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Dr. Vineeta Bajaj
Review Editor,
JoVE Journal

Dear Dr. Bajaj,

We submit a revised manuscript for JoVE59461 **“Evaluation of synaptic multiplicity using whole-cell patch-clamp electrophysiology”** .

As specified in Responses to Editorial Comments and Responses to Reviewers Comments, we believe that we thoroughly addressed both editorial and reviewers comments. Please note that changes made in the revised manuscript are **highlighted in gray**.

If possible, I would like to this article in press before March 22, 2019, in order to list this publication in my tenure application dossier.

Sincerely,

Wataru Inoue, PhD

Wataru Inoue

TITLE:

Evaluation of Synaptic Multiplicity Using Whole-Cell Patch-Clamp Electrophysiology

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KEYWORDS:

Whole-cell patch-clamp electrophysiology, *ex vivo*, synaptic transmission, synaptic gain, multiplicity, paraventricular nucleus of the hypothalamus, corticotropin-releasing hormone, hypothalamus

SUMMARY:

Here, we present a protocol for evaluating the functional synaptic multiplicity using whole-cell patch clamp electrophysiology in acute brain slices.

ABSTRACT:

In the central nervous system, a pair of neurons often forms multiple synaptic contacts and/or functional neurotransmitter release sites (synaptic multiplicity). Synaptic multiplicity is plastic and changes throughout the development and in different physiological conditions, being an important determinant for the efficacy of synaptic transmission. Here, we outline experiments for estimating the degree of multiplicity of synapses terminating onto a given postsynaptic neuron using whole-cell patch clamp electrophysiology in acute brain slices. Specifically, voltage-clamp recording is used to compare the difference between the amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) and miniature excitatory postsynaptic currents (mEPSCs). The theory behind this method is that afferent inputs that exhibit multiplicity will show large, action potential-dependent sEPSCs due to the synchronous release that occurs at each synaptic contact. In contrast, action potential-independent release (which is asynchronous) will generate smaller amplitude mEPSCs. This article outlines a set of experiments and analyses to characterize the existence of synaptic multiplicity and discusses the requirements and limitations of the technique. This technique can be applied to investigate how different behavioral, pharmacological or environmental interventions *in vivo* affect the organization of synaptic

contacts in different brain areas.

INTRODUCTION:

Synaptic transmission is a fundamental mechanism for communication between neurons, and hence, brain function. Synaptic transmission is also labile and can change its efficacy in an activity-dependent manner as well as in response to modulatory signals¹. Thus, examining the synaptic function has been a key focus of neuroscience research. Whole-cell patch clamp electrophysiology is a versatile technique that enables us to understand, by devising experimental designs and data analyses, in-depth biophysical and molecular mechanisms of synaptic transmission. A commonly used approach, perhaps owing to the simplicity of the technique and concept, is the measurement of miniature excitatory/inhibitory postsynaptic currents (mE/IPSCs) under the voltage clamp configuration^{2–6}. Individual mPSCs represent the flow of ions through postsynaptic ionotropic receptors (e.g. AMPA and GABA_A receptors) in response to the binding of their respective neurotransmitters released from the presynaptic terminal⁷. Because the recording is obtained in the presence of the voltage-gated Na⁺ channel blocker tetrodotoxin (TTX), the release is action potential-independent and normally involves a single synaptic vesicle that contains neurotransmitter. Based on this assumption, the average amplitude of mPSCs is widely used as a crude estimate for the quantal size, which represents the number and functionality of postsynaptic receptors opposing a single release site. On the other hand, the frequency of mPSCs is considered to represent a combination of the total number of synapses terminating onto the postsynaptic cell and their average release probability. However, these parameters do not measure another variable—multiplicativity of synapses, or synaptic multiplicity—which is important for the efficacy of synaptic transmission.

Based on the quantal theory of synaptic transmission^{7–9}, the strength of a given connection between a pair of neurons is dependent on three factors: the number of functional synapses (N), the postsynaptic response to the release of a single synaptic vesicle (quantal size; Q) and the probability of neurotransmitter release (P_r). Synaptic multiplicity is equivalent to N . The development of synaptic multiplicity or the pruning of multiplicative synapses is plastic throughout development and in different disease states^{3,4,6,10}. For this reason, characterizing synaptic multiplicity has important implications for understanding the efficacy of synaptic transmission in health and disease. Techniques, such as electron microscopy can identify structural evidence of synaptic multiplicity by detecting multiple synaptic contacts originating from the same axon onto the same postsynaptic neuron^{11–14}. However, these structurally identified multisynapses can be functionally silent^{15,16}. Precise functional examination of N requires technically challenging electrophysiological approaches, such as paired whole-cell recordings that can identify whether a given connection has multiple functional release sites and minimal stimulation approaches that aim to recruit a single putative axon.

In this protocol, we describe a simple method for estimating synaptic multiplicity by adopting a method originally developed by Hsia et al². This technique involves the measurement of spontaneous PSCs (sPSCs) and mPSCs using whole-cell patch clamp electrophysiology, which allows us to estimate the degree of synaptic multiplicity across all inputs to a given neuron. As previously defined, synaptic multiplicity reflects the number of synapses between a given pre-

and postsynaptic neuron. If multiple synapses are recruited in synchrony by an action potential, there will be a high probability of temporal summation of individual (i.e. quantal) PSCs, generating a greater amplitude PSC. In mPSC recordings (in which action potentials are blocked by TTX), the probability of temporal summation of individual (non-synchronous) mPSCs is low. Using this rationale, synaptic multiplicity can be estimated by comparing the sPSC amplitude (with action potential-dependent release) to the mPSC amplitude.

To examine the existence of multiplicity we describe four experiments and their analyses using glutamatergic EPSCs as an example. However, the same approach can be used for the fast GABAergic/glycinergic transmission (IPSCs). A brief rationale for each experiment is described below. First, as explained above, synaptic multiplicity can be estimated by comparing the amplitude of sEPSCs to mEPSCs. There are two requirements for this approach; 1) presynaptic axons must fire a sufficient number of action potentials during recording, and 2) P_r must be high so that multiple synapses release neurotransmitter upon the arrival of an action potential. In order to meet these requirements, sEPSCs are first recorded in low Ca^{2+} artificial cerebrospinal fluid (aCSF), and then recorded in the presence of a low concentration of the K^+ channel antagonist, 4-Aminopyridine (4-AP) to increase action potential firing and P_r . Then action potential firing is blocked by TTX and P_r decreased by a voltage-gated Ca^{2+} channel blocker Cd^{2+} . The amplitude of sEPSCs (with 4AP) is compared to that of mEPSC (with 4AP, TTX, and Cd^{2+}). In the second experiment, Ca^{2+} is replaced by equimolar Sr^{2+} in the aCSF to desynchronize vesicle release. As Ca^{2+} is required for the synchronous release of vesicles, replacement with Sr^{2+} should eliminate the large amplitude sEPSCs that are indicative of multiplicity. Third, mechanistically, multiplicity can result from either multiple synaptic contacts to the same postsynaptic neuron or multivesicular release (i.e. multiple vesicles released within a single synaptic contact)^{17,18}. To differentiate between the two types of multiplicity, the third experiment uses a low affinity, fast dissociating competitive antagonist of AMPA receptors, γ -D-glutamylglycine (γ -DGG)^{17,18} to determine whether large sEPSC are the result of the temporal summation of independent synapses or multivesicular release acting on an overlapping population of postsynaptic receptors. If the large amplitude events arise from multivesicular release, γ -DGG will be less effective at inhibiting larger compared to smaller sEPSCs, whereas large sEPSCs that arise from the temporal summation of multiple synaptic contacts will be similarly affected by γ -DGG. In the fourth experiment, a more physiological method is used to enhance action potential firing, namely afferent synaptic stimulation. Bursts of synaptic activity can transiently increase/facilitate the spontaneous action potential firing and release probability of the stimulated afferents. Therefore, this approach allows multiplicity to manifest in a more physiological manner.

The following protocol describes the method for conducting these experiments in mouse hypothalamic tissue. Specifically, corticotropin releasing hormone (CRH) neurons of the paraventricular nucleus of the hypothalamus (PVN) are used. We describe the procedures for conducting whole-cell patch clamp electrophysiology and explain the specific experiments to test for synaptic multiplicity.

PROTOCOL:

All animal experiments are approved by the Animal Care Committee of The University of Western

Ontario in accordance with the Canadian Council on Animal Care Guidelines (AUP#2014-031).

1. Solutions

1.1. Slicing solution

1.1.1. Refer to **Table 1** for the composition of the slicing solution.

1.1.2. Prepare a 20x stock solution in advance and store it at 4 °C for up to 1 month.

1.1.3. For 1x slicing solution, dissolve NaHCO₃, glucose, and sucrose in ddH₂O, and add the 20x stock. Ensure the osmolarity is between 315-320 mOsm and store the solution for no more than 1 week at 4 °C.

1.1.4. Fill two beakers with 100 mL of slicing solution and cover them with parafilm. Chill the solution in a freezer until the solution becomes partially frozen (approximately 20 min in -80 °C freezer). Using a gas dispersion tube, bubble both beakers of slicing solution with 95% O₂/5% CO₂ for 20 minutes on ice.

[Insert **Table 1** here]

1.2. aCSF (for slice recovery and maintenance)

1.2.1. Refer to **Table 1** for the composition of the aCSF.

1.2.2. Prepare a 20x stock solution in advance and store it at 4 °C for up to 1 month.

1.2.3. For 1x aCSF, dissolve NaHCO₃ and glucose in ddH₂O and add the 20x stock. Ensure the osmolarity is between 298-300 mOsm. Use the solution within 1 day.

1.3. aCSF (low Ca²⁺ for recording)

1.3.1. Refer to **Table 1** for the composition of the low Ca²⁺ aCSF.

1.3.2. Prepare a 20x stock solution in advance and store it at 4 °C for up to 1 month.

1.3.3. For 1x low Ca²⁺ aCSF, dissolve NaHCO₃ and glucose in ddH₂O and add 20x (CaCl₂ and MgCl₂ free) stock, CaCl₂ and MgCl₂ to specified concentrations. Ensure the osmolarity is between 298-300. Use the solution within 1 day.

1.4. aCSF (Sr²⁺ for recording)

1.4.1. Refer to **Table 1** for the composition of the Sr²⁺ aCSF.

1.4.2. Prepare a 20x stock solution in advance and store it at 4 °C for up to 1 month.

1.4.3. For 1x Sr^{2+} aCSF, dissolve NaHCO_3 and glucose in ddH₂O and add 20x (CaCl_2 and MgCl_2 free) stock, SrCl_2 and MgCl_2 to specified concentrations. Ensure the osmolarity is between 298-300. Use the solution within 1 day.

1.5. Internal solution

1.5.1. Refer to **Table 1** for the composition of the K-gluconate based internal solution.

1.5.2. To make 20 mL of internal solution, add 15 mL of molecular biology grade water to a 50 mL tube. Perform the subsequent steps on the ice.

1.5.3. Prepare the following solutions ahead of time to 1 M stock concentrations in molecular biology grade water. Add (in mL): 2.32 K-gluconate, 0.24 Na-gluconate, 0.20 HEPES, 0.16 KCl, 0.05 K₂-EGTA, 0.04 MgCl_2 to the 50 mL tube.

1.5.4. Add 100 μL of 0.3 M Na_3GTP .

1.5.5. Weigh 44.08 mg of K_2ATP in a 2 mL microcentrifuge tube and add 1 mL of molecular biology grade water, then add to 50 mL tube.

1.5.6. Adjust the pH to 7.2-7.4 with 1 M KOH. Ensure the osmolarity is between 283-289 mOsm.

2. Slice preparation

2.1. Prepare tools

2.1.1. Add 200 mL of aCSF to the recovery chamber (constructed from a 250mL beaker with 4 wells and netting) and place the recovery chamber in a water bath (35 °C).

2.1.2. Cover the chamber with a paraffin film and constantly bubble the aCSF with 95% O_2 /5% CO_2 using a glass dispersion tube for at least 20 min.

2.1.3. Prepare for the dissection by setting up the tools (scalpel, angled fine scissors, forceps, fine paint brush, plastic spoon).

2.1.4. Fill a 60 mL syringe with approximately 15 mL of the ice-cold slicing solution from step 1.1.4.

2.1.5. Prepare the dissection platform by placing a filter paper on the lid of a well plate.

2.1.6. Prepare the slicing chamber by placing it in the ice tray and filling the tray with ice.

2.1.7. Set up the vibratome by securing a disposable blade in the blade holder.

2.1.8. Make a transfer pipette by breaking the tip of a Pasteur pipette and placing a rubber bulb over the broken end.

2.2. Dissect mouse brain

2.2.1. Anesthetize the animal in a chamber saturated with 4% isoflurane until spinal reflexes are absent.

2.2.2. Decapitate the animal using a guillotine and quickly remove the brain.

2.2.2.1. Make a midline incision with a No. 22 scalpel blade from rostral to caudal.

2.2.2.2. Laterally peel the scalp on each side of the head.

2.2.2.3. Use fine scissors to cut the skull on one side from caudal to rostral (including the side of the frontal bones), using caution not to damage the brain.

2.2.2.4. Use forceps to lift the skull piece off the brain and quickly cool the brain with 15 mL of ice-cold slicing solution using the syringe from step 2.1.4.

2.2.2.5. Lift the brain out of the skull.

2.2.2.6. Place the brain in one of the beakers filled with ice-cold slicing solution (from step 1.1.4) bubbled with 95% O₂/5% CO₂.

2.3. Prepare slices of mouse hypothalamus

2.3.1. Block the brain for the desired brain area and cut angle (e.g., for coronal hypothalamic slices, trim off the tissue rostral to the optic chiasm and caudal to the pons using a blade and ensure the caudal block has a flat surface perpendicular to the base of the brain).

2.3.2. Using a cut piece of filter paper, pick up the brain from the anterior side and glue the posterior side to the holding plate using instant glue.

2.3.3. Quickly place the holding plate into the slicing chamber and fill the chamber with slicing solution from the second beaker in step 1.1.4.

2.3.4. Secure the slicing chamber and ice tray on the vibratome.

2.3.5. Define the slicing area (anterior and posterior to the brain) and begin slicing 250 µm coronal slices. Recommended parameters: speed 0.10 mm/s, amplitude 2 mm.

265 2.3.6. Trim the slices to the appropriate size for the desired brain area.

266
267 2.3.7. Recover the slices at 35 °C for 30-45 min. Then, remove the recovery chamber from the
268 warming bath and allow the slices to recover at room temperature for an additional 30 min.
269 Keep slices at room temperature for the rest of the day and continue to bubble the bath
270 constantly with 95% O₂/5% CO₂.

271 272 **3. Whole-cell patch clamp recording**

273 274 **3.1. Pull the patch pipettes**

275
276 3.1.1. Using the suggested parameters for the whole-cell recording from the pipette puller's
277 manual, pull patch pipettes from thick walled glass to a pipette resistance of 3-5 MΩ.

278
279 3.1.2. Using a microsyringe (commercial or homemade), fill a pipette tip with filtered internal
280 solution. To make a microsyringe, burn the tip of a 1 mL syringe and allow the tip to fall creating
281 a long fine tip.

282 283 **3.2. Obtain the whole-cell configuration**

284
285 3.2.1. Place the recording pipette just above the slice and offset pipette current in the voltage
286 clamp mode. Apply slight positive pressure to the pipette and lock the stopcock.

287
288 3.2.2. Select a healthy cell with an intact membrane and approach the cell with the pipette. The
289 positive pressure should cause a slight disturbance in the tissue (i.e. a slow wave in the tissue
290 when entering).

291
292 3.2.3. Slowly continue to bring the pipette closer to the cell using a diagonal motion until the
293 pipette forms a small dimple on the cell surface.

294
295 3.2.4. Release the positive pressure lock. The cell will begin to form a seal and the resistance
296 will increase above 1 GΩ. In voltage clamp, hold the cell at -68 mV.

297
298 3.2.5. Slightly pull away from the cell diagonally to remove excess pressure from the cell.

299
300 3.2.6. Compensate for the fast and slow pipette capacitance.

301
302 3.2.7. Apply a brief suction through the tube connected to the pipette holder to break through
303 the cell and obtain whole-cell configuration.

304
305 3.2.8. Switch to **Cell** mode on the membrane test window in an electrophysiology Data
306 acquisition and analysis software (e.g., Clampex).

3.2.9. Before each voltage clamp recording, perform a membrane test using the same software and record the relevant parameters in a lab book (membrane resistance, access resistance, and capacitance).

3.2.10. Maintain the temperature of the recording bath at 27–30 °C and the flow rate at 1.5–2.0 mL/min for subsequent experiments.

4. Multiplicity experiments

4.1. Experiment 1: estimating multiplicity using 4-AP

4.1.1. In voltage clamp, hold the cell at -68 mV. Using the same software, record the sEPSCs while perfusing the bath with low Ca^{2+} aCSF. Record for at least 5 min after the start of whole-cell configuration to ensure a stable baseline recording as the synaptic activity may be high shortly after the breakthrough of the membrane.

4.1.2. Using a micropipette, add 4-AP to the aCSF and bath apply 30 μM 4-AP. Record sEPSCs for at least 10 min to obtain the full drug effect.

4.1.3. Add 0.5 μM TTX and 10 μM Cd^{2+} to the aCSF with 4-AP and record the mEPSCs for at least 10 min.

4.1.4. For offline analysis, use the last 1 min of baseline immediately before the application of 4-AP (in low Ca^{2+} aCSF), the 10th min of 4-AP application and the 10th min of TTX application.

4.2. Experiment 2: desynchronize vesicle release using Sr^{2+}

4.2.1. While perfusing the bath with normal Ca^{2+} aCSF (the same as the bath aCSF) record sEPSCs for at least 5 min.

4.2.2. Switch from the normal Ca^{2+} aCSF and begin perfusing Sr^{2+} aCSF (from step 1.4) and record sEPSCs.

4.2.3. For offline analysis, to determine whether the large amplitude sEPSCs are due to the synchronous release of vesicles, compare the last 1 min of baseline (in normal aCSF) to the 10th minute of Sr^{2+} aCSF application.

4.3. Experiment 3: test for multivesicular release using γ -DGG

4.3.1. In low Ca^{2+} aCSF record sEPSCs for at least 5 min.

4.3.2. Add 30 μM 4-AP to the aCSF through the perfusion system. Record sEPSCs for at least 10 minutes.

4.3.3. Add 200 μ M γ -DGG to the aCSF with 4-AP and record the sEPSCs for at least 10 min.

4.3.4. As a control experiment in a separate cell, perform steps 1-3 but apply a low concentration of DNQX instead of γ -DGG.

4.3.5. For offline analysis, analyze the last minute of each drug application.

4.4. Experiment 4: Stimulate afferent inputs to increase action potential firing.

4.4.1. Record sEPSCs in normal Ca^{2+} aCSF.

4.4.2. Stimulate the afferents using a monopolar glass electrode filled with aCSF at a rate of 20 Hz for 2 s and repeat 10 times with an inter-burst interval of 20 sec.

4.4.3. For analysis, use the 5000 ms before the first stimulus as the baseline and compare to the 10-300 ms after the final stimulus and then take the average amplitude and frequency change over 10 trials.

5. Analysis

5.1. Analyze sEPSCs and mEPSCs using a program that detects and analyzes synaptic currents (e.g., Mini Analysis software).

5.1.1. Using this software, use the suggested detection parameters for detecting AMPA Receptor EPSCs (or GABA Receptor EPSCs if recording inhibitory currents).

5.1.2. Use the **Nonstop Analysis function** to detect EPSCs in the recording.

5.1.3. Manually scan each recording to ensure the program is accurately detecting each event (e.g., ensure events are not being missed or counted twice).

5.1.4. Export the event data by copying it to the clipboard and paste it into a data management software (e.g., Excel)

5.1.5. Calculate the average frequency and/or amplitude for each drug treatment and perform the relevant statistical analyses.

REPRESENTATIVE RESULTS:

The above protocol describes a method for using whole-cell patch clamp electrophysiology to examine the degree of synaptic multiplicity, using mouse hypothalamic neurons as an example. This slice preparation technique should yield healthy viable cells that do not have a swollen membrane or nucleus (**Figure 1**). Each step in the protocol is important for the health of the tissue and quality of the recordings.

[Place **Figure 1** here]

Figure 2 illustrates the rationale for identifying synaptic multiplicity using patch clamp electrophysiology. Synaptic multiplicity can result from either multiple synaptic contact between a pair of neurons (**Figure 2A left**), or by multivesicular release at a given synaptic site (**Figure 2A right**). In both of these situations, an action potential in the presynaptic neuron would elicit a large postsynaptic response (sEPSC) due to the temporal summation of multiple synaptic events. However, in the absence of presynaptic action potentials (e.g. in the presence of TTX and Cd^{2+} to block Na^{+} -dependent and Ca^{2+} -dependent action potential firing), vesicular neurotransmitter release is asynchronous. As a result, the postsynaptic response becomes smaller (**Figure 2B: AP-independent**). If two neurons do not exhibit multiplicity (i.e. have only one synaptic contact/no multivesicular release) there will be no difference in the postsynaptic response between the action potential-dependent and action potential-independent release of neurotransmitter (**Figure 2C**).

[Place **Figure 2** here]

In Experiment 1, 4-AP is applied to the bath to increase action potential firing and release probability. To ensure that 4-AP is increasing spontaneous action potential firing, the frequency of EPSCs can be compared between sEPSCs and mEPSCs (**Figure 3A, C**). Because sEPSCs are a combination of both action potential-dependent and -independent events, the difference in the frequency of sEPSC and mEPSC serves as a proxy for spontaneous action potential firing in the presynaptic axons. We use an arbitrary cut off of a $> 15\%$ difference in frequency between sEPSC to mEPSC to ensure that a sufficient number of action potential-dependent events are present for the analysis of multiplicity. If the EPSCs in the low Ca^{2+} condition and the TTX (mEPSC) condition are similar in frequency and amplitude (i.e. no spontaneous action potential firing in the low Ca^{2+} condition), the difference between the low Ca^{2+} baseline sEPSC and 4-AP can also be used for the analysis of multiplicity.

In an example result shown in **Figure 3**, 4-AP increases both the amplitude and frequency of sEPSCs. Subsequent application of TTX and Cd^{2+} decreases both the amplitude and frequency. As described above, the difference in the amplitude between sEPSCs and mEPSCs indicates synaptic multiplicity. In the hypothalamic neurons we examine here, the amplitude and frequency of the baseline and TTX conditions are the same (**Figure 3C, D**), suggesting that the baseline sEPSCs contain very few action potential-dependent EPSCs. Accordingly, subsequent experiments can compare the difference between baseline and 4-AP to measure multiplicity.

[Place **Figure 3** here]

The method described above estimates the average multiplicity of synapses terminating onto the postsynaptic neurons: it may not detect changes in multiplicity that occur to a small proportion of synapses. Nevertheless, in our recent study, this method revealed changes in the multiplicity of glutamate synapses at hypothalamic neurons between normal and chronically stressed conditions⁶.

Substitution of Ca^{2+} with equimolar Sr^{2+} in the aCSF desynchronizes action potential-dependent release of neurotransmitter vesicles^{19, 20}. Therefore, if the large amplitude sEPSCs are the summation of action potential-dependent synchronized vesicular neurotransmitter release (i.e., multiplicity), replacing Ca^{2+} with Sr^{2+} will decrease the amplitude of EPSCs. As seen in **Figure 4**, Sr^{2+} aCSF decreases the proportion of large amplitude events (**Figure 4B**), and as a consequence decreases the average amplitude (**Figure 4C**). When cells do not exhibit multiplicity, desynchronizing vesicle release will have no effect on the EPSC amplitude.

[Place **Figure 4** here]

γ -DGG, a fast dissociating competitive antagonist of AMPA receptors, can be used to determine whether multiplicity is due to the multivesicular release or multiple synaptic contacts. As multivesicular release acts on an overlapping population of postsynaptic receptors, the large amplitude EPSCs involves the pooling of glutamate in the synaptic cleft. In other words, the concentration of glutamate in the synaptic cleft is higher than that which results from unquantal release. On the other hand, multisynaptic contacts would have unquantal EPSCs at each synaptic site. If the large amplitude EPSCs arise from multivesicular release, the larger EPSCs will be less impacted by γ -DGG antagonism (due to higher glutamate concentration) compared to smaller amplitude EPSCs (**Figure 5A Right, B-D**). If the large amplitude EPSCs are due to the summation of synchronous unquantal EPSCs (multisynapse contacts), γ -DGG will similarly impact the amplitude of all EPSCs (**Figure 5A Left, E-G**). In contrast to γ -DGG, DNQX which is a high affinity, slow dissociating AMPA/kainate receptor antagonist causes a uniform decrease across all large and small amplitude EPSCs (**Figure 5H-J**). The sensitivity to γ -DGG and DNQX can be quantified as the ratio of the average EPSC amplitude divided by the maximum (the average of the largest 20 EPSCs) EPSC amplitude (**Figure 5K, L**).

[Place **Figure 5** here]

The strength of synaptic transmission can be transiently increased by bursts of synaptic activity. To investigate multiplicity under more physiological conditions, afferent stimulation can be used to increase action potential firing and release probability. If multiplicity is present, afferent stimulation should cause a brief increase in the EPSC amplitude (**Figure 6A-D**). If multiplicity is not present, activity driven increases in action potential firing will not increase the EPSC amplitude.

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Table 1. The composition of various solutions.

Figure 1. Healthy and unhealthy tissue following slice preparation. Slice electrophysiology

preparation of the PVN under differential interference contrast optics at 40x magnification. Red arrowheads indicate healthy cells, and black arrowheads indicate unhealthy cells.

Figure 2. Schematic diagram illustrating the consequences of different synaptic organizations on postsynaptic currents. A, B) In a synapse with multiplicity, an action potential and the ensuing Ca^{2+} influx triggers synchronous fusion of multiple synaptic vesicles that result in large, multiquantal EPSCs. Synaptic multiplicity can result from multisynapse contact or multivesicular release at a single synapse **(A)**. In the presence of TTX and Cd^{2+} , action potential-independent vesicle fusion is asynchronous and causes small unquantal EPSCs **(B)**. **C)** In synapses without multiplicity, both action potential-dependent and -independent vesicle fusion results in unquantal EPSCs. This figure has been modified from **Figure 1A** of our previous report⁶.

Figure 3. 4-AP application reveals synaptic multiplicity. A) Sample traces of sEPSCs recorded in low Ca^{2+} aCSF during baseline, and after 4-AP (30 μM) application, and subsequent TTX (0.5 μM) and Cd^{2+} (10 μM) application. **B)** The distribution of sEPSC amplitude from the recording shown in **(A)**. **C, D)** Summary of the mean frequency **(C)** and amplitude **(D)** between baseline (grey), 4-AP (red) and TTX + Cd^{2+} (blue). *** $P < 0.005$, ** $P < 0.01$. This figure has been modified from **Figure 2** of our previous report⁶.

Figure 4. Sr^{2+} desynchronizes large multiquantal events. A) Representative trace comparison between normal Ca^{2+} aCSF and Sr^{2+} aCSF. Sr^{2+} aCSF desynchronizes vesicle release and decreases the amplitude of multiquantal synchronous events as seen in a representative amplitude distribution **(B)** and the amplitude change for all cells **(C)** * $P < 0.05$. This figure has been modified from **Figure 3** of our previous report⁶.

Figure 5. Using γ -DGG to probe multivesicular release. A) Schematics illustrating two models of multiplicity. Left: temporal summation of unquantal transmission that targets independent populations of postsynaptic receptors. Large and small EPSCs are achieved by a similar glutamate concentration in the synaptic cleft and therefore are equally sensitive to γ -DGG. Right: multiquantal transmission that targets an overlapping population of postsynaptic receptors. Large EPSCs are caused by higher glutamate concentration in the cleft than smaller sEPSCs and are therefore less sensitive to γ -DGG. Values shown at bottom of model are hypothetical relative amplitudes. **B)** Sample traces from a recording in which there was an increase in mean sEPSC amplitude following 4-AP application (4-AP responder). **C)** cumulative plot for EPSC amplitude for the recording shown in **(B)**. **D)** Cumulative plot for normalized EPSC amplitude ($\text{EPSC}/\text{EPSC}_{\text{MAX}}$) before and after application of γ -DGG from the recording shown in **(B)**. **E)** Sample traces from a recording where there was no change in the mean sEPSC amplitude following 4-AP application (4-AP non-responder). **F)** Cumulative EPSC amplitude for the recording shown in **(E)**. **G)** Cumulative EPSC/ EPSC_{MAX} plot for the recording shown in **(E)**. **H)** Sample traces from a recording from baseline, as well as after 4-AP and DNQX application. **I)** The cumulative EPSC amplitude for the recording shown in **(H)**. **J)** Cumulative EPSC/ EPSC_{MAX} plot for the recording shown in **(H)**. **K)** Summary of mean EPSC/ EPSC_{MAX} after γ -DGG (in 4-AP responder and non-responder groups) or DNQX application normalized to pre- γ -DGG/DNQX (i.e. post-4-AP). **L)** Plots of post-4-AP mean EPSC amplitude (normalized to pre-4-AP) against post- γ -DGG/DNQX mean EPSC/ EPSC_{MAX}

(normalized to post-4-AP). *** $P < 0.005$. This figure has been modified from **Figure 4** of our previous report⁶.

Figure 6. High-frequency stimulation reveals synaptic multiplicity. **A)** Sample traces of sEPSCs before and after afferent synaptic stimulation. **B)** plot of sEPSC amplitude before and after synaptic stimulation (20 Hz, 2 s) from the recording shown in **(A)**. **C, D)** Summary of sEPSC frequency **(C)** and amplitude **(D)** changes following synaptic stimulation. *** $P < 0.001$, * $P < 0.05$. This figure has been modified from **Figure 5** of our previous report⁶.

DISCUSSION:

One important requirement for a successful patch clamp electrophysiology experiment is obtaining healthy slices/cells. Our described protocol is optimized for hypothalamic slices that contain PVN neurons. Other brain areas may require modified solutions and slicing methods²¹⁻²⁴. For the recording, it is critical to only accept stable recordings by constantly monitoring cell properties such as membrane resistance, capacitance and access resistance. An increase in access resistance can decrease EPSC amplitude and therefore confound amplitude measurements. Accordingly, cells with access resistance values that exceed 20 M Ω or increase by more than 20% during recording are discarded. Similarly, a decrease in (or a low) membrane resistance can result in poor space-clamp and, therefore, can decrease the amplitude. The neurons in our target system (parvocellular PVN neurons) have a high membrane resistance between 500 M Ω to 1 G Ω , and we discard cells with membrane resistances below 500 M Ω . Quality control cut-offs should be established for specific types of neurons under study. As this protocol relies on the difference in the amplitude before and after drug applications, it is important to ensure that the amplitude change is due to the drug application and not to the changes in membrane resistance and access resistance. The hypothalamic neurons we study in this protocol are small in size (cell capacitance is about 15 pF in mice and membrane resistance is around 1 G Ω), and K-gluconate based internal solution works well to obtain high quality EPSCs/IPSCs^{6,25}. For neurons with larger cell size and low input resistance, cesium based internal solution can be used to ensure a good space-clamp².

One specific requirement of using this method to measure the multiplicity is that the cells fire a sufficient number of spontaneous action potentials in order to compare action potential-dependent events to mEPSCs. This can be ensured by comparing the difference in the frequency of EPSCs in the absence and presence of TTX and Cd²⁺. In hypothalamic slices that contain the PVN, we have found that the application of 4-AP is efficient to elicit action potential firing. Another method, pioneered by Hsia and colleagues², uses high Ca²⁺ aCSF to increase action potential firing, rather than 4-AP. While this method was successful in hippocampal slices, we found that the high Ca²⁺ was less efficient than 4-AP in facilitating action potential firing in hypothalamic slices⁶. Indeed, it has been shown that high, extracellular Ca²⁺ concentration decreases intrinsic excitability of neurons and axons by altering Na⁺ conductance^{26, 27}. This may explain why in some slices, high Ca²⁺ aCSF is not effective in increasing EPSC frequency.

One limitation of this method, which is inherent to all slice patch clamp electrophysiology, is that many, long-range projections to the postsynaptic neurons are cut in slice preparations. In order

to observe action potentials, it is likely that the presynaptic axons and cell bodies need to be preserved in the slice. Therefore, the multiplicity measurement is skewed to synaptic connectivity that is preserved within the slice. Along the same line, the direction of slicing may cause certain populations of projections to be preserved while others are severed²⁸. These limitations have a general effect of underestimating the multiplicity of afferent inputs.

The described protocol provides a method for estimating the degree of synaptic multiplicity across all inputs to a given neuron. Other electrophysiology techniques, such as paired recordings or minimal stimulation of a single axon can identify whether a given connection has multiple contacts, but these experiments are often difficult and not possible in all systems. Further, they cannot give an overall indication of the organization of all of the inputs to a given neuron as they only isolate one pair of neurons. The present protocol uses basic patch-clamp electrophysiology methods to evaluate the degree of multiplicity across all inputs to a given neuron.

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DISCLOSURES:

The authors have nothing to disclose

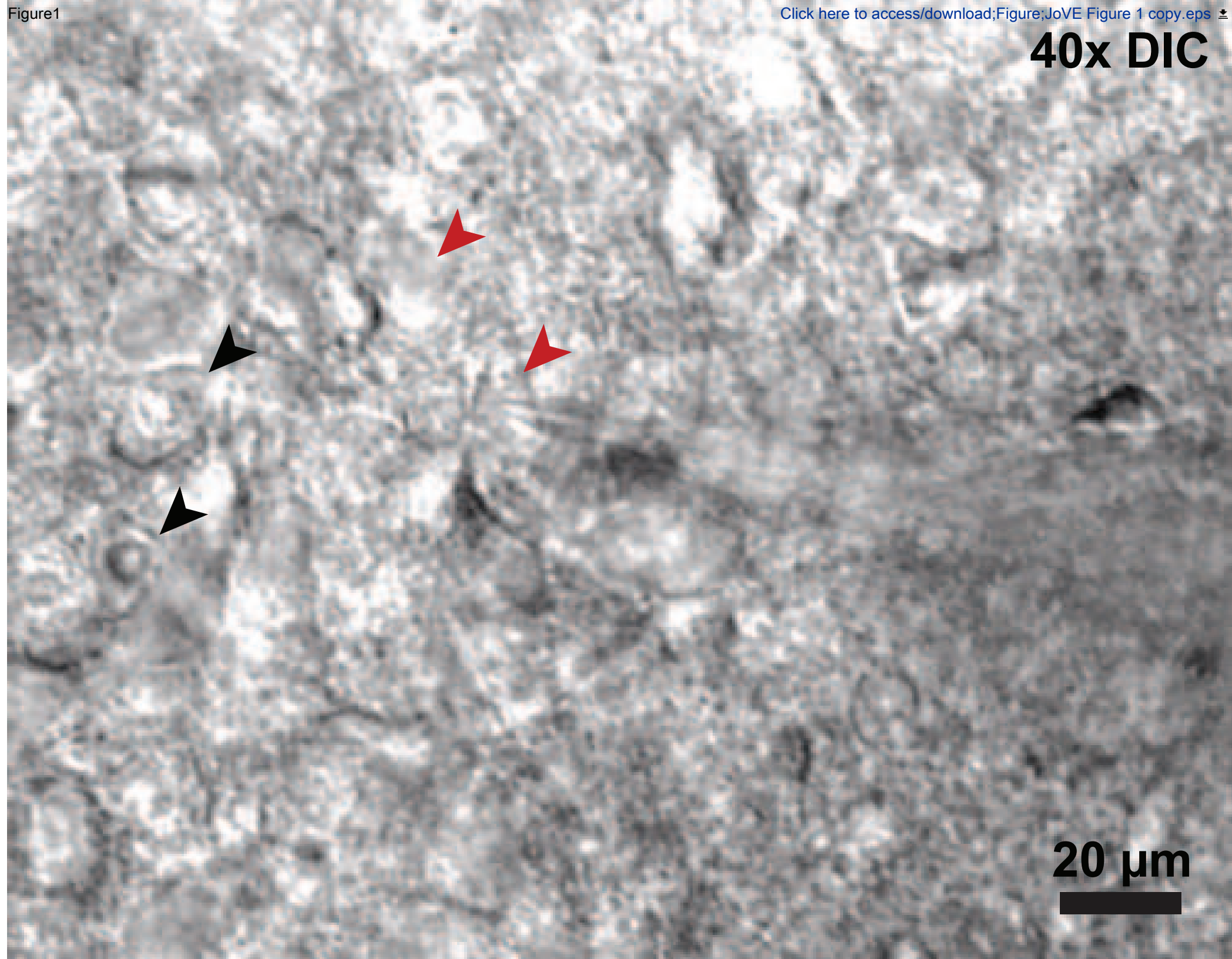
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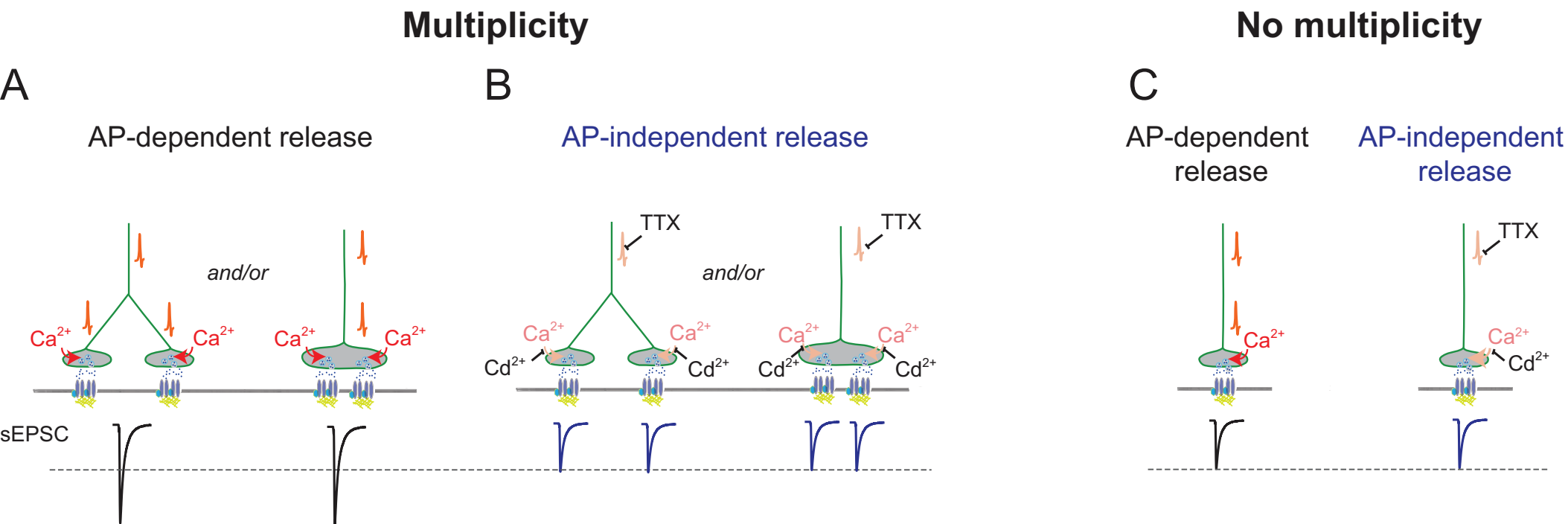
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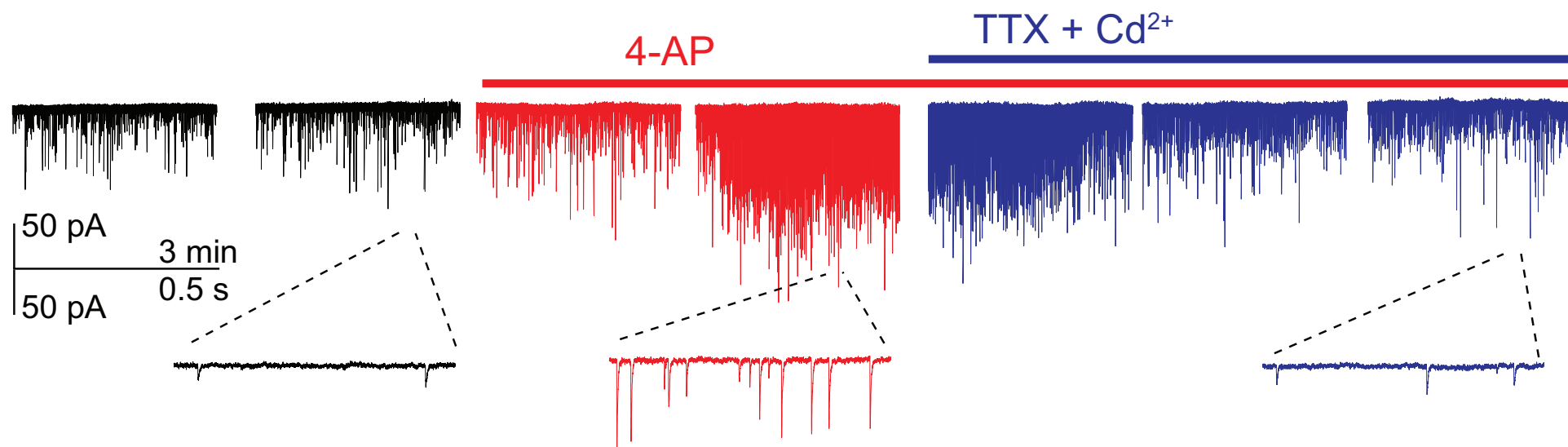


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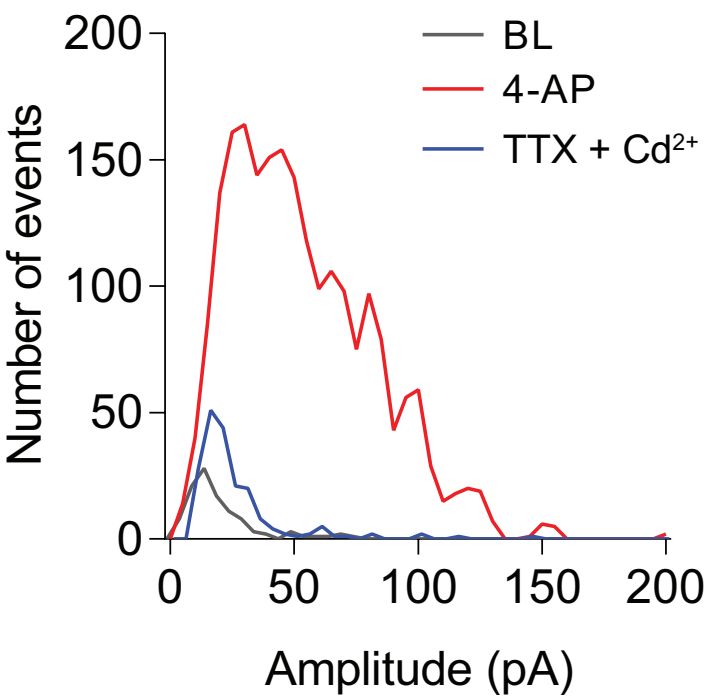




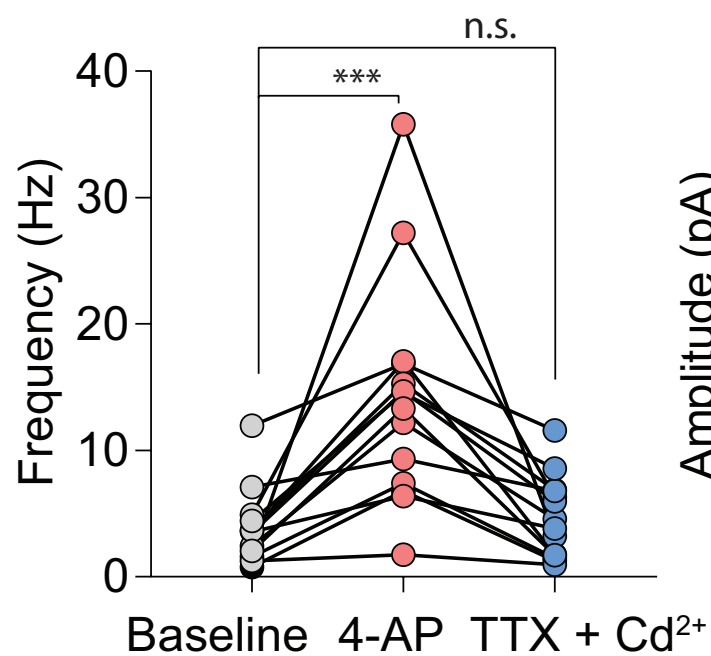
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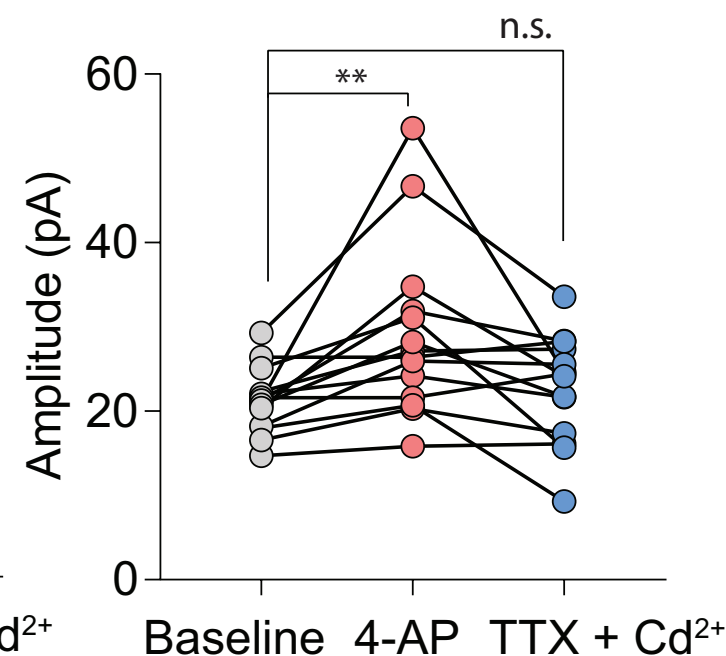
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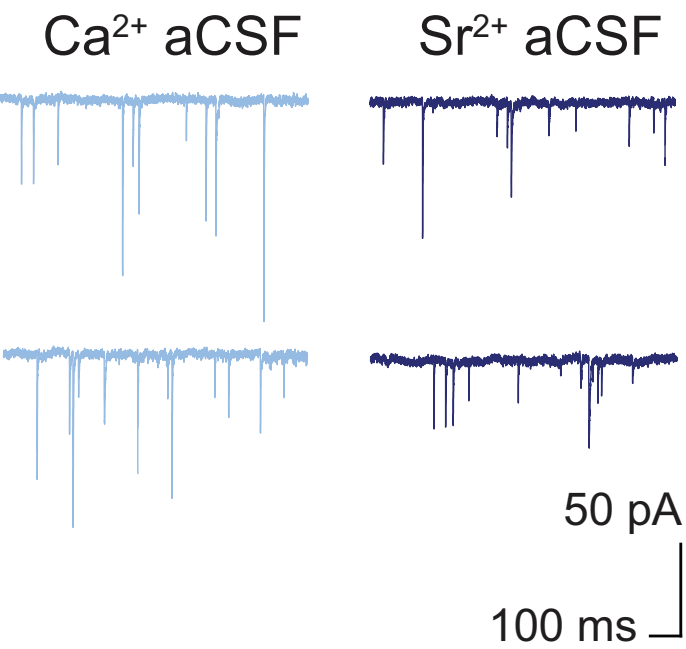
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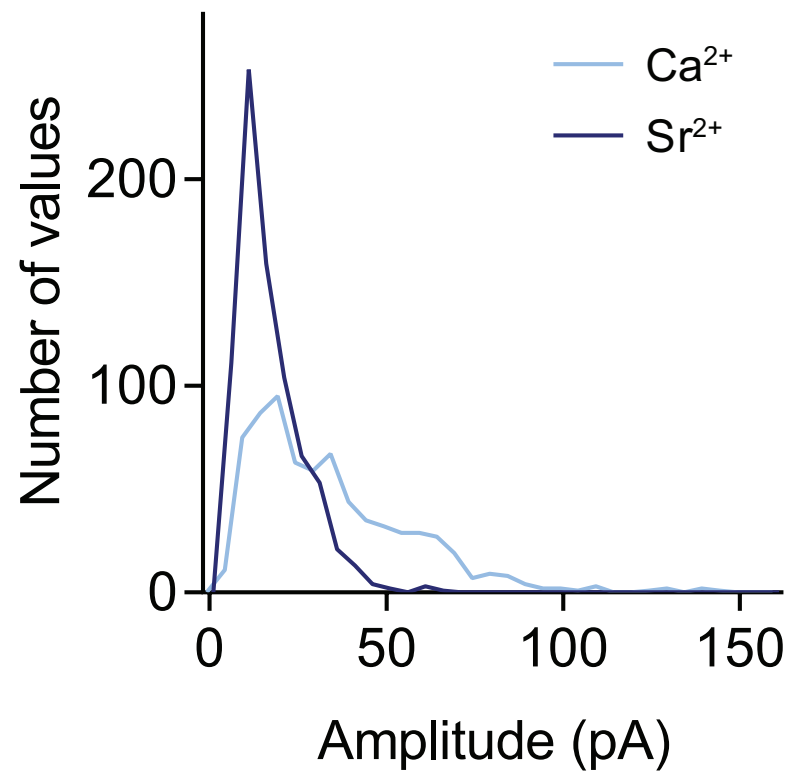
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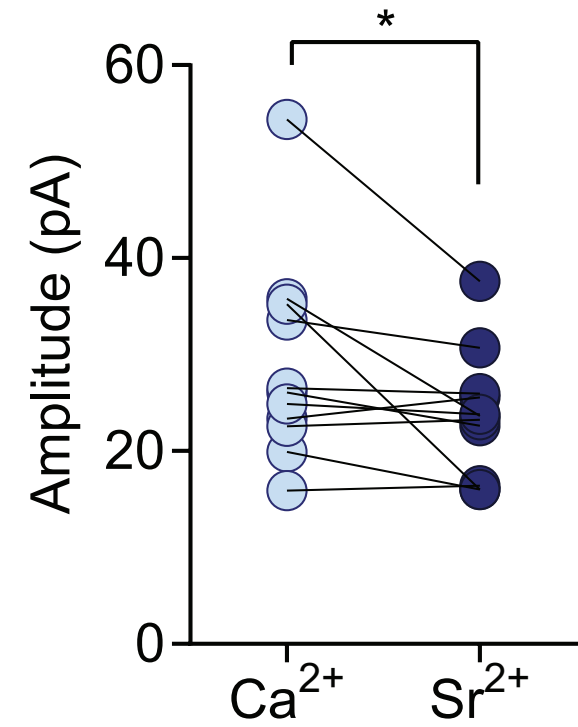
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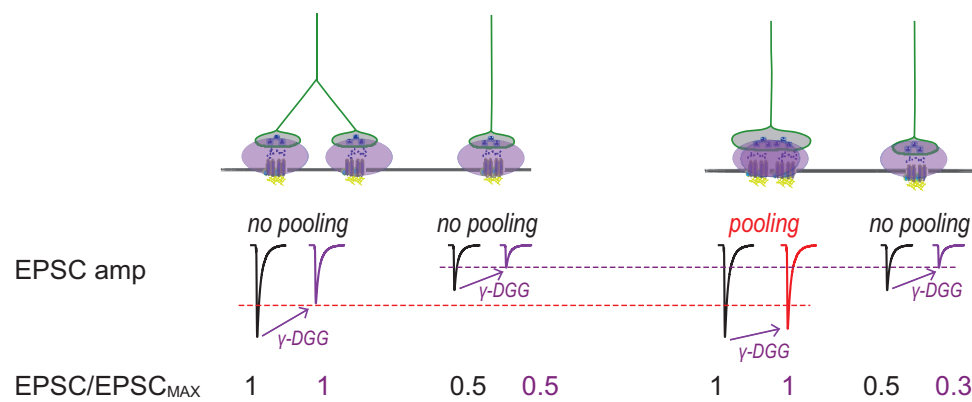


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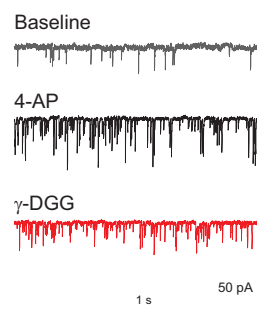


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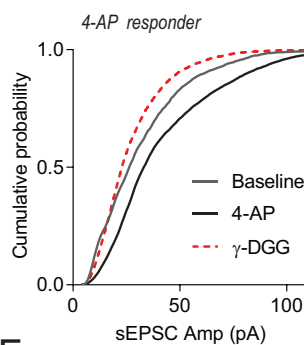




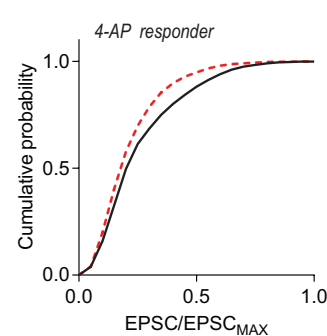
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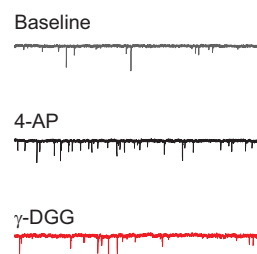
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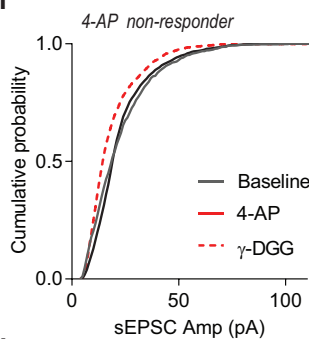
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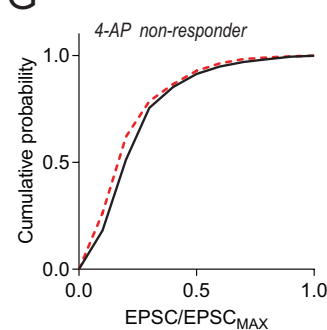
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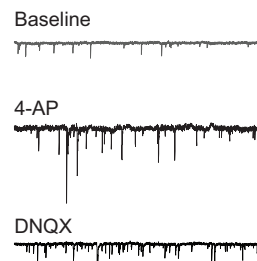
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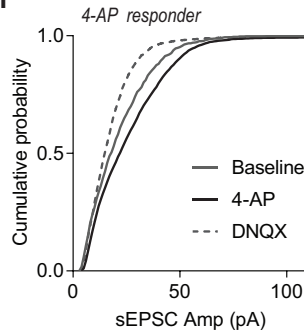
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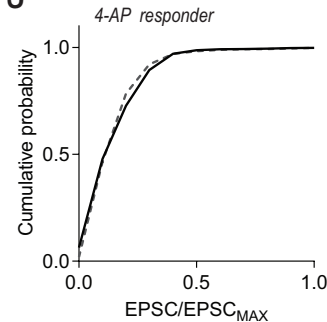
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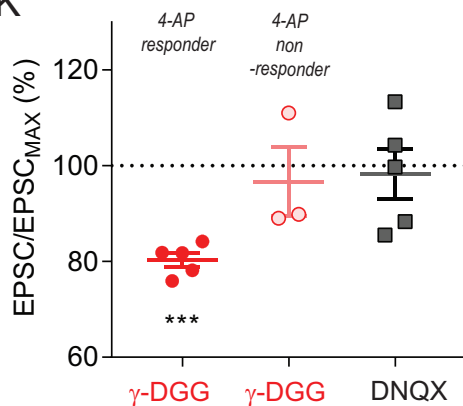
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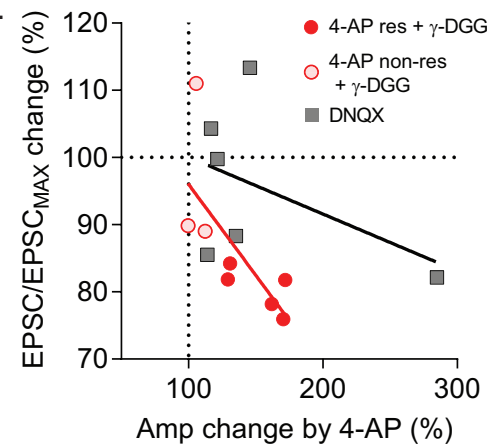
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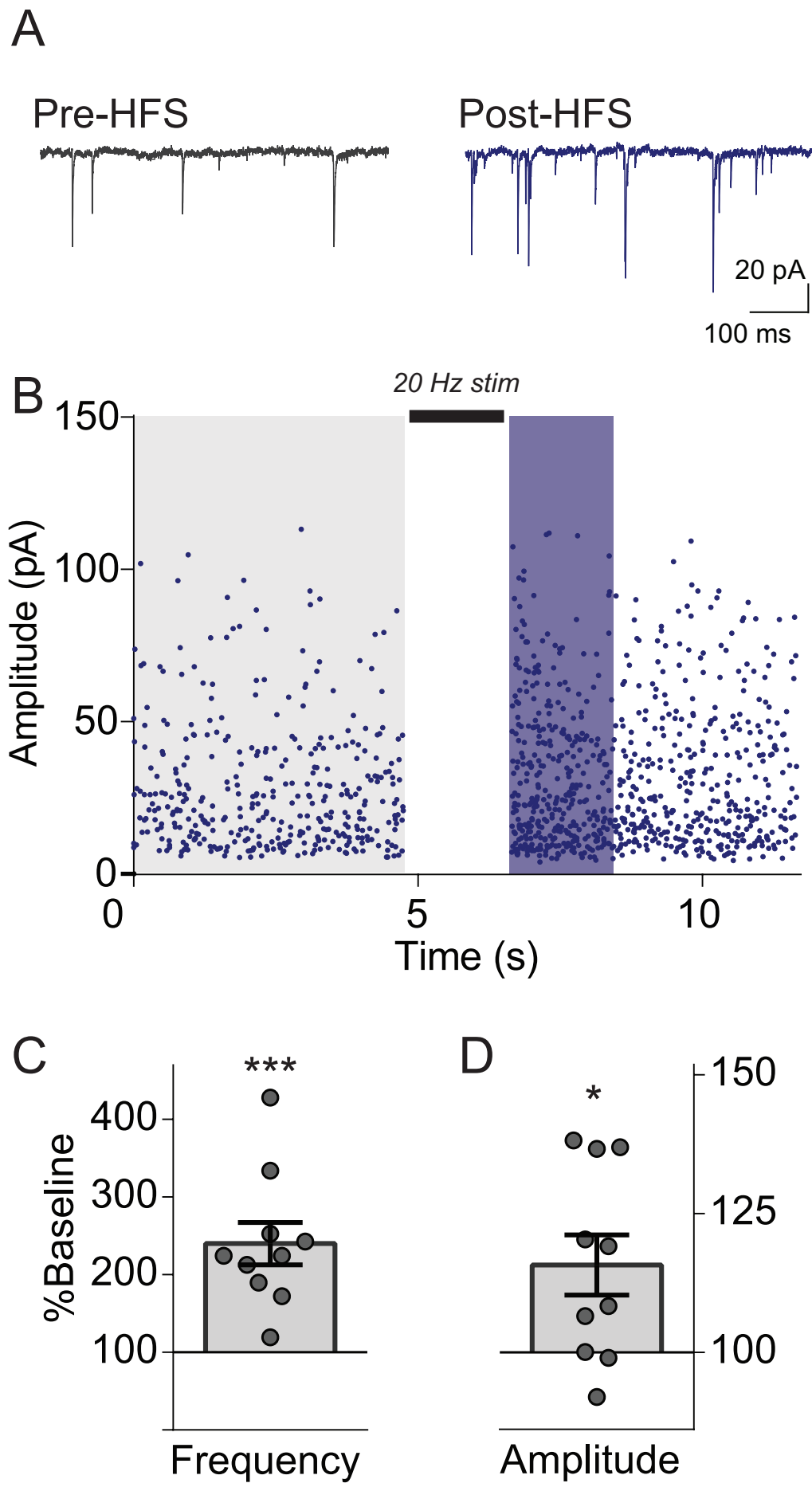


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	Solution Concentrations (mM)				
	Slicing	Normal aCSF	Low Ca ²⁺ aCSF	Sr ⁺ aCSF	Pipette/Internal
NaCl	87	126	126	126	-
KCl	2.5	2.5	2.5	2.5	8
CaCl ₂	0.5	2.5	0.5	-	-
SrCl ₂	-	-	-	2.5	-
MgCl ₂	7	1.5	2.5	1.5	2
NaH ₂ PO ₄	1.25	1.25	1.25	1.25	-
NaHCO ₃	25	26	26	26	-
Glucose	25	10	10	10	-
Sucrose	75	-	-	-	-
K-gluconate	-	-	-	-	116
Na-gluconate	-	-	-	-	12
HEPES	-	-	-	-	10
K2-EGTA	-	-	-	-	1
K2ATP	-	-	-	-	4
Na ₃ GTP	-	-	-	-	0.3

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 ml syringe	BD	309659	
10 blade	Fisher Scientific/others	35698	
22 blade	VWR/others	21909-626	
22 uM syringe filters	Milipore	09-719-000	
Adson forceps	Harvard Instruments	72-8547	
Angled sharp scissors	Harvard Instruments	72-8437	
Clampex	Molecular Devices	pClamp 10	
Double edge blade	VWR	74-0002	
Filter paper	Sigma/others	1001090	
Fine paintbrush	Fisher/various	15-183-35/various	
Gas Dispersion Tube	VWR	LG-8680-120	
Isoflurane	Fresenius Kabi/others	M60303	
Krazy glue	various	various	
Mini analysis	Synaptosoft	MiniAnalysis 6	
Osmomoter	Wescor Inc	Model 5600	
Parafilm	Sigma	PM-996	
Pasteur pipette	VWR	14672-200	
ph meter	Mettler Toledo	FE20-ATC	
Rubber bulb	VWR	82024-550	
Scalpel handle No. 3	Harvard Instruments	72-8350	
Scalpel handle No. 4	Harvard Instruments	72-8356	
Single edge blade	VWR	55411-050	
Vibratome slicer	Leica	VT1200S	
Water Purification System	Millipore	Milli-Q Academic A10	
Well plate lid	Fisher/various	07-201-590/various	
Chemicals/reagents			
4-AP	Sigma	275875	
BAPTA	molecular probes	B1204	
CaCl ₂ *2H ₂ O	Sigma	C7902	
CdCl ₂	sigma	202908	

DNQX	Tocris	189
EGTA	Sigma	E3889
glucose	Sigma	G5767
HEPES	Sigma	H3375
K2-ATP	Sigma	A8937
KCl	Sigma	P9333
K-gluconate	Sigma	G4500
MgCl2*6H2O	Sigma	M2670
Molecular biology grade water	Sigma	W4502-1L
Na3GTP	Sigma	G8877
NaCl	Bioshop	SOD001.1
Na-gluconate	Sigma	S2054
NaH2PO4	Sigma	71504
NaHCO3	Sigma	S6014
Picrotoxin	sigma	P1675
SrCl	Sigma	255521
sucrose	Bioshop	SUC507.1
TTX	Alamone Labs	T-550
γDGG	Tocris	6729-55-1



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Signature:		Date:	November 29, 2018

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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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please define all abbreviations before use.

We have defined the following abbreviations that were previously missed:

Line 115 γ -D-glutamylglycine (γ -DGG)

Line 405 differential interference contrast optics (abbreviation removed)

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

Changed to (line 27-28):

"Here, we present a protocol for evaluating functional synaptic multiplicity using whole-cell patch clamp electrophysiology in acute brain slices"

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. Please leave a single line space between each numbered step and substep of the protocol.

Done

5. 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.

The protocol has been updated according to points 5, 6 and 7. These changes are highlighted in grey in the protocol of the manuscript.

6. The Protocol should contain only action items that direct the reader to do something.

The protocol has been updated according to points 5, 6 and 7. These changes are highlighted in grey in the protocol of the manuscript.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

The protocol has been updated according to points 5, 6 and 7. These changes are highlighted in grey in the protocol of the manuscript.

8. Line 198: How do you prepare the dissection tool?

This point has been changed to:

"2.1.3. Prepare for dissection by setting up the tools (scalpel, angled fine scissors, forceps, fine paint brush, plastic spoon)."

9. Line 209: Please provide the concentration/percentage of the isoflurane used. Age, sex, strain specific bias if any. Please mention.

This point has been changed to:

“2.2.1. Anesthetize the animal in a chamber saturated with 4% isoflurane until spinal reflexes are absent.”

There are no age, sex, strain specific biases to report.

10. Line 221: Please mention the composition of the slicing solution? If mentioned before, please provide the step number here for clarity.

This point has been changed to:

“2.2.2.6. Place the brain in one of the beakers filled with ice-cold slicing solution (from step 1.1.4) bubbled with 95% O₂/5% CO₂.”

11. Line 224: How do you block the brain? What are the desired brain areas?

This has been changed to:

“2.3.1. Block the brain for the desired brain areas and cut angle (e.g. for coronal hypothalamic slices, trim off the tissue rostral to the optic chiasm and caudal to the pons using a blade and ensure the caudal block has a flat surface perpendicular to the base of the brain).”

The desired brain area will depend on the experimenter's area of interest.

12. Please ensure that the highlight is no more than 2.75 pages in length including heading and spacings.

The protocol highlight is less than 2 pages.

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14. Please alphabetically sort the table of materials.

Done

Reviewers' comments:**Reviewer #1:****Manuscript Summary:**

This manuscript describes a simple and direct in vitro protocol using patch-clamp electrophysiology in acute brain slices for estimating - synaptic multiplicity - a parameter reflecting the number of functional synaptic contacts onto a given neuron. The authors present specific experimental steps from composition of solutions to slicing and patch-clamp in their experimental preparation; to the rational of using voltage-clamp recordings comparing spontaneous and miniature excitatory and postsynaptic currents (sEPSPs / mEPSPs) in a set of four experimental conditions aimed at estimating synaptic multiplicity.

Major Concerns:

N/A

Minor Concerns:

Line 155, 164 and 172: In this section, it seems as if CaCl_2 and MgCl_2 are included in the stock solution for normal aCSF, but they are kept separate from the low Ca^{2+} aCSF and Sr^{2+} aCSF and added only when preparing the 1X solution. Please clarify if this is the case.

Yes, this is the case. For our normal calcium aCSF, CaCl_2 and MgCl_2 are included in the 20x stock solution. However, we prepare the modified calcium aCSF solutions from a calcium/magnesium-free stock and adjust the concentrations accordingly using 1M CaCl_2 and MgCl_2 solutions. This should not change the composition of the solution but allows for easier manipulation of calcium concentration (for example we can use the same stock for high calcium experiments). We thank the reviewer for pointing this out, and we have clarified this in the revised manuscript (line 160, 167).

Line 161 & 169: in phrase "the stock can be store for one month", store should be corrected for stored

Thank you, this has been corrected.

Line 274: It would be interesting to know the temperature and perfusion speed of recording medium in the recording chamber.

We agree that this is important information for this protocol. We have added point 3.2.10 as follows:

"3.2.10. Maintain the temperature of the recording bath at 27–30°C and the flow rate at 1.5–2.0 mL/min for subsequent experiments."

Line 294: Insert a space between using and γ -DGG

This has been corrected.

Line 381: ...that "which" results... would make a more complete sentence.

This has been corrected.

Line 434: *** $P < 0.001$ can be removed from legend since it is not found anywhere in Fig 4.

This has been corrected.

In Table of Material (first column): correct "Isoflurane"

Done

In Table of Material (2nd page): "Pasteur" pipette

Done

Reviewer #2:

Manuscript Summary:

This is an extremely interesting manuscript which provides a methodology which attempts to quantify synaptic 'multiplicity' by comparing spontaneous and miniature EPSCs under different experimental condition. It should provoke some interesting discussions on synaptic mechanisms. The methodology provides a simple probe which covers a number of other potential mechanisms, such as multivesicular release.

Major Concerns:

None

Minor Concerns:

My only query is how the experiments cover conditions where there is loose coupling between adjacent release sites such as via diffusion of Ca^{2+} .

Our protocol relies on the temporal summation of EPSCs in order to estimate the existence of synaptic multiplicity. In other words, if two asynchronous events (due to Ca^{2+} diffusion) temporally overlap and it increases the peak amplitude above their individual peaks, it contributes to the readout of multiplicity. If asynchronous events do not change the peak amplitude, they do not affect multiplicity. Thus, our protocol does not offer a means to tease out the specific contributions of Ca^{2+} diffusion to adjacent release sites and resulting asynchronous release. This type of question can be more precisely addressed by using different types of Ca^{2+} chelators (EGTA and BAPTA) in paired patch clamp recording to infuse the drugs into the presynaptic terminal¹. Such sophisticated experiments are beyond the scope of our protocol aimed to describe simple and relatively easy experiments to estimate synaptic multiplicity.

Reviewer #3:

Manuscript Summary:

This is very useful protocol. Synaptic multicity is a very difficult phenomenon to assess, and

patch clamp technique remains a very useful technique to evaluate fine changes in neuronal function.

This technical article is very welcome, well written, clear, and easy to follow.

While I have no major concerns, I would like to see a couple of points explained further.

Major Concerns:

None

Minor Concerns:

1. Page 4 (solutions/step 5): the authors use K-gluconate based internal solution. This is a bit surprising; to ensure a relatively good space-clamp, cesium-based (in contrast to potassium) internal solution are usually preferred. Please, explain why K-gluconate has been chosen, and whether Cs-based solution could be used to assess synaptic multiplicity.

We agree that cesium-based internal solution is more commonly used to achieve relatively better space-clamp. While we use K-gluconate based internal solution for the reasons specified below, cesium-based internal solution can be used to assess synaptic multiplicity². In the revised manuscript, we describe that cesium-based internal solution is compatible with the current method (line 475).

Our lab normally use K-gluconate based internal for two main reasons. First, we routinely characterize firing patterns of neurons under current clamp for cell-type characterization before voltage-clamp recording. K-gluconate based internal solution is required for this purpose. Second, the hypothalamic cells we study are small (capacitance near 15 pF for mice) and with high input-resistance (0.5-1 GΩ)^{3,4}. K-gluconate based internal solution is commonly used to record EPSCs³⁻⁵ and IPSCs^{3,6,7} in the PVN.

2. Page 10/discussion: authors say they "only accept stable recordings by constantly monitoring cell properties such as membrane resistance, capacitance and access resistance". Please, indicate from what percent change you decide aborting recordings or discarding the cell.

We thank the reviewer for the suggestion to include further information/clarification on these parameters. To address both point #2 and #3 the discussion on lines 465-475 have been added. The sentences that specifically address the reviewers 2nd point are highlighted below:

“One important requirement for a successful patch clamp electrophysiology experiment is obtaining healthy slices/cells. Our described protocol is optimized for hypothalamic slices that contain PVN neurons. Other brain areas may require modified solutions and slicing methods 21–24. For the recording, it is critical to only accept stable recordings by constantly monitoring cell properties such as membrane resistance, capacitance and access resistance. An increase in access resistance can decrease EPSC amplitude and therefore confound amplitude measurements. Accordingly, cells with access resistance values that exceed 20 MΩ or increase by more than 20% during recording are discarded. Similarly, a decrease in (or a low) membrane resistance can result in poor space-clamp and, therefore, can decrease the amplitude. The neurons in our target system (parvocellular PVN neurons) have a high membrane resistance between 500 MΩ to 1 GΩ, and we discard cells with membrane resistances below 500 MΩ. Quality control cut-offs

should be established for specific types of neurons under study. As this protocol relies on the difference in the amplitude before and after drug applications, it is important to ensure that the amplitude change is due to the drug application and not to the changes in membrane resistance and access resistance.

3. Related to the point #2, could you please give examples of why these parameters, particularly input and access resistance, must be monitored rigorously. For example, and as it has already been implied, a significant increase in access resistance leads to a decrease in EPSC amplitude, thereby leading to misinterpretation of data.

To address point #3 the following highlighted sentences have been added:

“One important requirement for a successful patch clamp electrophysiology experiment is obtaining healthy slices/cells. Our described protocol is optimized for hypothalamic slices that contain PVN neurons. Other brain areas may require modified solutions and slicing methods 21–24. For the recording, it is critical to only accept stable recordings by constantly monitoring cell properties such as membrane resistance, capacitance and access resistance. An increase in access resistance can decrease EPSC amplitude and therefore confound amplitude measurements. Accordingly, cells with access resistance values that exceed 20 MΩ or increase by more than 20% during recording should be discarded. Similarly, a decrease in (or a low) membrane resistance can result in poor space clamp and therefore decrease the amplitude. The neurons in our target system (parvocellular PVN neurons) have a high membrane resistance between 500 MΩ to 1 GΩ, and we discard cells with membrane resistances below 500 MΩ. Quality control cut-offs should be established for specific types of neurons under study. As this protocol relies on the difference in the amplitude before and after drug applications, it is important to ensure that the amplitude change is due to the drug application and not to the changes in membrane resistance and access resistance.

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1. Wang, L.-Y., Augustine, G.J. Presynaptic nanodomains: a tale of two synapses. *Frontiers in Cellular Neuroscience*. **8**, 455, doi: 10.3389/fncel.2014.00455 (2015).
2. Hsia, A.Y., Malenka, R.C., Nicoll, R.A. Development of Excitatory Circuitry in the Hippocampus. *Journal of Neurophysiology*. **79** (4), 2013–2024, doi: 10.1152/jn.1998.79.4.2013 (1998).
3. Gunn, B.G. *et al.* Dysfunctional astrocytic and synaptic regulation of hypothalamic glutamatergic transmission in a mouse model of early-life adversity: relevance to neurosteroids and programming of the stress response. *Journal of Neuroscience*. **33** (50), 19534–19554, doi: 10.1523/JNEUROSCI.1337-13.2013 (2013).
4. Salter, E.W., Sunstrum, J.K., Matovic, S., Inoue, W. Chronic stress dampens excitatory synaptic gain in the paraventricular nucleus of the hypothalamus. *The Journal of Physiology*. **596** (17), 4157–4172, doi: 10.1113/JP275669 (2018).
5. Kuzmiski, J.B., Marty, V., Baimoukhametova, D. V., Bains, J.S. Stress-induced priming

- of glutamate synapses unmasks associative short-term plasticity. *Nature Neuroscience*. **13** (10), 1257–1264, doi: 10.1038/nn.2629 (2010).
6. Wamsteeker Cusulin, J., Füzesi, T., Inoue, W., Bains, J. Glucocorticoid feedback uncovers retrograde opioid signaling at hypothalamic synapses. *Nature Neuroscience*. **16** (5), 596–604, doi: 10.1038/nn.3374 (2013).
 7. Inoue, W. *et al.* Noradrenaline is a stress-associated metaplastic signal at GABA synapses. *Nature Neuroscience*. **16** (5), 605–612, doi: 10.1038/nn.3373 (2013).

Editorial Comments

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

2. Please address all the specific comments marked in the manuscript.

Line 31: Needs more clarity

Changed to: “Synaptic multiplicity is plastic **and changes** throughout development”

We were unsure whether this referred to the use of the word “plastic” which is commonly used in the field to refer to changes in synaptic activity. We have added “and changes” to clarify this idea.

Lines 49-51: Citation?

We have added a citation for the following review on synaptic plasticity: (Abbot and Nelson, 2000)

Line 250: Maybe this can be highlighted for the cohesive story.

Explaining all of the preparation steps would form a more cohesive story, but our slice preparation protocol is a generic one: there are JoVe papers covering this part. Considering that the focus of our manuscript is patch clamp experiments to study multiplicity, we did not include this part in the highlight.

Line 257: Krazy glue is trademarked. Please use generic term and move this to the table of materials.

We have changed this to “instant glue”.

Line 259: Fill it with the slicing solution in the other beaker? Or fill with the other beaker of slicing solution?

We have changed this to: “Quickly place the holding plate into the slicing chamber and **fill the chamber with slicing solution from the second beaker in step 1.1.4.**”

Line 317: How?

We have changed this to: “Before each voltage clamp recording, perform a membrane test **using the Clampex software** and record the relevant parameters **in a lab book** (membrane resistance, access resistance, and capacitance).”

Line 329: What volume? Record how?

For this part the volume is not important because the bath is being continually perfused with the solution at the flow rate described in 3.2.10. We have changed this to the following to reflect that more clearly:

“Using the Clampex software, record the sEPSCs **while perfusing the bath with** low Ca^{2+} aCSF.”

Line 344: What volume? How do you record?

For this part the volume is not important because the bath is being continually perfused with the solution. We have changed this to the following to reflect that more clearly:

“While perfusing the bath with normal Ca^{2+} aCSF (the same as the bath aCSF) record sEPSCs for at least 5 min.”

To avoid repetition we have not repeated the recording program each time we instruct to record EPSCs. All are recorded using the Clampex software in voltage clamp as explained in point 4.1.1.

Line 390: What is this. Please explain how to do the step. Please provide all the button clicks, Graphical user interface, scripting (scripting steps can be uploaded as a supplemental file)

We have added multiple steps to clarify the analysis of EPSCs using the MiniAnalysis program as follows:

“5.1 Analyze sEPSCs and mEPSCs using a program that analyzes synaptic currents (e.g., Mini Analysis).

5.1.1 .In Mini Analysis, use the suggested detection parameters for detecting AMPA Receptor EPSCs (or GABA Receptor EPSCs if recording inhibitory currents).

5.1.2. Use the Nonstop Analysis function to detect EPSCs in the recording.

5.1.3. Manually scan each recording to ensure the program is accurately detecting each event (e.g., ensure events are not being missed or counted twice).

5.1.4. Export the event data by copying it to the clipboard and paste it into a data management software (e.g., Excel)

5.1.5. Calculate the average frequency and/or amplitude for each drug treatment and perform statistical analyses.”

Line 395: How do you visually identify this?

We have added the following example of what to look for:

“5.1.3. Manually scan each recording to ensure the program is accurately detecting each event (e.g., ensure events are not being missed or counted twice).”

3. For the protocol section, Please ensure you answer the “how” question, i.e., how is the step performed?

Please see the tracked changes in the protocol section of the manuscript for updates that further clarify how steps are performed.

4. Once all the changes are done, Please ensure that the highlight is no more than 2.75 pages including headings and spacings.

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