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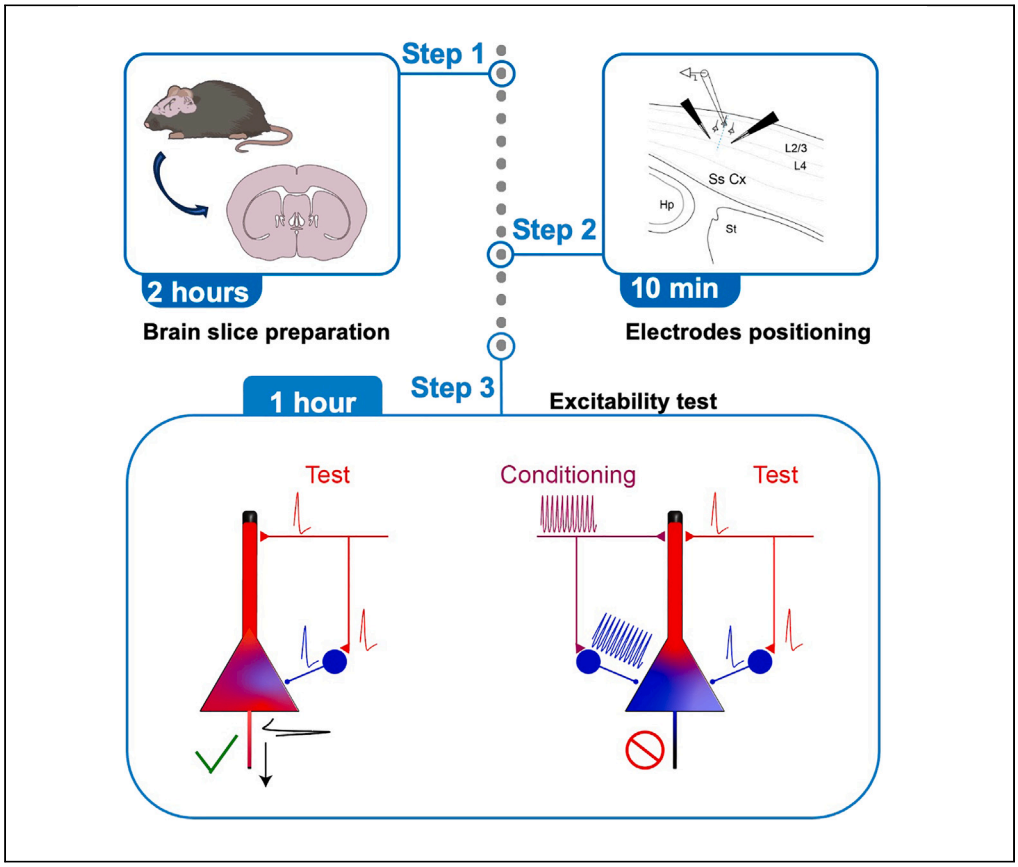
Protocol for quantifying pyramidal neuron hyperexcitability in a mouse model of neurodevelopmental encephalopathy



Here, we present a protocol for quantifying pyramidal neuron hyperexcitability in a mouse model of STXBP1 neurodevelopmental encephalopathy (*Stxbp1*^{hap}). We describe steps for preparing brain slices, positioning electrodes, and performing an excitability test to investigate microcircuit failures. This protocol is based on recording layer 2/3 cortical pyramidal neurons in response to stimulation of two independent sets of excitatory axons that recruit feedforward inhibition microcircuits.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Slice preparation for the brain of a mouse model of STXBP1 encephalopathy

Step-by-step protocol for stimulating cortical feedforward inhibition microcircuits

Hyperexcitability quantification of principal neurons from *Stxbp1*^{hap} animals

List of parameters to tune the protocol to different models

dos Santos et al., STAR Protocols 5, 102954
June 21, 2024
https://doi.org/10.1016/j.xpro.2024.102954

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Protocol for quantifying pyramidal neuron hyperexcitability in a mouse model of neurodevelopmental encephalopathy

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SUMMARY

Here, we present a protocol for quantifying pyramidal neuron hyperexcitability in a mouse model of STXBP1 neurodevelopmental encephalopathy (*Stxbp1*^{hap}). We describe steps for preparing brain slices, positioning electrodes, and performing an excitability test to investigate microcircuit failures. This protocol is based on recording layer 2/3 cortical pyramidal neurons in response to stimulation of two independent sets of excitatory axons that recruit feedforward inhibition microcircuits.

For complete details on the use and execution of this protocol, please refer to Dos Santos et al.[1]

BEFORE YOU BEGIN

Institutional permissions

All the experiments were performed in accordance with the relevant institutional and national guidelines and regulations, which might differ between institutes and countries. Note that some authorization might be required and approved before the work starts. All procedures performed here were conducted in mice and were carried out according to Danish animal welfare legislation, and breeding of mice was approved by the Animal Experiments Inspectorate (2018-15-0202-00157 and 2023-15-0202-00197).

For this protocol mice (P18 – 23) of both sexes were used. Heterozygous C57BL/6 *Stxbp1*mice (*Stxbp1*$^{+/-}$) were crossed with wild type C57BL/6 Stxbp1$^{+/+}$ mice to obtain *Stxbp1*$^{+/-}$ animals and control *Stxbp1*$^{+/+}$ littermates. Here, we refer to these mice as *Stxbp1*^{hap} (*hap* for haploinsufficiency) and *Stxbp*^{wt} (*wt* for wild type). The *Stxbp1*^{hap} mouse line was described before.[2,3] All animals were PCR genotyped before and after experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Alexa Fluor 488 hydrazide	Sigma-Aldrich; Catalog number: A10436	https://www.thermofisher.com
Alexa Fluor 594 hydrazide	Sigma-Aldrich; Catalog number: A10438	https://www.thermofisher.com

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
C57BL6 *Stxbp1*$^{+/-}$	Described in Verhage et al.[2] Same original line as RRID:IMSR_JAX:006381	N/A
Software and algorithms		
Clampfit 10.7	Molecular Devices	https://support.moleculardevices.com
Inkscape	Inkscape	https://inkscape.org/
Prism	GraphPad Software	https://www.graphpad.com/

MATERIALS AND EQUIPMENT

Preparation of solutions

Timing: 2 h

Slicing solution (1 L)		
Reagent	Final concentration (mM)	Amount
NaCl	111	6.487 g
$NaHCO_3$	25	2.1 g
NaH_2PO_4	1.25	0.15 g
KCl	3	0.224 g
$CaCl_2$ (1 M)	0.5	0.5 mL
Glucose monohydrate	11	2.180

Note: The slicing solution should be saturated with carbogen (5% CO_2) for at least 15 min before use and then be cooled for 30 min in a −20°C Freezer; all compounds are available at Sigma-Aldrich.

Artificial cerebrospinal fluid (aCSF) (1 L)		
Reagent	Final concentration (mM)	Amount
NaCl	111	6.487 g
$NaHCO_3$	25	2.1 g
NaH_2PO_4	1.25	0.15 g
KCl	3	0.224 g
$CaCl_2$ (1 M)	2.5	2.5 mL
$MgCl_2$ (1 M)	1.3	1.3 mL
Glucose monohydrate	11	2.18 g

Note: aCSF should be saturated with carbogen and heated to 28°C; all compounds are available at Sigma-Aldrich.

Internal pipette solution (20 mL)		
Reagent	Final concentration (mM)	Amount
K-gluconate	122	571 mg
Na_2ATP	5	55.51 mg
$MgCl_2$ (1 M)	2.5	50 µL
$CaCl_2$ (1 µM)	0.0003	6 µL
Mg-gluconate	5.6	45.3 mg
K-HEPES	5	27.7 mg
HEPES	5	23.8 mg
EGTA	1	7.6 mg
Alexa Fluor 488 hydrazide	0.1	1.1 mg

Note: The pH of the internal pipette solution should be adjusted to 7.2 by adding KOH. The osmolarity needs to be verified to ensure it falls within the range of 280–290 mOsm. All compounds are available at Sigma-Aldrich, except Alexa Fluor 488 hydrazide, from Invitrogen.

STEP-BY-STEP METHOD DETAILS

Brain slices preparation

Timing: 2 h

The quality of brain slices determines the success of the protocol. Therefore, rapid slicing is essential for minimizing tissue damage, preserving cellular integrity, and maximizing the viability of the brain tissue. The time between decapitation and cutting the first slice should not exceed 2 min.

1. Decapitate the mouse and extract the brain by means of a spatula. Put it in ice-cold slicing solution.
2. Trim the brain to exclude the cerebellum with a razor blade.
3. Cut the brain in two halves along the midline of the sagittal plan with a razor blade.
4. Glue the sagittal side of one hemisphere on a vibratome stage with cyanoacrylate. Cover the hemisphere with carbogenated slicing solution.
5. Cut 300 μm thick slices from rostral to caudal by means of a vibratome. We recommend an amplitude of 1.0 mm and a blade travel speed of 0.2 mm/s.
6. Collect cortical brain slices containing the somatosensory cortex (or other region of interest).
7. Transfer slices to an interface chamber filled with aCSF saturated with carbogen (5% CO_2) and heated at 28°C.
8. Incubate the slices in an interface chamber for at least 1 h before experiments.

⚠ CRITICAL: It is important to continuously bubble the aCSF with carbogen during the entire slice preparation process. We strongly advocate for storing brain slices in an interface chamber prior to their experimental implementation. This approach surpasses submerged chambers in safeguarding the integrity of synapses.[4] Alternative protocols that produce viable slices from older brains can also be considered.[5,6]

Positioning stimulation electrodes on L4

Timing: 10–15 min

The current protocol is specifically designed to illustrate and quantify the hyperexcitability of cortical principal neurons from *Stxbp1*^{hap} mice, but it can be readily adapted to other models of disorders where basket cell recruitment is aberrant. Monitoring the membrane potential of layer 2/3 pyramidal neurons in response to the stimulation of two distinct excitatory inputs that recruit feedforward inhibition microcircuits serves as a pivotal tool for distinguishing *Stxbp1*^{hap} from *Stxbp1*^{wt} phenotypes.[1] This is accomplished by means of two stimulation electrodes positioned on the input layer of the cortex (L4) (Figure 1). In this step, it is important to identify the different cortical layers and to position the stimulation electrodes 250–300 μm apart.

9. Choose a slice cut between 1.8 and 3.2 mm from the midline.
   a. place it in a recording chamber under the objective of an upright microscope equipped with epifluorescence.

Note: The chamber must be continuously perfused with aCSF saturated with carbogen (5% CO_2).

10. Employ a 4× objective to pinpoint the somatosensory cortex (Ss cortex).

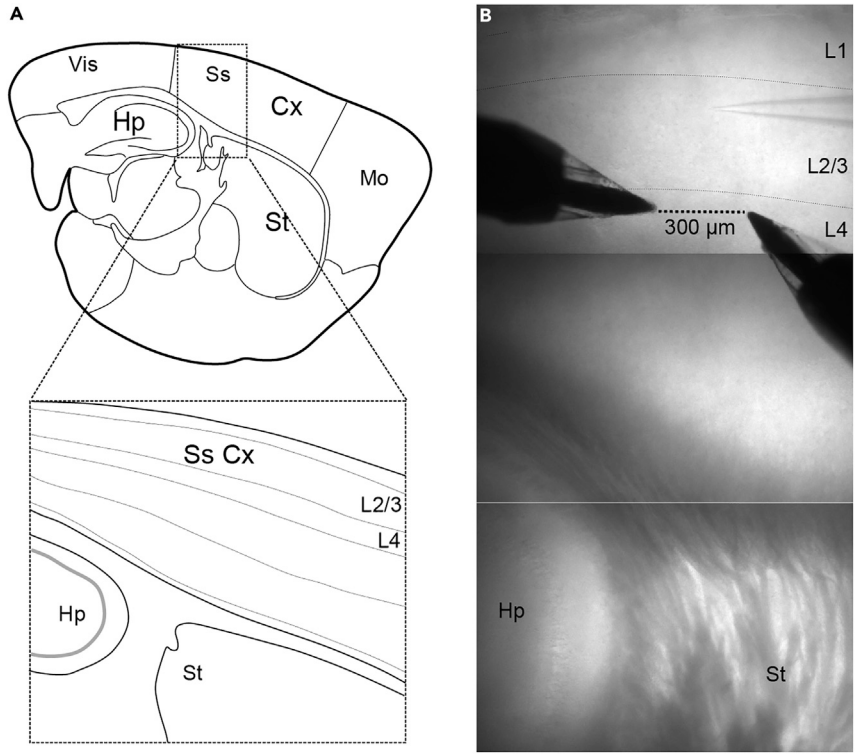

Figure 1. Positioning the stimulation electrodes on L4 from the somatosensory cortex

(A) Schema of a sagittal section from the mouse brain. Cx: Cortex, Hp: Hippocampus, Mo: Motor, Ss: Somatosensory, St: Striatum, Vis: Visual. Inset: enlargement of the somatosensory cortex.

(B) Picture of somatosensory cortex and deeper structures from mouse observed through a X4 objective. Notice the two stimulation electrodes positioned 300 µm apart in L4 and the patch clamp recording electrode in L2/3.

Note: This can be achieved by first identifying the hippocampus (Hp) and the striatum (St). The Ss cortex lies along a line that bisects the distance between the Hp and the St (Figures 1A and 1B). To ensure accuracy, it is advisable to verify the location using a reference brain atlas.

11. Utilize a 40× immersion objective to discern cortical layers 1 to 4 (Figure 1).
    a. Gradually shift the field of view from superficial to deeper layers.

Note: Layer 1 is a thin layer (~125 µm) distinguished by sparse and small neuron somas. Layer 2/3, a thick layer (~300 µm), harbors large neurons with somas that are pyramidal in shape. Layer 4 is a thin layer (~125 µm) containing a higher density of small, spiny stellate neurons compared to other cortical layers. The boundary with layer 5 is evident by a stark morphological shift towards larger, distinctly pyramidal neurons with a lower density.[7]

12. Position two bipolar stimulation electrodes on layer 4 (i.e., within the barrel cortex), 250–300 µm apart at a depth of 50–70 µm in the slice (Figure 1B).

Note: We recommend tungsten electrodes with a tip diameter of 2–3 µm (e.g., TM33CCNON; World Precision Instruments, Sarasota, FL, USA).

    a. The electrodes should be connected to a stimulus isolator (e.g., A365RC, WPI, UK)) controlled by a digitizer (e.g., Digidata 1550B, Molecular Devices, Sunnyvale CA, USA).

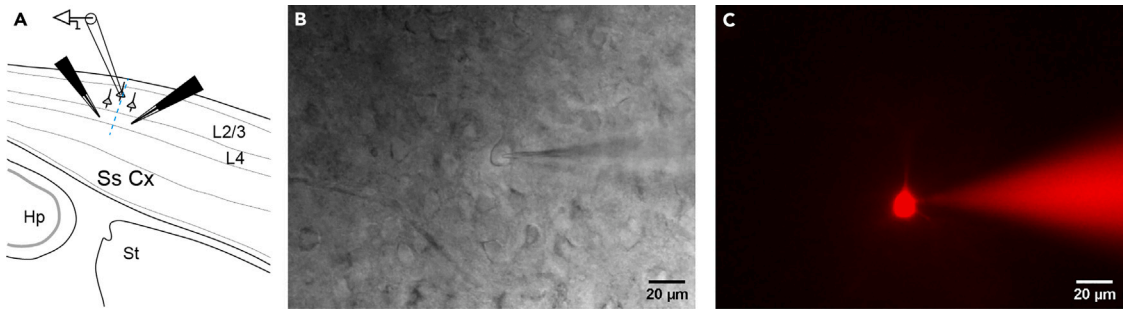

Figure 2. Identifying and recording L2/3 pyramidal neuron from the somatosensory cortex

(A) Schema of a sagittal section from the mouse brain illustrating the somatosensory cortex (Ss Cx). Hp: Hippocampus, St: Striatum. Notice the two stimulation electrodes on L4 and the patch clamp recording electrode in L2/3. The blue dot line indicates the perpendicular bisector of the segment connecting the stimulations electrodes.

(B) Picture of the Ss Cx taken with a 40× immersion objective. Notice the patch clamp electrode positioned on the soma of a putative pyramidal cell.

(C) Fluorescent picture showing the same cell as in B recorded in whole-cell configuration. Notice the single apical dendrite projecting towards the cortical surface and multiple basal dendrites pointing laterally.

Finding and recording of pyramidal neurons from L2/3

This step aims at recording a layer 2/3 pyramidal cell with the patch-clamp technique, in whole-cell configuration. The morphology and the electrophysiological properties of the cell are used to confirm the identity of the recorded neuron.

13. Prepare patch-clamp electrodes (resistance of 4–7 MΩ) on a horizontal or vertical pipette puller (e.g., BF150-86-7.5 borosilicate glass capillaries, Sutter Instrument, USA; pulled with P-1000 micropipette Puller, Sutter Instrument, USA).
14. Inspect layer 2/3 of the somatosensory cortex by means of the 40× objective (bright field illumination) in a region that remains within 50 µm from perpendicular bisector of the segment connecting the two stimulation electrodes in L4 (Figure 2A).
    a. Identify putative pyramidal neurons by their triangular-shaped somata (Figure 2B).
15. Position a patch clamp electrode onto the membrane of a potential cell candidate and establish a stable seal to record in whole-cell configuration.

Note: More details about the patch clamp technique can be found in classical papers.[8,9]

    a. Allow several minutes (5–10) for the fluorophore (e.g., Alexa 488 or Alexa 594) to diffuse throughout the cell.
    b. Illuminate the brain slice with epifluorescence and visualize the somato-dendritic arborization of the recorded cell.

Note: Pyramidal neurons exhibit a single apical dendrite extending towards the cortical surface and multiple basal dendrites projecting laterally (Figure 2C).

16. The identification of pyramidal neurons can be confirmed definitively by analyzing their firing properties.
    a. In current clamp mode, deliver 0.5–2 s depolarizing current pulses of increasing amplitude to the cell. Pyramidal neurons exhibit repetitive firing patterns with a maximum firing frequency of approximately 25 Hz.[10] Their action potentials half-width ranges from 1–1.5 ms. These characteristics distinguish them from interneurons.[11,12]

Confirmation of independent recruitment of two excitatory axon populations by stimulation electrodes

This step aims at verifying that the two pathways stimulated in L4 do not overlap. The two sets of axons are designated as test and conditioning. Excitatory axons originating from L4 project to

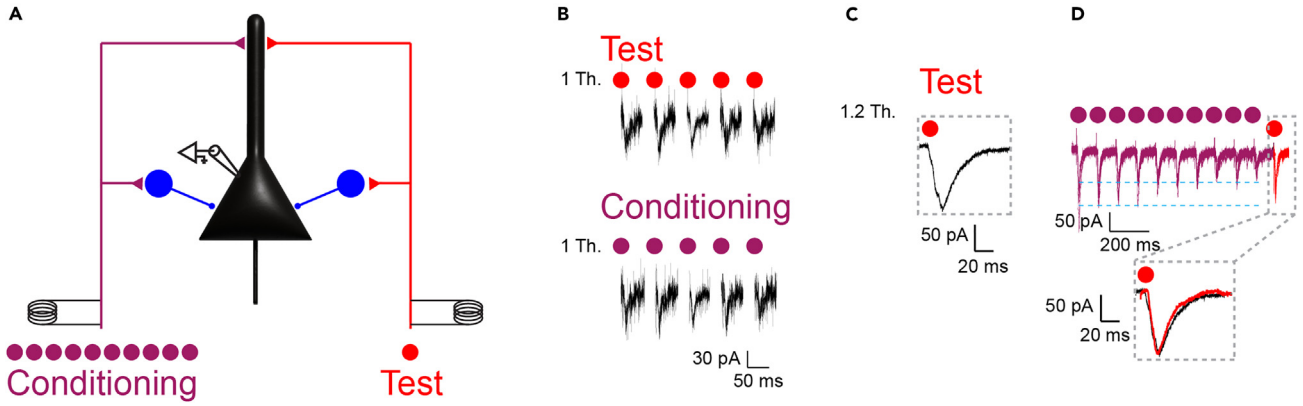

Figure 3. Confirmation of independence of the two sets of axons stimulated in L4

(A) Schema of the experimental protocol. Stimulation of two independent sets of L4 excitatory axons that activate feedforward inhibition microcircuits in L2/3.

(B) Voltage clamp recording of a L2/3 pyramidal neuron in response to the stimulation of the test and conditioning pathways at the minimal intensity necessary for inducing five consecutive EPSCs (1 Th.). 10 s between each stimulation. Vh = −70 mV.

(C) EPSCs induced by the test stimulation at 1.2 Th (average of 5 sweeps).

(D) EPSCs induced by a train of 10 shocks at 10 Hz at 1.2 Th. Applied on the conditioning pathways followed by a single shock at 1.2 Th. on the test pathway. Notice the depression of EPSCs during repetitive stimulation (blue dot lines). Inset: Superimposition of the test pathways EPSCs evoked before and after the conditioning train (averages of 5 sweeps). Similar time courses and amplitudes indicate the independency of the pathways.

L2/3 pyramidal neurons, while collateral branches stimulate inhibitory basket cells, which then form an inhibitory synapse on the pyramidal neuron, resulting in feedforward inhibition[1,13] (Figure 3A). The independence of the two groups of axons is insured by demonstrating that a conditioning stimulation does not affect the amplitude of the test synaptic response. Follow the steps below to complete the protocol.

17. Record the pyramidal neuron in voltage-clamp mode while holding the membrane potential at a value in between the reversal potential of excitatory and inhibitory synapses (e.g., near −40 mV). Apply a single current shock with a bipolar electrode (same characteristic as mentioned previously) and verify that it activates a feedforward inhibition microcircuit.[1,13,14]

    Note: The access resistance of the patch-clamp recording should be monitored regularly by means of a membrane test. If the access resistance increases more than 20%, the recordings should be discarded. Voltage-clamp recordings should be interpreted cautiously as inadequate space clamp, an inherent problem when recording cells with long processes, may lead to significant distortion of synaptic current due to deviation of the dendritic potential from that imposed by the somatic electrode.[15]

    a. The response should consist of an EPSC immediately followed by an inhibitory postsynaptic current (IPSC) (see Figure 1A from[1]).
    b. The latency of the EPSC should be fixed, while the latency of the IPSC should fluctuate.
    c. The minimal stimulation necessary for inducing the EPSC should be slightly lower than for the IPSC.
    d. Blocking EPSCs with AMPA and NMDA receptor antagonists should also inhibit the IPSC.
18. Record the pyramidal neuron in voltage-clamp mode while holding the membrane potential close to the reversal potential of inhibitory synapses (i.e., near −70 mV).
19. For the test and conditioning pathways separately, determine the stimulation threshold for evoking excitatory post synaptic current (Th EPSC) in the pyramidal neuron.
    a. Apply single shocks every 10 s at the lowest possible intensity. Gradually increase the stimulation intensity until the appearance of EPSCs.
    b. The threshold is the minimal intensity that evokes 5/5 EPSCs without failures (Figure 3B).

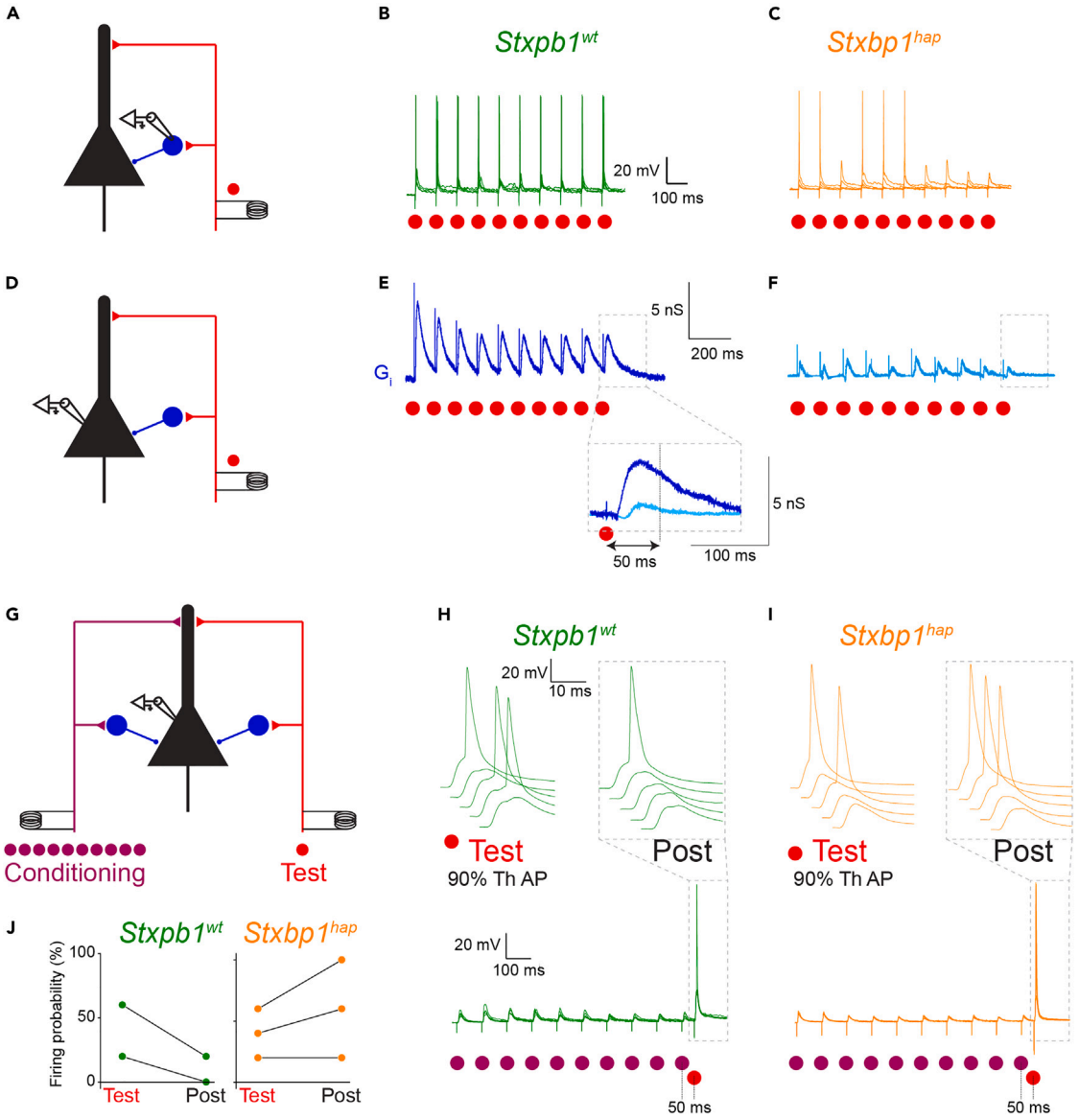

Figure 4. Hyperexcitability of pyramidal neurons in *Stxbp1*^{hap} animals compared to *Stxbp*^{wt}

(A) Schema of the experimental protocol. Recording of the membrane potential of a basket cell in response to L4 stimulation.

(B) Response of a basket cell from a *Stxbp1*^{wt} animal to the repetitive stimulation of L4 excitatory axons (10 shocks at 10 Hz, 5 superimposed sweeps). The cell keeps firing action potentials during the whole protocol duration.

(C) Response of a basket cell from a *Stxbp1*^{hap} animal to same stimulation protocol. The cell stops firing APs after few shocks due to the failure of excitatory synapses (dos Santos et al., 2023).

(D) Schema of the experimental protocol. Recording of the membrane potential of a L2/3 pyramidal neuron in response to L4 stimulation.

(E) Inhibitory synaptic conductance (Gi) of a L2/3 pyramidal neuron from a *Stxbp1*^{wt} animal during repetitive stimulation of L4 axons (10 shocks at 10 Hz). Gi was calculated using a method described elsewhere (House et al., 2011; Dos Santos et al., 2023).

(F) Same as in (E), from a *Stxbp1*^{hap} animal. Inset: superimposition of Gi induced by the last of the 10 shocks for *Stxbp1*^{wt} and *Stxbp1*^{hap} animals. Notice that 50 ms after the shock (dashed line) Gi is still significant for *Stxbp1*^{wt} but not for *Stxbp1*^{hap} animals.

(G) Schema of the experimental protocol. Recording of a L2/3 pyramidal neuron in response to the stimulation of two independent sets of L4 excitatory axons that activate feedforward inhibition microcircuits in L2/3.

(H) Up left: Response of a *Stxbp1*^{wt} pyramidal cell to a single stimulation of the test pathway at 90% of Th AP (3 action potentials evoked out of 5 trials). Response of the same neuron to the repetitive stimulation of the conditioning pathway (1.2 Th EPSC) followed 50 ms later by a single shock applied on the test pathway (90% Th AP). Only 1 action potential was evoked out of 5 trials, demonstrating a decrease in excitability.

Figure 4. *Continued*
(I) Same set up from a *Stxbp1*^{hap} animal. The pyramidal neuron responded by 2 action potentials in control condition and by 3, after the conditioning train. The excitability was not decreased.
(J) Paired diagram illustrating the firing probability of the same pyramidal neurons as in I in response to different intensity stimulations applied before and after the conditioning train.

c. Record the Th EPSC value.

20. Set the stimulation intensity to 1.2 * Th for each pathway.
21. Stimulate the test pathway with a single shock (Figure 3C). Repeat five times, waiting at least 10 s between each stimulation and calculate the average response.
22. Apply a train of 10 shocks at 10 Hz on the conditioning pathway, followed with a single shock on the test pathway, 100 ms after the last conditioning shock (Figure 3D). Verify that the synaptic response gets depressed during the conditioning train (Figure 3D).
23. Compare the average amplitudes of EPSCs induced by the test stimulations before and after the conditioning trains.
    a. If the test and conditioning pathways overlap, the depression of synaptic transmission should affect the test pathway and the EPSC should have a smaller amplitude.
    b. If the amplitude and the time course of the test EPSC remains the same after the conditioning train, it indicates that the conditioning and test pathways are independent (Figure 3D).

Note: The results from this step should be analyzed before going further. The independence of the test and conditioning pathways is necessary for interpreting the excitability test.

The depression of synaptic transmission during the conditioning train is necessary to establish the lack of overlap between the pathways. If this is not the case, check troubleshooting problem 1.

If the test and conditioning pathways overlap, check troubleshooting problem 2.

Excitability test

Herein, we present a protocol to demonstrate and quantify the hyperexcitability of L2/3 pyramidal neurons from *Stxbp1*^{hap} animals. This protocol utilizes the repetitive activation of feedforward inhibition microcircuits to induce a prolonged inhibition of pyramidal neurons lasting over 100 ms in *Stxbp*^{wt} animals (Figure 4E). During this inhibition window, the responses triggered by the test pathway are diminished. In contrast, in *Stxbp1*^{hap} animals, the weakened excitatory synapses fail to fully engage basket cells during repetitive stimulation, leading to an absence of persistent inhibition in pyramidal neurons (Figures 4G–4I).[1] Here, excitability refers to the ability of pyramidal neurons to generate action potentials in response to synaptic stimulation. Because the protocol relies on the activation of feedforward inhibition microcircuits, the excitability depends both on synaptic strength and membrane properties.

24. Switch the recording of the pyramidal cell to Current-clamp mode and compensate series resistance by bridge balance. Record the resting membrane potential without injecting any bias current.

Note: We recommend discarding cells that have a membrane potential more depolarized than −55 mV.

25. For the **test pathway**, identify the minimum stimulation intensity required to consistently evoke action potentials (five action potentials in five consecutive trials).

Note: this value is different from the Th EPSC used for checking the independency of the pathways. We recommend the following procedure:

a. Start by stimulating at an intensity sufficient to evoke action potential every time (use 2–3× the Th EPSC as reference).

b. Gradually decrease the intensity until the minimal value necessary to evoke 5 action potentials out of 5 consecutive trials.
c. Record this value as the Threshold for Action Potentials (Th AP).

Note: Setting Th AP should be done cautiously and may require more than five trials to be accurate, especially if stimulation intensities engage only few axon terminals.

26. To execute the excitability test:
    a. Sequentially set the **test** pathway stimulation intensity to 90%, 85% and 80% of the Th AP.
    b. Record 5 sweeps in response to a single shock at each of the stimulation intensities (inter-sweep interval >10 s) and calculate the firing probability for each stimulation intensity.
    c. Set the stimulation intensity of the **Conditioning** pathway at 1.2× Th EPSC.
    d. Apply a train of 10 shocks at 10 Hz on the **conditioning** pathway followed by a single **test** shock after a delay of 50 or 100 ms (Figures 4G–4I). Repeat the procedure five times.
    e. Apply this procedure for each stimulation intensities of the test pathway (90%, 85%, 80% Th AP).
    f. Count the number of action potential induced by the **test** stimulation and determine the firing probability for each stimulation intensity.
27. Quantify the results.
    a. For each stimulation intensity of the **test** pathway, compare the firing probability before and after the conditioning train.
    b. Display these values on a paired diagram (Figure 4J) and apply an appropriate statistical paired test to determine if the firing probability is significantly changed after the **conditioning** train.

Note: The parameters provided here have specifically been optimized for comparing *Stxbp1*^{hap} and *Stxbp*^{wt} animals and may require further adjustment for other experimental models. These parameters encompass the number of sweeps per stimulation intensity, the number and frequency of shocks in the conditioning train, and the delay for the test stimulation following the train (see below).

EXPECTED OUTCOMES

This protocol was developed to demonstrate and quantify the hyperexcitability of principal neurons in the neocortex of *Stxbp1*^{hap} mice. In *Stxbp*^{wt} animals, repetitive stimulation of L4 excitatory afferents consistently engages L2/3 basket cells (Figures 4A and 4B), triggering a prolonged inhibition of pyramidal neurons that lasts for over 100 ms (Figures 4D and 4E). Consequently, the probability of a test stimulus eliciting an action potential is substantially reduced 50 ms after the conditioning train (Figures 4G and 4H). In *Stxbp1*^{hap} mice, excitatory synapses are severely compromised,[1] and cannot maintain the recruitment of basket cells during 10 Hz trains (Figure 4C). For this reason, inhibition of pyramidal neurons is absent after the conditioning train and the probability that a test stimulation evokes an action potential is not affected 50 ms after the train and is even increased after 100 ms.[1]

Critically, the conditioning train was calibrated to evoke prolonged inhibition of pyramidal cells in control animals but not in mutant mice. To validate this point, we determined the inhibitory synaptic conductance (Gi) in the pyramidal cell (Figures 4D and 4F) using a method meticulously described in.[1,13] Alternatively, recording the inhibitory synaptic current by clamping the pyramidal neuron at the reversal potential for excitatory synapses (~0 mV) should also be sufficient for comparing inhibition levels. The conditioning train (number of shocks and frequency) must be fine-tuned through trial and error to ensure that it induces prolonged inhibition in control but not in mutant animals. The delay for the test stimulus following the train should be carefully selected to ensure that inhibition is present in control animals but not in mutant (i.e., 50 ms in our study; Figures 4H and 4I). We believe that similar protocols can be adapted to quantify the hyperexcitability of cortical neurons in other

disease models characterized by impairments in basket cell firing, such as Dravet syndrome[16] or absence epilepsy.[17]

Importantly the protocol can be used at preclinical level to test the ability of compounds to rescue the phenotype of mutant animals. We demonstrated this point by showing that positive allosteric modulators for AMPA receptors bring the excitability of pyramidal cells back to normal in *Stxbp1*^{hap} mice.[1]

LIMITATIONS

Our experimental protocol was designed to suit the unique characteristics of a specific mouse model of *STXBP1* encephalopathy, namely, the heterozygous mouse model developed by[2] (see also[3]). It is imperative to bear in mind that this protocol may necessitate adjustments when applied to other models of the same disease.

We anticipate that the protocol holds potential utility for investigations involving neurodevelopmental disorders that specifically impact the recruitment of basket cells, such as Dravet syndrome or absence epilepsy. Nevertheless, we wish to underscore that, at this stage, we cannot definitively predict whether the protocol would allow the quantification of cortical hyperexcitability.

Crucially, it is essential to recognize that our protocol is based on slice preparations, and any results obtained should be interpreted with caution. Extrapolating these findings to physiological conditions may present challenges, and therefore, a careful consideration of these limitations is advised. Further studies and validation efforts are warranted to enhance the robustness and generalizability of the protocol across different experimental settings.

TROUBLESHOOTING

Problem 1

No depression of synaptic transmission during the conditioning train when testing the independence of excitatory pathways.

Potential solution

Increase the frequency and / or the number of shocks applied during the train until a clear synaptic depression is observed.

Problem 2

If the test and conditioning pathways overlap (response to test stimulation after the conditioning train displays a reduction of more than 10%).

Potential solution

Change the position of one of the stimulation electrodes in L4 of few μm and repeat the protocol. Alternatively, patch another pyramidal cell and repeat the protocol.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Altair Brito dos Santos (altair.santos@sund.ku.dk).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Altair Brito dos Santos (altair.santos@sund.ku.dk).

Materials availability

Materials generated in this study that are not commercially available will be made available on request addressed to the lead contacts.

Data and code availability

Any data requested will be made available upon request.

ACKNOWLEDGMENTS

We thank Matthijs Verhage for providing the *Stxbp1* heterozygous mouse model. The project was funded by a Large Thematic Project grant awarded by the Lundbeck Foundation (R277-2018-802 to J.B.S. and J.-F.P.).

AUTHOR CONTRIBUTIONS

A.B.d.S. collected data, wrote the manuscript, and made figures. S.D.L. conceptualized the excitability test. C.D.G. collected data and created figures. J.B.S. participated in the elaboration of the protocol. J.-F.P. wrote the manuscript and created figures. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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