate, ascorbic acid, and glucose. Maintain solution on ice and saturate with oxygen by bubbling with 95%/5% (O_2/CO_2) for 20 min before the experiment.

Modified artificial cerebrospinal fluid *or* Efflux buffer for efflux experiments – Ensure that this is prepared fresh on the day of experimentation

This is the *Dissection buffer* supplemented with pargyline, and $CaCl_2$: 116 mM NaCl, 3 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.4 mM ascorbic acid, 11 mM D-glucose, 1.8 mM $CaCl_2$, 0.01 mM pargyline, pH 7.4. Maintain solution at 37°C and saturate with oxygen by bubbling with 95%/5% (O_2/CO_2) for 20 min before the experiment.

High $^+$K Efflux Buffer - Ensure that this is prepared fresh on the day of experimentation

80 mM NaCl, 39 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.4 mM ascorbic acid, 11 mM D-glucose, 1.8 mM $CaCl_2$, 0.01 mM pargyline, pH 7.4. Maintain solution at 37°C and saturate with oxygen by bubbling with 95%/5% (O_2/CO_2) for 20 min before the experiment.

Lysis buffer

20 mM HEPES, 125 mM NaCl, 10% Glycerol, pH 7.6, containing 1% Triton X-100 and protease inhibitors (AEBSF 500 µM, Aprotinin 150 nM, E-64 1 µM, EDTA 1 mM, EGTA 1 mM, Leupeptin 1 µM).