Journal of Visualized Experiments

Quantifying Spontaneous Ca2+ Fluxes and their Downstream Effects in Primary Mouse Midbrain Neurons

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61481R1
Full Title:	Quantifying Spontaneous Ca2+ Fluxes and their Downstream Effects in Primary Mouse Midbrain Neurons
Keywords:	neuroscience; primary cell culture; in vitro; excitotoxicity; dopaminergic neurons; Parkinson's disease; high content screen; caspase; apoptosis; glutamate; NMDA; AMPA
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bryan TX, USA

TITLE:

Quantifying Spontaneous Ca^{2+} Fluxes and their Downstream Effects in Primary Mouse Midbrain Neurons

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KEYWORDS:

Neuroscience, primary cell culture, in vitro, excitotoxicity, midbrain, dopaminergic neurons, Parkinson's Disease, high content screen, caspase, apoptosis, glutamate, NMDA, AMPA

SUMMARY:

Here we present a protocol to measure in vitro Ca^{2+} fluxes in midbrain neurons and their downstream effects on caspase-3 using primary mouse midbrain cultures. This model can be employed to study pathophysiologic changes related to abnormal Ca^{2+} activity in midbrain neurons, and to screen novel therapeutics for anti-apoptotic properties.

ABSTRACT:

Parkinson's disease (PD) is a devastating neurodegenerative disorder caused by the degeneration of dopaminergic (DA) neurons. Excessive Ca^{2+} influx due to the abnormal activation of glutamate receptors results in DA excitotoxicity and has been identified as an important mechanism for DA neuron loss. In this study, we isolate, dissociate, and culture midbrain neurons from the mouse ventral mesencephalon of ED14 mouse embryos. We then infect the long-term primary mouse midbrain cultures with an adeno-associated virus (AAV) expressing a genetically encoded calcium indicator, GCaMP6f under control of the human neuron-specific synapsin promoter, hSyn. Using live confocal imaging, we show that cultured mouse midbrain neurons display spontaneous Ca^{2+} fluxes detected by AAV-hSyn-GCaMP6f. Bath application of glutamate to midbrain cultures causes abnormal elevations in intracellular Ca^{2+} within neurons and this is accompanied by caspase-3 activation in DA neurons, as demonstrated by immunostaining. The techniques to identify glutamate-mediated apoptosis in primary mouse DA neurons have important applications for the high content screening of drugs that preserve DA neuron health.

INTRODUCTION:

Parkinson's Disease (PD) is the second most common neurodegenerative disorder worldwide, with no known cure. Estimates suggest that PD prevalence will continue to increase and is projected to surpass 1 million diagnoses by the year 2030 in the United States alone[1]. With few effective treatments currently available to combat PD, there is a pressing need to develop more effective therapies. PD is characterized by a rapid and progressive loss of midbrain dopamine (DA) neurons[2]. The mechanisms that underlie neurodegeneration in PD are poorly understood. Evidence suggests a likely convergence of multiple mechanisms, such as oxidative stress, mitochondrial dysfunction, etc. that contribute to the initiation of apoptotic signaling cascades and eventual cell death[3].

One such convergent mechanism, glutamate-mediated excitotoxicity has been implicated in multiple neurodegenerative diseases, including PD[4]. While glutamate-mediated excitotoxicity is thought to work mainly through stimulation of NMDA receptors via an excessive increase in intracellular Ca^{2+} concentration and eventual initiation of apoptosis, Ca^{2+}-permeable AMPA receptors have also been implicated in the excitotoxic response[5-7]. Therefore, it is of interest to determine the contribution of AMPA receptors to glutamate-mediated apoptosis within a PD model. This can be achieved using NBQX, an AMPA and kainate blocker, which at micromolar concentrations is selective for AMAPR receptors[8]. Glutamate-mediated excitotoxicity and apoptotic signaling cascades are an ideal downstream target to measure the extent of cell death, and a potential target for therapeutic intervention. Therefore, developing a high-content method for assessing glutamate-mediated modulation of calcium activity and associated downstream signaling in primary ventral mesencephalic (VM) neurons would be valuable for screening novel treatment methods on their ability to preserve neuronal health.

Here, we have developed a protocol in which we express the genetically encoded calcium indicator (GECI), GCaMP6f, using AAV2/5 with the human synapsin (hSyn) promotor to measure the Ca^{2+} activity of mouse VM primary neurons in response to glutamate application can be measured at the physiological and molecular level. This high-content screening can be adapted for discovering pharmaceuticals or treatments that modulate Ca^{2+} activity to preserve the health of VM neurons. We propose that this primary culture model is an effective way to screen for novel PD interventions, based on their ability to preserve the health of VM neurons and mitigate the progression of PD.

PROTOCOL:

All procedures involving the use of animal subjects have been approved by the Texas A&M University Institutional Animal Care and Use Committee (25th Nov 2019; AUP# 2019-0346).

NOTE: Preparation of cell culture solutions should be done using sterile procedure in a biological safety cabinet and filtered at 0.2 µm to prevent contamination.

1. **Preparation of solutions and culture medium**

1.1. Prepare laminin coating solution by diluting 20 µL of 1 mg/mL laminin stock into 2 mL of sterile distilled H_2O. Prepare on the day of dissection.

1.2. Prepare 10% equine (horse) serum stop solution by adding 5 mL of ES to 45 mL of 1x Hank's Balanced Salt Solution (HBSS). Sterile filter using a 0.2 µm filter system or syringe filter tip. Store at 4 °C.

1.3. Prepare 4% bovine serum albumin (BSA) stock solution by adding 2 g of BSA powder to 1x phosphate-buffered saline (PBS) and bringing to a final volume of 45 mL. Sterile filter using a 0.2 µm filter system or syringe filter tip. Store at 4 °C.

1.4. Prepare papain stock solution by diluting papain to 3 mg/mL in 1x HBSS. Sterile filter using a 0.2 µm filter system or syringe filter tip. Store at -20 °C.

1.5. Prepare deoxyribonuclease (DNase) solution by adding 20 mg of DNase powder to sterile H_2O and bringing to a final volume of 20 mL. Sterile filter using a 0.2 µm filter system or syringe filter tip. Store at -20 °C.

1.6. Prepare ascorbic acid stock solution by adding 352 mg of ascorbic acid to sterile distilled H_2O and bringing to a final volume of 20 mL. Heat in 37 °C bath to dissolve if necessary. Sterile filter using a 0.2 µm filter system or syringe filter tip. Store at -20 °C.

1.7. Prepare Cell Culture Medium by adding the following to 50 mL of Neurobasal medium: 500 µL of Glutamax (100x), 500 µL equine serum, 1 mL of B-27, 100 µL of ascorbic acid, 500 µL of penicillin-streptomycin, 50 µL of kanamycin and 50 µL of ampicillin. Sterile filter using a 0.2 µm filter system. Store at 4 °C.

1.8. Prepare 0.01% Triton X-100 Solution by adding 1 mL of Triton X-100 into 9 mL of 1x PBS to make a 10% solution. As Triton X-100 is viscous, pipette slowly to allow tip to fill completely. Heat in 37 °C bath to dissolve if necessary. Store at 4 °C.

1.9. To dilute 10% stock to 0.01%, perform 3 serial 1:10 dilutions. Dilute 1 mL of 10% stock into 9 mL of 1x PBS to make 1% solution. Dilute 1 mL of 1% solution into 9 mL of 1x PBS to make 0.1% solution. Dilute 1 mL of 0.1% solution into 9 mL of 1x PBS to make a 0.01% solution.

1.10. Prepare 10% and 1% normal goat serum (NGS) solution by adding 1 mL of NGS to 9 mL of 1x PBS for a 10% solution. Add 100 µL of NGS to 9.9 mL of 1x PBS to make 1% solution.

1.11. Prepare glutamate stock solution (100 mM) by adding 735 mg of L(+)-Glutamic acid to sterile distilled H_2O and bring to a final volume of 50 mL. Solubility at this concentration will be an issue. Adding small volumes (100 µL) of 1 M hydrochloric acid is sufficient to increase solubility.

1.12. Prepare NBQX stock solution (10 mM) by adding 50 mg of NBQX to sterile distilled H_2O and bring to a final volume of 13 mL.

2. **Preparation of culture dishes and coverslips** (**Done the day before dissection**)

NOTE: We have found that combining three coating agents, poly-L-lysine, poly-L-ornithine, and laminin allows for ideal cell adhesion and viability.

2.1. Place 10 35 mm Petri dishes in a biological safety cabinet. Place two circular 12 mm coverslips in each dish and fill with 70% EtOH for 10 min. Use a vacuum line to aspirate the remaining EtOH from each dish, allowing the EtOH to evaporate completely.

2.2. Pipette ~90-100 µL of 0.1% poly-L-lysine solution onto each coverslip, making sure that the entire coverslip is covered by the poly-L-lysine solution. Cover the dishes with lids and place in a 37 °C incubator for 1 h.

2.3. Aspirate remaining poly-L-lysine solution from each coverslip and rinse with sterile H_2O.

2.4. Repeat steps 2.2 – 2.3 with 0.1% poly-L-ornithine solution.

2.5. Again, repeat steps 2.2 – 2.3 with 0.01% laminin solution. Place in a 37 °C/5% CO_2 incubator until ready for cell plating.

3. **Mouse embryonic dissections**

NOTE: We use between 4 to 6 timed pregnant mice per culture. While much of the dissection process occurs outside of a biological safety cabinet it is still important to maintain sterile procedure. Plentiful use of 70% EtOH on surfaces near the dissection microscope and on surgical tools is ideal. A mask may also be worn during the dissection to further prevent contamination. Additionally, we use 4 separate antibiotics in the culture medium, so contamination is unlikely. However, if use of antibiotics is problematic, this dissection setup could be moved inside a sterile hood. To preserve cell viability all dissection solutions should be pre-chilled at 4 ˚C, and dissections should be completed as quickly as possible. We do not perform the dissections on ice. The method for dissection of mouse embryonic midbrain neurons is identical to previously described methods[9,10].

3.1. Prepare a space on a bench near a dissection microscope with an absorbent pad and spray liberally with 70% EtOH.

3.2. Spray two 100 x 15 mm glass Petri dishes and one 50 x 10 mm glass Petri dish with 70% EtOH and allow EtOH to evaporate. Once evaporated, place 50 mL of sterile 1x HBSS into each 100 x 15 mm Petri dish.

3.3. Submerge surgical scissors, forceps, and microtome blade in 70% EtOH for 10 min minimum to sterilize. Place instruments on the absorbent pad to dry.

3.4. Using CO_2 followed by cervical dislocation, euthanize 2-3 month old timed pregnancy mice on embryonic day 14.

3.5. Spray the abdomen of the euthanized mice with 70% EtOH. Using forceps grab the lower abdomen and open the abdominal cavity using surgical scissors. Start cutting near where the forceps are holding the abdomen, making lateral cuts on each side until the abdominal wall can be folded back and the uterus is clearly visible.

3.6. Using surgical scissors, cut both ends of the uterine horn. Then remove the uterus and place into Petri dish with 1x HBSS.

3.7. Using straight-tip forceps carefully remove embryos from the uterus. Leave embryos in HBSS throughout this process. Using either the forceps or a microtome blade, quickly decapitate embryos by cutting near the neck. Making as level a cut as possible.

3.8. Under a dissecting microscope, move an embryo head to a dry 50 mm Petri dish and place on the ventral side. Stabilize the head with forceps by placing and penetrating near the eyes/snout. Forceps should be angled downward at ~45° to avoid penetrating the mesencephalon.

3.9. Using the forceps in the other hand, carefully remove the translucent layer of skin and skull just before the prominent ridge of the mesencephalon. Start near the midline and remove skin and skull caudally until the mesencephalon is fully exposed.

3.10. Hold the forceps perpendicular to the exposed mesencephalon with one tip between the cortex and mesencephalon and the other near the cerebellum. Press down and pinch the forceps together to remove the entire midbrain. The midbrain segment should be approximately 0.5 mm thick. Place the midbrain segment into the second Petri dish filled with fresh 1x HBSS. Repeat this process for each embryo.

3.11. Using the dissection microscope, position the brain segment with the ventral side facing up. If the meninges are still attached, carefully remove it by grabbing with the forceps and lifting up and away from the brain segment.

3.12. The brain segment should have 4 visible quadrants. Place the segment in such a manner that the two smaller quadrants are positioned superior to the two larger quadrants. There is a prominent ridge separating the superior two (small) quadrants from the inferior two (large) quadrants.

3.13. Using the forceps pinch and separate the superior quadrants from the inferior quadrants, and then discard the superior quadrants. The remaining inferior quadrants will have excess tissue laterally on the dorsal side, this tissue will look less opaque than the remaining ventral tissue. Remove the less dense dorsal tissue and discard. The remaining segment should contain both the Substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA).

3.14. Using the forceps cut the remaining ventral tissue segment into 4 smaller pieces and using a 1mL wide bore pipette transfer these segments in a 15 mL conical tube with 1x HBSS. Keep the conical tube with brain segments on ice throughout the procedure.

3.15. Repeat this process for all remaining brain segments.

4. **Dissociation of cells**

4.1. Enzymatic digestion of cells

4.1.1. Carefully aspirate the HBSS from the 15 mL conical tube containing midbrain segments, leaving the segments at the bottom of the tube.

4.1.2. Add ~800 µL of papain solution to the tube and place in a 37 °C incubator for 7 min. Resuspend cells by flicking the tube and replace to the 37 °C incubator for an additional 7 min.

4.1.3. With a wide-bore 1 mL pipette tip remove only the midbrain segments into a 1 mL aliquot of DNase. Allow the segments to reach the bottom of the aliquot or about 1 min of exposure.

4.1.4. With a wide-bore 1 mL pipette tip remove only the midbrain segments into a 15 mL conical tube containing 2 mL of stop solution. Allow segments to settle at the bottom of the tube and repeat the rinse in an additional conical tube filled with stop solution.

4.2. Mechanical trituration of cell suspension

4.2.1. In the second stop solution rinse tube, using a wide-bore 1 mL pipette tip, pipette the cells up and down 10 times until there are no large tissue segments visible. It is important to avoid over trituration for minimal cell lysis.

4.2.2. Slowly pipette 300 µL of 4% BSA solution to the bottom of the 15 mL conical tube containing brain segments. Carefully remove the pipette tip to maintain a suspension layer. Centrifuge at 0.4 x *g* for 3 min. Then carefully aspirate the supernatant and resuspend cells in 400 µL of cell culture medium.

5. **Plating the cells**

NOTE: Based on experience, about 100,000 viable cells per embryo are collected. 2-3 month old timed pregnant mice typically have litter sizes of 8-10 embryos; therefore, a rough estimate for total yield of cells per timed pregnant mouse is approximately 1 million cells.

5.1. Using a hemocytometer preform a cell count and then dilute the suspension to 2,000 cells/µL using cell culture medium. Triturate briefly to mix.

5.2. Remove coverslips with laminin solution from step 2 from the incubator and aspirate the remaining laminin solution from the coated coverslips using a vacuum. Plate quickly to avoid the coverslips from drying completely. Pipette 100 µL (2.0 x 10^5 cells/coverslip) onto each coverslip and place Petri dishes into a 37 °C incubator for 1 h.

5.3. Carefully add 3 mL of cell culture medium to each dish and place back into the 37 °C incubator. Preform half medium changes 2 times per week for 2 weeks.

6. Infection of cell culture at 14 DIV with adeno-associated viral (AAV) vectors

6.1. For each dish prepare 1 mL of serum free DMEM medium with 1 µL of hSyn-GCaMP6f AAV (1.0 x 10^{13} titer)

6.2. Aspirate the cell culture medium from each dish and replace with 1 mL of serum free DMEM containing hSyn-GCaMP6f. Place dishes back into the 37 °C incubator for 1 h.

6.3. Aspirate the serum free medium containing AAVs and replace with 3 mL of cell culture medium. Place dishes back into the 37 °C incubator. We have found that 5-7 days of AAV infection allows for ideal levels of GCaMP expression. Continue to change medium every 2-3 days throughout this period of viral infection.

7. Live confocal Ca^{2+} imaging between 19-21 DIV

NOTE: As mentioned in step 6.3, imaging can be done between 5-7 days following viral infection. This is the ideal window to achieve visible expression of the fluorophore at levels which allow for detection of spontaneous Ca^{2+} activity.

7.1. Preparation of recording buffers

7.1.1. To make 1 L of HEPES recording buffer, add: 9.009 g of NaCl, 0.3728 g of KCl, 0.901 g of D-glucose, 2.381 g of HEPES, 2 mL of 1 M $CaCl_2$ stock solution, and 500 µL of 1 M $MgCl_2$ stock solution to 800 mL of sterile distilled H_2O. Bring the pH to 7.4 with NaOH. Bring to a final volume of 1 L.

7.1.2. To make 200 mL of 20 µM glutamate recording buffer, dilute 40 µL of 100 mM glutamate stock solution into 200 mL of HEPES recording buffer described above.

7.1.3. To make 200 mL of 10 µM NBQX recording buffer, dilute 200 µL of 10 mM NBQX stock solution into 200 mL of HEPES recording buffer.

7.2. Confocal imaging

7.2.1. Fill a sterile 35 mm Petri dish with 3 mL of recording buffer.

7.2.2. Remove a 35 mm Petri dish with infected cultures from the 37 °C incubator. Using fine tip forceps, carefully grab the edge of one coverslip and transfer it quickly into the Petri dish filled with recording buffer. Place the remaining coverslip in medium back into the 37 °C incubator. Transport the dish with recording buffer to the confocal microscope.

7.2.3. Start the imaging software. Proceed to next step while it initializes.

7.2.4. Start the peristaltic pump and place the line into the recording buffer. Calibrate the speed of flow to be 2 mL/min.

7.2.5. Transfer the infected coverslip from the 35 mm Petri dish into the recording bath.

7.2.6. Using the 10x water immersion objective and BF light, find the plane of focus and look for a region with a high density of neuron cell bodies. Switch to the 40x water immersion objective and using BF light refocus the sample.

7.2.7. In the “Dyes list” window within FluoView select AlexaFluor 488 and apply it.

7.2.8. AAV expression can be variable; therefore, in order to prevent overexposure and photobleaching of the fluorophores, start with low HV and laser power settings. For the AlexaFluor 488 channel, set the high voltage (HV) to 500, the gain to 1x, and offset to 0. For the 488 laser line set the power to 5%. In order to increase the effective volume imaged in the z-plane, increase the pinhole size to 300 µm. Use the “focus x2” scanning option to optimally adjust emission signals to sub-saturation levels. From here, settings can be adjusted until ideal visibility of each channel is achieved.

NOTE: To accurately capture the full range of Ca^{2+} fluxes with GCaMP, adjust the baseline HV and laser power settings in order to allow for an increase in fluorescent intensity without oversaturating the detector.

7.2.9. Once microscope settings are optimized, move the stage in order to locate a region with multiple cells displaying spontaneous changes in GCaMP6f fluorescence and focus to the desired plane for imaging.

7.2.10. Use the “Clip rect” tool to clip the imaging frame to a size that can achieve a frame interval of just under 1 second. This is necessary to set the imaging interval at 1 frame per second.

7.2.11. Set the “Interval” window to a value of 1.0 and the “Num” window to 600.

NOTE: In order to deliver different recording buffers at the desired time point (300 s), it is important to calibrate the latency of the pump to deliver the new solution to the bath. This will be dependent on the solution perfusion rate (2 mL/min) and the length of the line used to pump solution.

7.2.12. To capture a t-series movie select the “Time” option and then use the “XYt” scanning option to begin imaging.

7.2.13. Watch the imaging progress bar and move the line from the HEPES recording buffer into the 20 µM glutamate recording buffer at the appropriate time point (e.g., if the latency of the pump is calibrated to deliver solution at 60 s, move the line into the glutamate buffer at 240 frames in order to deliver glutamate at 300 s).

7.2.14. When imaging is complete, select the **Series Done** button and save the finished t-series movie. Continue to perfuse 20 µM Glutamate for an additional 5 min, so that the cultured neurons have been exposed to glutamate for a total of 10 min. Repeat this process for each coverslip to be imaged.

7.2.15. Following the additional 5 min exposure to 20 µM Glutamate, remove the coverslip from the bath and place back into the 35mm Petri dish containing recording buffer until the day of imaging is completed. When finished, proceed to step 8.

7.3. Ca^{2+} trace analysis

7.3.1. Perform image analysis in ImageJ. Install the BIO-FORMATS plugin for ImageJ, which will allow .OIB image files to open.

7.3.2. In the ImageJ toolbar, click **Analyze | Set Measurements**, and select the box for Mean gray value (MGV).

7.3.3. In ImageJ, open a t-series movie as a hyperstack.

7.3.4. Drag the slider for the movie and identify the frame with maximal glutamate response to visualize all neurons that respond to glutamate. Use the polygon tool to trace all visible neuron cell bodies, adding their ROIs to the “ROI manager” list.

7.3.5. When finished tracing and adding ROIs, select all ROIs within the **ROI Manager** window and use the **Multi measure** selection in the **more** list of options. Copy and paste these data into a spreadsheet. Complete this process for all movies to be analyzed.

7.3.6. For each ROI, convert the raw MGV data from each frame to $\Delta F/F_0$ values using the equation: $\Delta F/F_0 = [F(t) - F_0] / F_0$. Where $F(t)$ = MGV of any given frame, and F_0 = average baseline MGV of ~10 frames where no Ca^{2+} fluxes are present.

7.3.7. Using a statistical software such as OriginPro 2020, converted $\Delta F/F_0$ traces can be made into line graphs. The “Peak analyzer” function can be used (or similar function if using a different software) to measure the peak amplitude of glutamate response, latency to respond to glutamate, and area under the curve.

8. **Immunostaining of cultures**

NOTE: Following fixation with formalin, coverslips can be stored in 1x PBS at 4 °C until ready to be processed for immunostaining. Primary and secondary antibody incubation was done in a serial manner, as such incubation with anti-Caspase-3 primary antibody and its complementary secondary antibody preceded incubation with the anti-TH primary antibody and its complementary secondary antibody.

8.1. Immediately following glutamate exposure, place the coverslip back into its 35 mm Petri dish, aspirate the recording buffer, and add 3 mL of 10% formalin. Let sit for 40 min at room temperature (RT).

8.2. Rinse the dish 3 times with 1x PBS.

8.3. Aspirate the PBS and permeabilize cells in 1 mL of 0.01% Triton X-100 in PBS for 2 min.

8.4. Rinse the dish 3 times with 1x PBS.

8.5. Aspirate the PBS and block cells in 1 mL of 10% NGS in PBS.

8.6. Rinse the dish 3 times with 1x PBS.

8.7. Add 1 µL of rabbit anti-Caspase-3 primary antibody to 1 mL of 1% NGS in PBS (1:1000 dilution). Aspirate PBS from the dish and replace with the primary antibody solution. Place on a shaker and incubate for 1.5 h at RT.

8.8. Rinse the dish 3 times with 1x PBS.

8.9. Add 1 µL of goat anti-rabbit AlexaFluor 488 secondary antibody to 1 mL of 1% NGS in PBS (1:1000 dilution). Aspirate PBS from the dish and replace with the secondary antibody solution. Place on a shaker and incubate for 1 h at RT. Moving forward protect the samples from light.

8.10. Rinse the dish 3 times with 1x PBS.

8.11. Repeat steps 8.7 – 8.10, but using the chicken anti-TH primary antibody in step 8.7 and the goat anti-chicken AlexaFluor 594 secondary antibody in step 8.9.

8.12. Following the final PBS rinse, place 30 µL of mounting medium onto a microscope slide. Using forceps grab a coverslip from the 35 mm Petri dish and place the coverslip with the cells facing down into the mounting medium. Both coverslips will fit on a single microscope slide if placed correctly. Place in a dry, dark area and allow the mounting medium to dry overnight.

9. **Confocal imaging of immunostained cultures**

9.1. Confocal imaging

9.1.1. Start the imaging software. Place the sample onto the microscope stage.

9.1.2. With the microscope eyepieces, using a 20x magnification objective and epifluorescent light with a TRITC filter, focus the sample and search for a TH+ cell body.

9.1.3. Once locating a TH+ cell body, center it in the field of view and then move to the 60x magnification objective.

9.1.4. Select the AlexaFluor 488 and AlexaFluor 594 dyes in the “dye list” window.

9.1.5. As with live imaging, start with low HV, gain, offset, and laser power settings to prevent photobleaching. Use the “focus x2” scan option to assess the fluorescent intensity of each channel and adjust accordingly. As these images will later be quantified for fluorescent intensity it is necessary to keep the imaging settings consistent across all fields of view. Therefore, it is best to look at a few examples of each condition to get an idea of the range of fluorescent intensity across samples.

9.1.6. Once ideal imaging settings are determined, center the cell in the field of view and apply a 3x digital zoom. Next, select the “focus x2” scan option and move the cell of interest into the center of the field of view. Increase the digital zoom to 3x using the “zoom” slider.

9.1.7. Using the focus knob, find the plane of focus with the brightest fluorescence and capture a single plane XY image. Save the image to finish.

9.1.8. Switch back to the 20x magnification objective to search for another TH+ cell. Repeat this process until the desired number of cells have been sampled from each condition.

9.2. Image analysis

9.2.1. In the ImageJ toolbar, click **Analyze | Set Measurements**, and select the boxes for Area, Integrated density, and Mean gray value.

9.2.2. Open an image as a hyperstack with each channel separated by dragging and dropping into the ImageJ toolbar or selecting the image via the file menu.

9.2.3. Use the TH channel (594 nm) to draw ROIs around the cell body. Using the polygon tracing tool in ImageJ, closely trace the outer edge of the cell body. Where the distance between the cell membrane and cell nucleus is the smallest, trace a straight line through the cytosol to the edge of the nucleus and then closely follow the outline of the nucleus in order to exclude it. Then trace a straight line back to the external membrane, bordering the initial line as closely as possible, and continue to follow the outline of the cell body until the ROI is complete.

9.2.4. Using the keyboard shortcut “T” or using the toolbar menu path **Analyze | Tools | ROI Manager**, open the ROI manager and add the ROI that was just drawn to the list.

9.2.5. Select the window of the caspase-3 channel (488 nm), and then select the added ROI in the "ROI Manager" list.

9.2.6. In the **ROI Manager** window, select the **Measure** button. The results window will appear with the measurements set previously. Copy these to a spreadsheet and repeat this process for each cell.

REPRESENTATIVE RESULTS:

Following initial culturing of cells, we treated VM culture dishes at 14 DIV with 1 µL of AAV hSyn-GCaMP6f and allowed for 5 days of viral expression. On the day of imaging HEPES recording buffer was prepared fresh. We used two conditions; in one condition 20 µM glutamate was applied for 10 min, while in the other condition 5 min of 10 µM NBQX application preceded a 10 min co-application of 10 µM NBQX + 20 µM glutamate. In both conditions, we observed heterogenous and spontaneous changes in GCaMP6f fluorescence, which indicate spontaneous Ca^{2+} fluxes, as shown in the representative traces (**Figure 1A,B, Supplemental Movie 1-2**). Application of 20 µM glutamate generated a robust and sustained Ca^{2+} response in both spontaneously active and quiescent neurons (**Figure 1A, Supplemental Movie 1**). Application of 10 µM NBQX reduced spontaneous activity, and partially blocked the glutamate response (**Figure 1B, Supplemental Movie 2**). The extent to which glutamate application stimulated a Ca^{2+} response in each condition was quantified using area under the curve, peak amplitude, and latency to respond. Both area under the curve and peak amplitude were similar for both the glutamate and NBQX + glutamate treated conditions (**Figure 1C**), while latency to response was significantly increased in the NBQX + glutamate condition (**Figure 2A,B**)**.** In addition to quantifying the Ca^{2+} response to glutamate treatment, we fixed and stained samples with an anti-caspase-3 antibody as a measure of glutamate-mediated apoptosis. We observed a range of caspase-3 activation across the conditions (**Figure 3A,B**). Caspase-3 activation was quantified by measuring area and mean caspase-3 intensity. When compared to untreated control cells, the average area of cells with caspase-3 activation under glutamate and NBQX + glutamate conditions trended towards significance (**Figure 3B**). Mean caspase-3 intensity was significantly higher in the glutamate and NBQX + glutamate conditions as compared to untreated controls (**Figure 3B**). Together, these results demonstrate a high-content framework in which apoptosis of neurons can be measured by quantifying Ca^{2+} responses to excitotoxic agents and followed up with an analysis of downstream apoptotic events such as caspase-3 activation in the same set of cultures.

FIGURE AND TABLE LEGENDS:

Figure 1: Cultured ventral mesencephalic neurons display spontaneous Ca^{2+} activity and are robustly stimulated by glutamate application. (**A**) Representative traces of spontaneous Ca^{2+} activity in VM neurons and their response to 20 µM Glutamate application. (**B**) Representative traces of spontaneous Ca^{2+} activity in VM neurons and their response to 10 µM NBQX + 20 µM Glutamate application. (**C**) Population data showing area under the curve and peak amplitude of Ca^{2+} traces.

Figure 2: AMPAR blockade with NBQX delays response to glutamate application in cultured

ventral mesencephalic neurons. (**A**) Representative Ca^{2+} traces of glutamate (gray) and NBQX + glutamate (blue) evoked responses. Average Ca^{2+} traces of glutamate (black) and NBQX + glutamate (red) are shown overlaid. (**B**) Population data showing latency to response for glutamate and NBQX + glutamate evoked responses. Percent change between glutamate and NBQX + glutamate conditions is displayed in the right panel.

Figure 3: Glutamate application increases caspase-3 expression in tyrosine hydroxylase (TH) positive ventral mesencephalic neurons. (**A**) Representative confocal images of VM cultures immunostained for caspase-3 (green) and TH (red), scale bar = 10 µm. (**B**) Population data showing DA neuron area and mean gray value of caspase-3 expression in each condition.

Supplementary Movie 1: Spontaneous Ca^{2+} activity and response to glutamate application. Spontaneous Ca^{2+} fluxes in the presence of HEPES recording buffer (0-300 s) followed by application of 20 µM glutamate (301-600 s). Scale bar = 50 µm.

Supplementary Movie 2: Spontaneous Ca^{2+} activity and response to NBQX + glutamate application. Spontaneous Ca^{2+} fluxes in the presence of HEPES recording buffer (0-300 s) followed by application of 10 µM NBQX (301-600 s), and 10 µM NBQX + 20 µM glutamate (601-900 s). Scale bar = 50 µm.

DISCUSSION:

We describe a long-term primary ventral mesencephalic (VM) cell culture system for high-content analysis of glutamate-mediated apoptosis in neurons. Studies have employed primary midbrain dopaminergic cultures to elucidate excitotoxic mechanisms in the context of PD models[11,12]. In this study, we employ a combinatorial approach using GECIs to measure Ca^{2+} activity and further associate this activity with downstream molecular changes, such as initiation of apoptotic signaling cascades[4]. The method has multiple advantages to other similar cell culture systems. As we have particular interest in excitotoxicity within the context of Parkinson's disease, using primary VM cell cultures is ideal. By using different field relocation techniques, such as gridded coverslips or a motorized XY microscope stage combined with TH immunostaining, we can directly study the cell type specific effects of glutamate-mediated apoptosis in DA neurons. Additionally, the 3-week cell culture model allows for neurons to develop their full, mature molecular profile, reflecting adult DA neurons[9]. Previous methods have mainly focused on molecular changes following glutamate-mediated excitotoxicity[13,14]. The model is unique in its ability to correlate acute changes in neuronal physiology with downstream molecular events in identified cell types. One limitation of the primary culture model is that the dissection technique captures the entire ventral midbrain, including DA and GABAergic neurons as well as neurons from the SNc and VTA. Evidence now suggests that DA neurons of the SNc have selective vulnerability to calcium and eventual cell death compared to DA neurons of the neighboring VTA[15]. Unfortunately, differentiating SNc from VTA neurons in embryonic cultures has proven difficult with few anatomical landmarks to define these structures in the embryonic brain.

We demonstrate that the primary culture technique allows for quantification of heterogenous spontaneous Ca^{2+} activity (**Figure 1**). Therefore, this is an ideal cell culture system model to study tonically active cells, such as pacemaking dopaminergic neurons of the midbrain, neocortical neurons, and GABAergic neurons of the suprachiasmatic nucleus (SCN)[16,17]. In most applications, Ca^{2+} imaging does not achieve the same temporal resolution as electrophysiology. Therefore, it is likely that a single Ca^{2+} event is analogous to a burst of neuronal action potentials. This can be interpreted to mean that Ca^{2+} imaging allows for relatively accurate measures of abnormal bursting activity in pacemaking cells and is therefore appropriate for a high content screen of Ca^{2+}-mediated excitotoxic cell death.

To achieve and maintain spontaneous Ca^{2+} activity, it is important to address two key points in the protocol. First is the plating density of the cells following dissection. For primary VM neurons, previous studies have used around 100,000 cells/cm^2 [9,10]. We have adapted the protocol to plate a density of 200,000 cells/cm^2, which creates a heterogenous range of spontaneous activity and increases the number of dopaminergic VM neurons present on each coverslip. Since different pacemaking neurons have distinct firing properties[16], the plating density needs to be customized to the cell type being studied and optimized in order to achieve ideal levels of spontaneous activity. Second is the incubation time following viral infection of AAVs. Like plating density, this will be dependent on the specific context of the research question and type of AAV being used. For the specific AAV, 5 days of incubation following viral infection is ideal to achieve the desired protein expression levels, which allows for dynamic changes in GCaMP fluorescence in order to record Ca^{2+} activity. Many factors determine how quickly and efficiently an AAV will express its cargo, much of which is outside the scope of this method, but briefly, it is important to consider promoter activity and the rate at which the cargo protein matures and folds.

Another advantage of the method is that it allows for considerable flexibility in format, expression vectors, use of imaging equipment, and the range of scientific questions that can be addressed. In addition, the method enables inquiry into a wide range of specific questions that surround glutamate-mediated excitotoxicity in PD, and other models of nervous system dysfunction. For example, glutamate-mediated excitotoxicity involves multiple receptors and signaling cascades[5]. By using the method, and as demonstrated with the AMPAR blocker, NBQX in **Figure 1**, it is possible to dissect out specific components of the excitotoxic glutamate response at a physiological and molecular level. Conceivably, a similar approach using inhibitors of second messenger systems could be used to determine their contribution to excitotoxicity. Additionally, the AAVs used here could be adapted to express genetically coded calcium indicators (GECIs) with cell-specific promoters or AAV-expressed optogenetic sensors could be used to measure other parameters such as neurotransmitter release.

Apart from primary embryonic dissections and confocal imaging, much of the protocol uses basic laboratory skills that do not require specialized training. Therefore, the limitations to the model include the difficulty of the embryonic dissection technique, the length of time the cells must be cultured to reach maturity, and access to a confocal microscope, or similar imaging apparatus. The many benefits and flexibility of the method outweighs these limitations, making this an ideal model to study the role glutamate-mediated excitotoxicity in nervous system disorders. Finally, this model could be an effective tool to screen novel compounds for anti-apoptotic effects and their ability to preserve DA neuron health.

ACKNOWLEDGMENTS:
Supported by grants from the American Parkinson Disease Association (APDA) and NIH R01NS115809-01 to RS. We thank the Texas A&M Institute for Genomic Medicine (TIGM) for providing timed pregnant mice to generate primary dopaminergic cultures.

DISCLOSURES:
The authors have nothing to disclose

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Figure 1

Click here to access/download;Figure;Glut_excitotoxicity_figure1_version3.tif

Figure 1

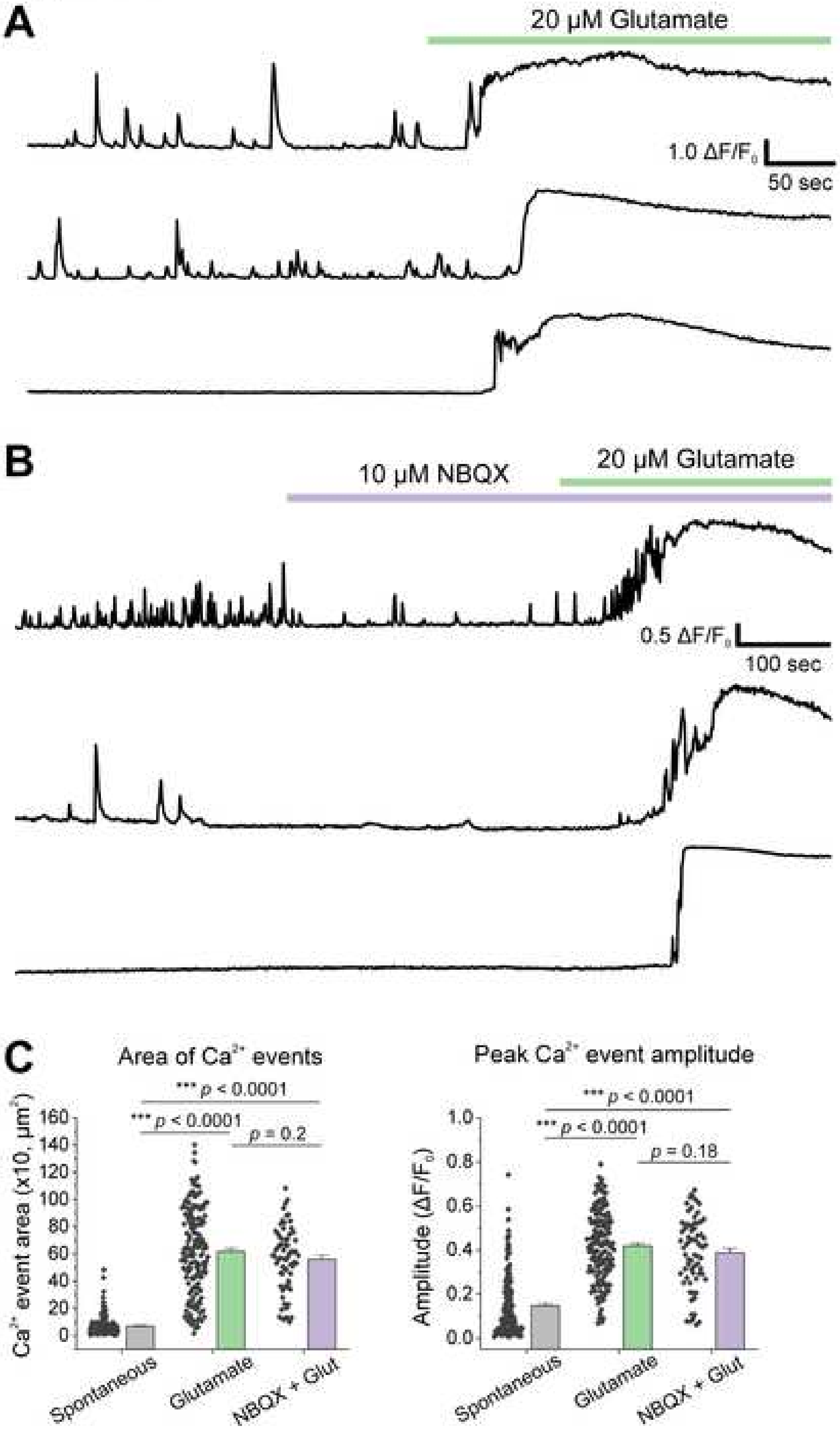


Figure 2

Figure 2

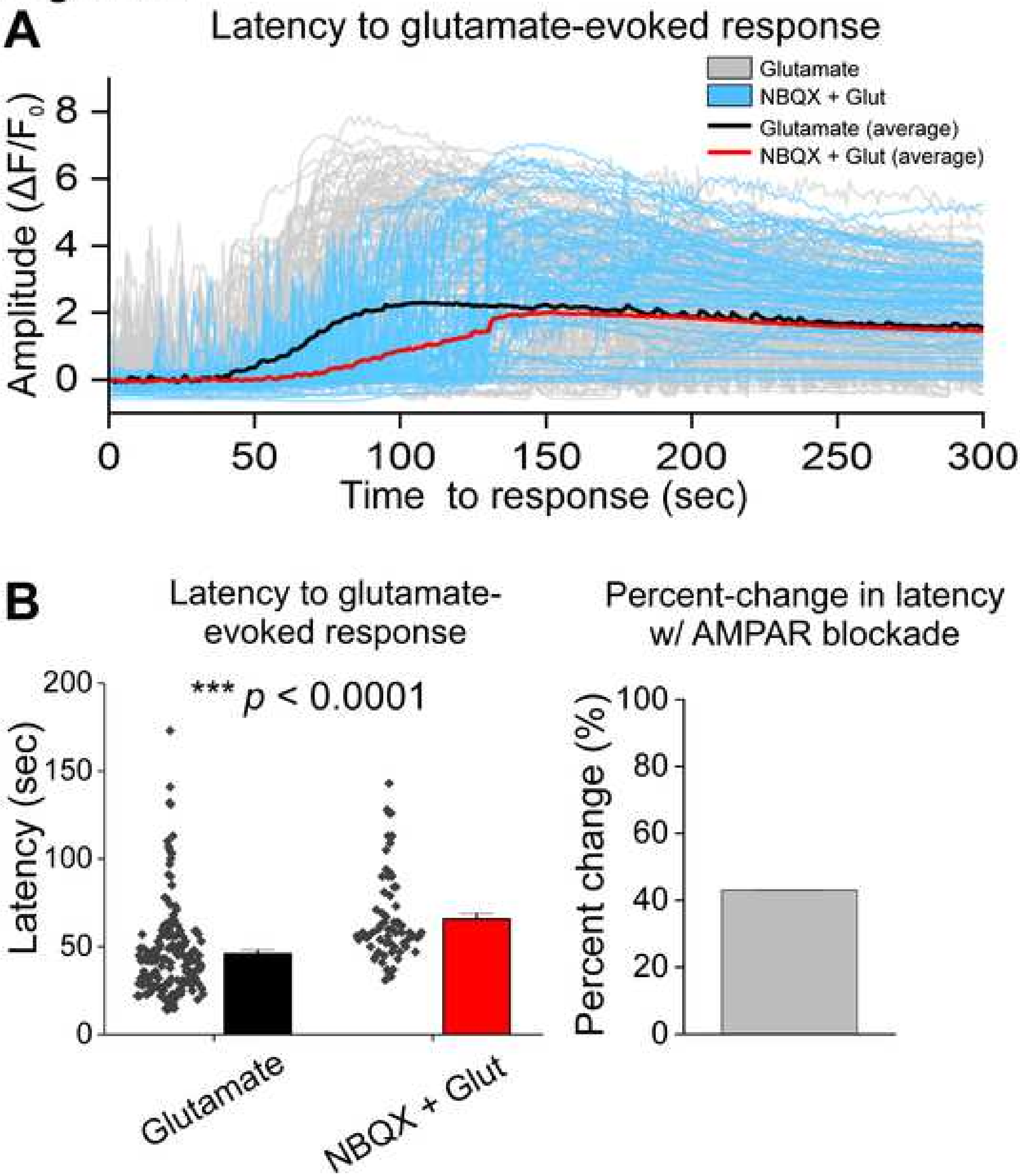
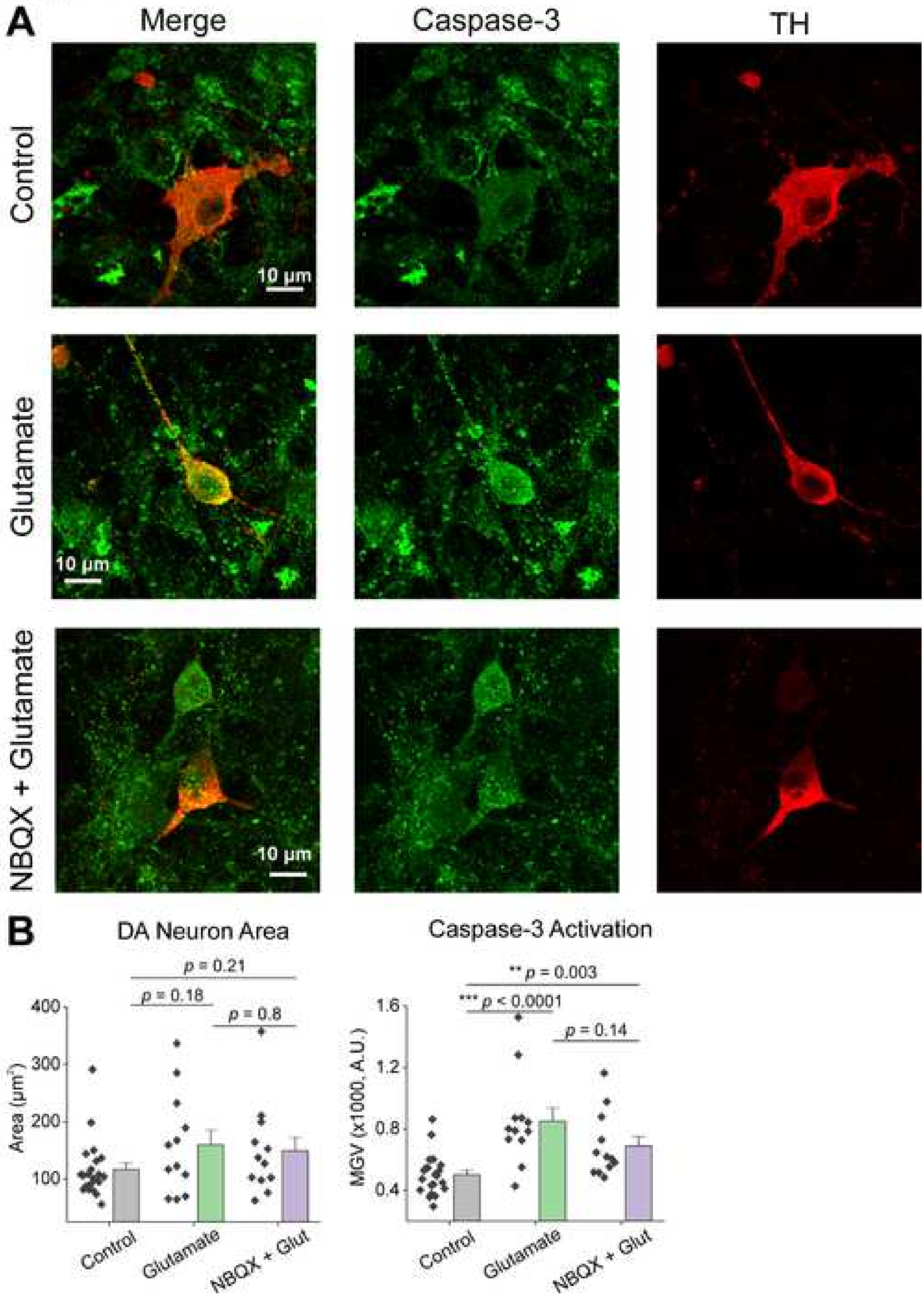


Figure 3

Click here to access/download;Figure;Glut_excitotoxicity_figure3_version2.tif

Figure 3



Supplementary movie 1

Click here to access/download
Video or Animated Figure
Supplementary Movie 1_glutamate_ver 2.avi

Supplementary movie 2

Click here to access/download

Video or Animated Figure

Supplementary Movie 2_NBQX+glut.avi

Name of Material/ Equipment	Company	Catalog Number
10% Formalin/PBS	VWR	100496-506
10X NA 0.3 water-immersion objective	Olympus	UMPLFLN10XW
12 mm circular cover glass No. 1	Phenix Research Products	MS20-121
20X NA 0.85 oil-immersion objective	Olympus	UPLSAPO20XO
35 mm uncoated plastic cell culture dishes	VWR	25382-348
40X NA 0.3 water-immersion objective	Olympus	LUMPLFLN40XW
60X NA 1.35 oil-immersion objective	Olympus	UPLSAPO60XO
Ampicillin (sodium)	Gold Bio	A-301-25
B-27 supplement	ThermoFisher	17504044
Binolcular Microscope	Kent Scientific	KSCXTS-1121
Bovine serum albumin (BSA)	Sigma-Aldrich	A7030
Calcium Chloride ($CaCl_2$), anhydrous	Sigma-Aldrich	746495
Chicken polyclonal anti-Tyrosine Hydroxylase	Abcam	ab76442
Deoxyribonuclease I (DNase)	Sigma-Aldrich	DN25
D-glucose, andydrous	Sigma-Aldrich	RDD016
DMEM + GlutaMAX medium	ThermoFisher	10569010
Equine serum	ThermoFisher	26050088
Fiber Optic Illuminator, 100V	Kent Scientific	KSC5410
Filter System, PES 22UM 250ML	VWR	28199-764
Fluoview 1000 confocal microscope	Olympus	
Fluoview 1200 confocal microscope	Olympus	
GlutaMAX supplement	ThermoFisher	35050061
Goat polyclonal anti-chicken Alexa Fluor 594	Abcam	ab150176
Goat polyclonal anti-rabbit Alexa Fluor 594	Abcam	ab150077
Hanks-balanced Salt Solution (HBSS) 1x	ThermoFisher	14175095
HEPES	VWR	101170-478
HeraCell 150 CO_2 incubator	Heraeus (ThermoFisher)	
ImageJ v1.52e	NIH	
IRIS-Fine Scissors (Round Type)-S/S Str/31*8mm/13cm	RWD	S12014-13

Kanamycin monosulfate	Gold Bio	K-120-25
Laminin	Sigma-Aldrich	L2020
L-Ascorbic acid	Sigma-Aldrich	A7506
L-glutamic acid	VWR	97061-634
Magnesium Chloride ($MgCl_2$), andydrous	Sigma-Aldrich	M8266
MPII Mini-Peristaltic Pump, 115/230 VAC, 50/60 Hz	Harvard Apparatus	70-2027
MULLER Micro Forceps-Str, 0.15mm Tips, 11cm	RWD	F11014-11
NBQX	Hello Bio	HB0443
Neurobasal medium	ThermoFisher	21103049
Normal goat serum (NGS)	Abcam	ab7481
Origin 2020	OriginLab	
pAAV.Syn.GCaMP6f.WPRE.SV40	Addgene	100837-AAV1
Papain	Worthington Biomedical Corporation	LS003126
Penicillin streptomycin	ThermoFisher	15140122
Phosphate-buffered saline (PBS) 1x	ThermoFisher	10010049
Poly-L-lysine	Sigma-Aldrich	P4832
Poly-L-ornithine	Sigma-Aldrich	P4957
Potassium Chloride (KCl), anhydrous	Sigma-Aldrich	746436
Pump Head Tubing Pieces For MPII	Harvard Apparatus	55-4148
Rabbit monoclonal anti-caspase-3	Abcam	ab32351
Sodium Chloride (NaCl), anhydrous	Sigma-Aldrich	746398
Sucrose	Sigma-Aldrich	S7903
Time-pregnant female C57BL/6 mice	Texas A&M Institue for Genomic Medicine	
Triton X-100	Sigma-Aldrich	X100
Wide-bore blue pipette tips P1000	VWR	83007-380

Comments/Description

50x stock

500 mL
heat-inactivated

500 mL

500 mL

Titer: 1.00E+13 gc/ml

10,000 U/mL
500 mL

BioXtra, ≥99.5% (GC)

500 mL

Jove Reviewer Comments

We appreciate editorial and reviewer comments and have made the necessary changes. We believe the manuscript is much improved as a result of these revisions. All changes to the manuscript have been highlighted in cyan. Below is a point-wise response to editorial and reviewer comments.

Editorial comments:

Comment: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version**.**

Response: We have taken to time to review the manuscript for spelling and grammar issues. We are happy with the current state of the manuscript.

Comment: Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: The summary has been rephrased as suggested and now fits within the 10-50 word limit.

Comment: 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.

Response: For protocol steps lacking detail or the “how” questions we have taken time to elaborate on these steps adding more descriptive information on how to actively perform the task.

Comment: 2: Please specify the concentrations of all solutions used in the manuscript.

Response: All missing concentrations of solutions have been added where necessary.

Comment: 2.1: Please specify the desired amount. We need specific values in order to film.

Response: We have specified the exact amount of 35mm petri dishes to be used at this step.

Comment: What is the age/sex/gender of the mouse used?

Response: The age of the timed pregnant mice has been added. As these are pregnant mice, they are female.

Reviewer #1 comments:

Comment: The introduction (lines 58-67) mentions that glutamate-mediated excitotoxicity occurs mainly through NMDA receptor activation. Yet, the authors used NBQX, a blocker of AMPA receptors to mitigate the calcium response of midbrain neurons challenged with glutamate. What is the rational for that?

Response: We have added references and an explanation for the involvement of AMPARs in glutamate-mediated excitotoxicity. Our rational was to determine the AMPAR component of glutamate-mediated excitotoxicity within a cell culture model of midbrain neurons.

Comment: The authors mentioned the fact that "while much of the previous work on excitotoxicity has been done within cell culture models, many studies have used cortical neurons or other, more easily cultured, cell lines" (lines 558-560). This is only partially true. See for instance the following references [Douhou A, J Neurochem. 2001 78(1):163-74; Lavaur J, et J Neurochem. 2017 142(1):14-28]. What is true, however, is that genetically encoded calcium indicators have not been commonly used for monitoring calcium when midbrain cultures are used to assess excitotoxic processes.

Response: We appreciate this insight and have included the references listed in the comment above. We have rewritten this section (lines 587 to 594) to focus on the novelty of using GECIs to monitor calcium activity of midbrain neurons following glutamate challenge.

Comment: Calcium measurements were performed on the whole population of midbrain neurons and not specifically on dopamine neurons, which most likely represent a small percentage of neuronal cells in this model system. Therefore, the authors should mention the fact that it would be also technically possible to perform calcium imaging specifically in dopamine neurons using field relocation protocols after TH immunocytochemistry.

Response: We have added a short explanation of the potential for differentiating TH+ from TH- neurons within our culture model as follows: "By using different field relocation techniques, such as gridded coverslips or a motorized XY microscope stage, combined with TH immunostaining, we can directly study the cell type specific effects of glutamate-mediated excitotoxicity in DA neurons"

Comment: Section 6. (line 269) "Infection of cell culture with adeno-associated viral (AAV) vectors": It is not clearly stated at what stage of maturation, cultured cells are transduced with viral vector particles. Besides, the age of the cultures, which are used for calcium imaging, is mentioned nowhere. This is of importance, as the response to a glutamate challenge will vary dramatically as a function of the degree of maturation of these cultures.

Response: We have now added information on the number of days *in vitro* for the cells in section 6 and section 7. In the discussion we also mention that by 2-3 weeks, the timeframe of AAV infections and Ca^{2+} imaging, the cells are fully matured based on their molecular profile. Appropriate references are included.

Comment: Section 3.12 (lines 209-216) An illustration for the dissection protocol would be welcome. Alternatively, cite previously described protocols for this section.

Response: A previously published protocol of the dissection process is referenced at the beginning of section 3 as it outlines the entire process from 3.1 through 3.14

Comment: As a perspective, the authors could also mention the possibility of comparing the impact of test compounds on glutamate-evoked responses to their rescuing effect for dopamine neurons.

Response: We completely agree and have added the following sentence at the end of the discussion: "this model could be an effective tool to screen novel compounds for anti-apoptotic effects and their ability to preserve DA neuron health.".

Comment: In the abstract (line 33), the authors describe the degeneration of dopaminergic neurons as "rapid" which is probably not true. It may be rapid at the level of a single neuron but slow if considering the whole population of vulnerable dopamine neurons.

Response: We agree that the use of the word rapid here is slightly misleading about the progressive nature of PD *in vivo*. We have removed the word.

Comment: In the abstract (line 39): "under control of the neuron-specific promoter, hSyn" hSyn stands for what? Synapsin?

Response: We have added the full spelling of the promotor to prevent any confusion.

Reviewer #2 comments:

Comment: The authors claim that this is a model for evaluate excitotoxic cell death in dopaminergic neurons. There are not enough results about cell viability/intoxication/death, the unique result related to cell intoxication is the evaluation of Caspase 3 activation by imaging techniques. In order to make this method suitable to evaluate dopaminergic cells toxicity and death, more experiments need to be added to the method, e.g. TH expression evaluation and cell counts. However, considering that the method is suitable for measuring Ca flux, instead of adding further experiments the authors could modify the title and the discussion focusing on the efficacy as a method for screening drugs that interfere with Ca flux.

Response: We agree that Caspase-3 activation alone is not a complete measure of cell death. We have reworded title as follows: "A method to quantify spontaneous Ca^{2+} fluxes and their downstream effects in primary mouse midbrain neurons". We have also reworded parts of the introduction and discussion to focus on the downstream effects of Ca^{2+} influx in midbrain neurons, rather than excitotoxic cell death.

Comment: It's appropriate to add a brief description of NBQX effects in the introduction.

Response: We agree and have added a short description of NBQX pharmacology along with a reference.

Comment: The approx total number of the cells obtained (adjusted to the number of embryos) could be an useful data.

Response: We agree and have added this information at the beginning of protocol step 5 as follows: "Note: Based on our experience, about 100,000 viable cells per embryo are collected. 2-3 mo old timed pregnant mice typically have litters sizes of 8-10 embryos, therefore, a rough estimate for total yield of cells per timed pregnant mouse is approximately 1 million cells."

Comment: Many substances are dissolved in H2O, the authors mean distilled water?

Response: We have clarified throughout the protocol that distilled water was used in these steps.

Comment: In step 4.2.1 the approx number of "up and down" with pipette should be indicated.

Response: We have added the exact number of trituration steps to perform.

Reviewer #3 comments:

Comment: Why is the dissection done outside a biological safety cabinet as it increases the risk of contamination? Did the authors consider adding an antibiotic to the HBSS.

Response: We use 4 separate antibiotics in our culture medium therefore the risk of contamination is minimal. However, if the use of antibiotics is unwarranted the dissection setup can be moved into a sterile hood. We have addressed this issue in section #3 of the protocol as follows: "Plentiful use of 70% EtOH on surfaces near the dissection microscope and on surgical tools is ideal. A mask may also be worn during the dissection to further prevent contamination. Additionally, we use 4 separate antibiotics in our culture medium, so contamination is unlikely. However, if use of antibiotics is problematic, this dissection setup could be moved inside a sterile hood".

Comment: For this method, is it necessary to use 3 coating reagents (PDL, PLO, laminin) for the plate?

Response: While it is not necessary, it is ideal. We have added a brief note at the beginning of section #2 of the protocol as follows: "Note: We have found that combining three coating agents, poly-L-lysine, poly-L-ornithine, and laminin allows for ideal cell adhesion and viability".

Comment: concentration of hydrochloric acid that is best? Adding "small amount" is a bit ambiguous

Response: We have provided more accurate information on the volume and concentration of hydrochloric acid used.

Comment: It is not clear where to leave the embryos after collecting them (HBSS?). Were embryos kept on ice throughout collection and midbrain dissection?

Response: In the note at the beginning of protocol step 3# we have added more descriptive information as follows: “To preserve cell viability all dissection solutions should be chilled at 4˚C, and dissections should be completed as quickly as possible. We do not perform the dissections on ice.” We have also clarified the method to preserve dissected embryos by adding the following statement in the manuscript “Embryos should be left in HBSS throughout this process”

Comment: what is the control? Would it be a no glutamate addition, or no virus addition? Point 6.2 states two AAVs, but 6.1 only describes 1. This is confusing.

Response: The control condition are cultures infected with the AAV but with no glutamate exposure. We apologize for the error in step 6.2 suggesting there are two AAVs. Only one AAV, hSyn-GCaMP6f was added and we have made changes to the protocol to reflect this.

Comment: How do researchers know if viral expression is ready after 5 or 7 days? Are there any steps for confirmation of effective viral expression? "Continue to change the medium every 2-3 days" does this mean during the 5-7 days of viral expression? If so this should be made clear

Response: We have clarified the question by adding the following sentence to the manuscript: “We have found that 5-7 days of AAV infection allows for ideal levels of GCaMP expression. Continue to change medium every 2-3 days throughout this period of viral infection”.

Comment: why are 2 lasers being used during the calcium recording? Are they going to be recording different things or is this specific for the AAV used? As this part is not being conducted on cells that have been stained with different antibodies then why the two wavelengths. A general reader will not understand

Response: Again we apologize for this mistake. Only was laser was used to visualize GCaMP and the protocol has been updated to reflect this.

Comment: are there still 2 coverslips per 35mm dish at this point? How is the formalin addition managed at this point. If there are multiple coverslips being imaged are there any points at which the coverslips can be kept in solution (such as in PBS wash) so that staining canbe performed at the same time?

Response: As mentioned in step 7.2.2. only one coverslip is brought down to the microscope at a time. We have made a few changes to this step to make this more clear. We have also modified step 7.2.15 to read, “Following the additional 5 min of 20 µM Glutamate exposure the coverslip can be removed from the bath and placed back into the 35mm petri dish containing recording buffer until the day of imaging is completed. When finished, proceed to step 8. Additionally, in the note following step 8 we have added the sentence, “Following fixation with formalin, coverslips can be stored in 1x PBS at 4°C until ready to be processed for immunostaining.

Comment: anti-caspase-3 antibody is not a measure of excitotoxicity but a measure of apoptosis. Suggestion would be to alter this phrase to excitotoxicity-mediated apoptosis.

Response: We agree with the reviewer that caspase-3 activation is a measure of apoptosis, not excitotoxicity. We have changed the sentence to read, “In addition to quantifying the Ca^{2+} response to glutamate treatment, we fixed and stained samples with an anti-caspase-3 antibody as a measure of glutamate-mediated apoptosis.” We have also reworded the manuscript to state “glutamate-mediated apoptosis” rather than “glutamate-mediated excitotoxicity”

Comment: "as we can study the cell type specific effects of glutamate-mediated excitotoxicity in dopaminergic neurons directly." Have the authors examined the purity of the cultures for dopamine neurons? Only the apoptosis was confirmed in TH positive cells. Without purity information is it possible the calcium imaging is picking up differences in non dopaminergic cells?

Response: We appreciate this insight and while yes, the cultures are not pure, we have addressed this issue in lines 588-592, “As we have particular interest in excitotoxicity within the context of Parkinson’s disease, using primary VM cell cultures is ideal. By using different field relocation techniques, such as gridded coverslips or a motorized XY microscope stage combined with TH immunostaining, we can directly study the cell type specific effects of glutamate-mediated excitotoxicity in DA neurons.” While we did not differentiate cell type for calcium imaging data in this study we outline above how this can be achieved.

Comments: Is a 2 week period considered a long-term cell experiment, given this timeframe is standard for neurons. Can the authors clarify what they mean by "shorter-term" methods and provide a more appropriate reference as current reference used describes a 3 week protocol.

Response: We apologize for the confusion and have reworded this section for clarity, “Additionally, our 3-week cell culture model allows for neurons to develop their full, mature molecular profile, reflecting adult DA neurons [9]”. The protocol we describe here takes place across 3-weeks. As PD typically manifests late in life, more mature neurons would be most reflective of the PD condition. Based on the reference listed, at 3 weeks *in vitro* the neurons have fully matured and contain the molecular machinery of a typical adult neuron.

Reviewer #4 comments:

Comment: The cultures contain both GABAergic and dopaminergic neurons, all of which are expressing GCaMP after infection. Both cell types can pace spontaneously and respond to glutamate. Furthermore, you have at least two types of dopamine neurons, those of the VTA and those of the SNc. Those dopamine neurons are fundamentally different in their calcium handling and sensitivity to excitotoxicity. Dopamine neurons of the SNc are sensitive to calcium dependent apoptosis while those of the VTA are resistant. Therefore, the results of the calcium imaging part of the experiment is from a mixture of three different populations of neurons.

Response: We appreciate your insight and are aware of this limitation. To address the issue of differentiating GABAergic neurons, we have added a brief explanation of possible methods to differentiate DA vs. GABA neurons, "By using different field relocation techniques, such as gridded coverslips or a motorized XY microscope stage combined with TH immunostaining, we can directly study the cell type specific effects of glutamate-mediated excitotoxicity in DA neurons." Unfortunately with the dissection technique we use it is near impossible to differentiate SNc vs VTA DA neurons in our cultures. We agree that this is one of the main limitations as their physiology is quite distinct. We have added the following passage to address this, "One limitation of our primary culture model is that our dissection technique captures the entire ventral midbrain, including DA and GABAergic neurons as well as neurons from the SNc and VTA. Evidence now suggests that DA neurons of the SNc have selective vulnerability to calcium and eventual cell death compared to DA neurons of the neighboring VTA {Surmeier, 2017}." Differentiating SNc from VTA neurons in embryonic cultures has proven difficult with few anatomical landmarks to define these structures in the embryonic brain.

Comment: In lines 522-525 "Together………in the same cells", suggests that the same cells measured in the calcium imaging were measured for caspase-3 and TH positive. By the description in the protocol, it is not clear that the same cells/fields of view were aligned and measured for calcium signal, caspase-3 fluorescence and tyrosine hydroxylase fluorescence. Unless you are aligning the same cells in the calcium images and caspase-3/TH images, the dopamine neuron signals of the calcium imaging cannot be separated from those of the GABA (see 1). Please clarify if "the same cells" are the exact same cells/fields of view or if they are just from the same coverslip.

Response: We apologize and agree that this sentence is confusing. We have now changed the sentence to read, "Together, these results demonstrate a high-content framework in which apoptosis of neurons can be measured by quantifying Ca^{2+} responses to excitotoxic agents and followed up with an analysis of downstream apoptotic events such as caspase-3 activation in the same set of cultures." As mentioned in the above comment, we discus possibilities to allow for alignment of Ca^{2+} imaging and capase-3 activation data at a single cell resolution.

Comment: what are the concentrations of poly-L-lysine and poly-L-ornithine?

Response: We apologize for this oversight and have added the concentrations of both poly-L-Lysine and poly-L-ornithine where appropriate.

Comment: "mice" should be mouse unless you use more than one pregnant mouse per culture

Response: We have included the statement “We use between 4 to 6 timed pregnant mice per culture” in section 3. Therefore, we have retained the wording as mice.

Comment: What is the thickness of the section? Give an estimate.

Response: We have added a rough estimate to the thickness of the initial midbrain segments, “The midbrain segment should be approximately 0.5 mm thick.”

Comment: tissue will be less dense" what do you mean by this?

Response: We appreciate this comment and have reworded this sentence in an attempt to be more visually descriptive, “The remaining inferior quadrants will have excess tissue laterally on the dorsal side, this tissue will look less opaque than the remaining ventral tissue.”

Comment: Aspirate the remaining laminin" is the laminin left on the coverslips until use or are the coverslips in the incubator in rinse water? Please clarify.

Response: We have rewritten step 5.2 to reiterate that laminin solution should be left on the coverslips at the end of section 2 of the protocol, “Remove coverslips with laminin solution from step 2 from the incubator and aspirate the remaining laminin solution from the coated coverslips using a vacuum.”

Comment: "both AAVs" is there more than hSyn-GCaMP6f AAV? Please clarify

Response: We thank the reviewers for catching this mistake and have corrected it as follows, “Aspirate the cell culture medium from each dish and replace with 1 mL serum free DMEM containing hSyn-GCaMP6f. Place dishes back into the 37 °C incubator for 1 h.”