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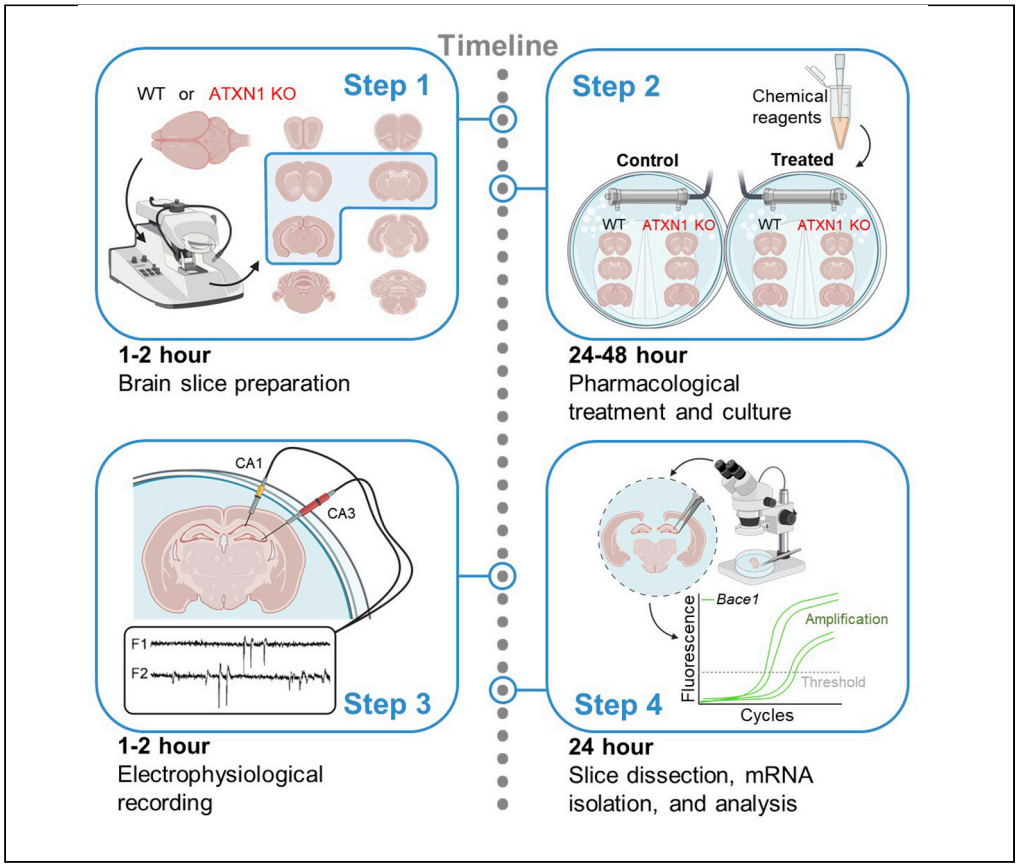
Analysis of brain region-specific mRNA synthesis and stability by utilizing adult mouse brain slice culture



Utilization of live animals for mechanistic study is challenging yet pivotal to elucidate pathogenesis of neurological diseases. Here, we present a protocol that employs cultured brain slices derived from adult mice to examine mRNA metabolism. We describe the preparation of acute brain slices and the treatments of RNA synthesis inhibitor and nucleotide analog to examine the effects of ataxin-1 loss-of-function on *Bace1* mRNA stability and transcription in cortex. This protocol also includes electrophysiological recording of spontaneous neuronal activity in hippocampus.

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Highlights

Protocol for acute brain slice cultures derived from adult mice for RNA metabolism study

Assessment of gene knockout effects on target mRNA stability in selected brain regions

Analysis of mRNA synthesis in cultured brain slices by utilizing Click-iT reaction

Electrophysiological analysis of spontaneous neuronal activity in hippocampal slices

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Analysis of brain region-specific mRNA synthesis and stability by utilizing adult mouse brain slice culture

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SUMMARY

Utilization of live animals for mechanistic study is challenging yet pivotal to elucidate pathogenesis of neurological diseases. Here, we present a protocol that employs cultured brain slices derived from adult mice to examine mRNA metabolism. We describe the preparation of acute brain slices and the treatments of RNA synthesis inhibitor and nucleotide analog to examine the effects of ataxin-1 loss-of-function on *Bace1* mRNA stability and transcription in cortex. This protocol also includes electrophysiological recording of spontaneous neuronal activity in hippocampus.

For complete details on the use and execution of this protocol, please refer to Suh et al. (2019).

BEFORE YOU BEGIN

Mice

This protocol below describes specific steps to use 10-month-old ataxin-1 knockout (KO) and wild type (WT) mice for brain slice cultures and subsequent mRNA analysis. We have also used this protocol with 3- and 14-month-old mice. Ataxin-1 KO mice are generated in C57BL/6 genetic background (Matilla et al., 1998) and housed under specific pathogen free condition in an animal facility at Massachusetts General Hospital (MGH). Mouse generation, husbandry, and experimental procedures are approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (MGH SRAC) and conform to the NIH guidelines of the care and use of laboratory animals.

Chemical agent and buffer preparation

Timing: 1–2 h

This section lists the stock and working solution of reagents needed for experiments. It is strongly recommended to prepare the working solution immediately before the experiment.

1. Make stock and working artificial cerebrospinal fluid (aCSF) solution as the recipes in "materials and equipment."
2. Make stock solution for Actinomycin D (Act-D) and 5-ethynyl uridine (5EU) as the recipes in "materials and equipment."

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Actinomycin D	Sigma	Cat# A9415
TRIzol Reagent	Invitrogen	Cat# 15596026
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	Cat# 10977-023
UltraPure™ Glycogen	Thermo Fisher Scientific	Cat# 10814-010
RNAseOUT™ Recombinant Ribonuclease Inhibitor	Thermo Fisher Scientific	Cat# 10777-019
Critical commercial assays		
iScript Advanced cDNA Synthesis Kits for RT-qPCR	Bio-Rad	Cat# 1725037
SsoAdvanced Universal Probes Supermix	Bio-Rad	Cat# 172-5280
Click-iT Nascent RNA Capture Kit	Thermo Fisher Scientific	Cat# C10365
SuperScript VILO cDNA synthesis kit	Invitrogen	Cat# 11754-050
qPCR assay for *Bace1* mRNA expression	Applied Biosystems	Mm00478664_m1
qPCR assay for *Atxn1* mRNA expression	Applied Biosystems	Mm00485928_m1
qPCR assay for *Gapdh* mRNA expression	Applied Biosystems	Mm99999915_g1
Experimental models: Organisms/strains		
Ataxin-1 KO mice (10 months old, male and female)	Matilla et al. (1998)	N/A
WT (C57BL/6) mice (10 months old, male and female)	The Jackson Laboratory	Stock # 000664
Software and algorithms		
Axoscope	Molecular Devices	AxoScope 10.7 https://www.moleculardevices.com
Origin	OriginLab Corporation	OriginPro 2018 https://www.OriginLab.com
pClamp	Molecular Devices	pClamp 10.7 https://www.moleculardevices.com
Other		
Vibratome	Leica	VT-1000S
Micro-Bubbler Ceramic Air Stone (2 inch)	Aquarium Pharmaceuticals	RENA, B0002566UQ
Real-Time PCR Detection System	Bio-Rad	CFX96
Tungsten Microelectrodes	California Fine Wire Company	100211
Low-noise Data Acquisition System	Molecular Devices	Axon DigiData 1550B
Low-noise Bioamplifier Chassis (Iso-DAM8A)	World Precision Instruments	74030
Duran Aspirator Bottle (1,000 mL)	DURAN	Duran 5399-09

MATERIALS AND EQUIPMENT

aCSF stock solution (10×, stock at 4°C for at least 1 month)		
Reagent	Final concentration (10×)	Amount or volume
NaCl	1,260 mM	147.4 g
KCl	35 mM	5.2 g
$NaH_2PO_4 \cdot H_2O$	12 mM	3.3 g
$MgCl_2 \cdot 6H_2O$	13 mM	5.28 g
Add deionized H_20 to 1,800 mL and mix well to dissolve all salts		
$CaCl_2 \cdot 2H_2O$	20 mM	5.88 g
deionized H_2O	n/a	To 2,000 mL
Total	n/a	2,000 mL

aCSF working solution (1×), freshly prepared (processing of two mice)		
Reagent	Final concentration	Amount or volume
D-glucose	11 mM	4.36 g
$NaHCO_3$	25 mM	4.2 g
deionized H_2O	n/a	To 1,800 mL
aCSF stock solution	1×	200 mL
Total	n/a	2,000 mL (pH 7.4)

Actinomycin D (Act-D) stock solution, 20 mM (stock at –20°C up to 3 months, protected from light)		
Reagent	Final concentration	Amount or volume
Actinomycin D powder	20 mM	12.55 mg
DMSO	n/a	500 µL
Total	n/a	500 µL

5-ethynyl uridine (5EU) stock solution, 200 mM (stock at −20°C up to 12 months. 5EU is a component of Click-iT Nascent RNA Capture Kit)		
Reagent	Final concentration	Amount or volume
5-ethynyl uridine powder	200 mM	5 mg
DMSO	n/a	93 µL
Total	n/a	93 µL

STEP-BY-STEP METHOD DETAILS

Preparation of acute mouse brain slices from adult mice

Timing: 1–2 h per mouse

This section describes how to prepare mouse brain slices before applying chemical agents to the cultures. In this protocol, two mice (one WT and the other ataxin-1 KO) are used to prepare brain slices.

Note: The overall goal of this procedure is to prepare healthy brain slices from mature adult mice that are suitable for pharmacology and electrophysiology experiments. This brain slice preparation, from mice of virtually of any age, allows researchers to explore changes in gene expression up to 2 days after application of drugs/chemicals in the slice culture.

⚠ CRITICAL: Good handling and quick transition between the steps of the procedure is desired. Individual slices should reach their new culture dish quickly without much time outside of the oxygenated aCSF. Once the slices are in their temporal incubation chambers the next mouse may be started at step 8 allowing for the sectioning of multiple mouse brains for a single experiment. Entire time from anaesthetizing a mouse for decapitation (step 8) to transfer for brain slice to temporal dish (step 14) may be completely within 30 min. Two mouse brains can be sliced and processed in a single experiment without compromising culture quality.

1. Place 500 mL aCSF in −20°C freezer and chill until ice-cold (< 4°C). Avoid over-freezing as this can change osmolarity of the solution.
2. Prepare all sterile instruments for surgery and dissection (Figure 1A).
3. Place a large glass or plastic Petri dish (150 mm-diameter) on ice, place the round filter paper on the bottom.

Figure 1. Preparation of brain slices from adult mice

(A) Photograph of microtome (left), slice culture dishes (middle), and surgery equipment (right). One big culture dish is for temporal incubation and two small dishes are for incubation up to 2 days.

(B) Stone cylinder producing air bubbles in aCSF solution.

(C) 5-ports manifolds with female luer locks controlling oxygenation level in the culture.

(D) Folded filter paper that keep brain slices from different mice separate.

(E) Brain slice sectioning within ice-cold chamber. *, bubbling blue tube to oxygenate in the chamber.

(F) Coronally sectioned brain slices.

a. Set a small micro-bubbler oxygen diffuser connected to a gas tank via flexible tubing and 5-ports manifold (Figures 1A and 1C).
b. Add 150 mL of ice-cold aCSF. Oxygenate the aCSF solution with 95% O_2/5% CO_2 gas.
c. This Petri dish is used for brain dissection only.

4. Install one additional glass or plastic Petri dish (150 mm-diameter) as described in step 3. This large Petri dish is used for temporal incubation of brain slices (Figure 1B).

Note: Oxygenation level in the culture dish is persistently controlled by 5-ports manifold with female luer locks to avoid excessive bubbling (Figure 1C).

5. In the dish prepared in step 4, put a round filter paper that is folded to make a separation barrier in the middle, as shown in Figure 1D. This folded-barrier is useful to separate brain slices from WT to those from ataxin-1 KO mice.
6. Prepare two additional plastic Petri dishes (100 mm-diameter) as described in steps 4–5 for a long-term slice incubation.
   a. Dilute 37.5 μL stock Act-D solution in 75 mL aCSF solution to make 10 μM final concentration.
   b. Dilute 37.5 μL DMSO solution in 75 mL aCSF solution to make vehicle control.
   c. In each Petri dish, pour either the vehicle (DMSO) or Act-D CSF solution.

Figure 2. Brain slice culture chemical agent treatment and incubation

(A) Slices arranged from anterior to posterior side of a mouse brain. Size bar = 1 cm.

(B) WT and ataxin-1 KO brain slices are treated with vehicle control (left) or chemical agent (e.g., Act-D; right) and incubated with oxygenation.

d. Cover Petri dishes with corresponding plastic lids. Cover the entire culture dishes with dark fabric and/or aluminum foil to prevent light exposure and keep them at room temperature (20°C–22°C).

Note: The plastic lids should have a small open space at the edge to allow plastic tubing that supplies oxygen to the culture to pass though.

7. Set a vibratome (Leica VT-1000S) to cut brain at 375–400 μm thickness (375 μm for older, 400 μm for younger mice).
   a. Use the cutting brain speed at ~ 0.25 mm/s and blade sectioning (vibration) frequency ~ 90 Hz.
   b. Blade return stroke is performed at constant speed of 5 mm/s.
8. Fill the slicing chamber of the vibratome with ice-cold aCSF solution and oxygenate it carefully (Figure 1E).
   a. Fill the tray surrounding the slicing chamber with a slurry of ice and water to keep it cool.
   b. Place a razor blade in the blade holder.
9. Anaesthetize one adult WT mouse with isoflurane, decapitate and remove the brain rapidly.
10. Place the brain in the dissection Petri dish on ice that contains oxygenated ice-cold aCSF. For coronal sectioning, cut off the cerebellum and a small prefrontal cortex part (~ 3 mm) with a razor blade.
11. Attach the brain onto the specimen disc of vibratome with fast adhesive glue at cerebellum-cut side (Figures 1E and 1F). Orient dorsal side to face the razor blade.

Note: A piece of agar fixed with adhesive glue on the specimen disc and placed on the ventral-side of brain provides additional support during slice cutting (Figure 1F). It is recommended that the agar block is 1–2 mm taller than the dissected brain to provide support.

12. Define the start and stop positions of the slicing, and slice down the brain with the vibratome. Usually, one brain produces 8–12 coronally sectioned 375–400 μm-thick slices (Figure 1F).
13. Upon completion of the sectioning, gently transfer individual brain slices with wide-mouthed transfer pipet to the temporal incubation dish filled with oxygenated prechilled aCSF (prepared at step 4).
14. In the temporal incubation dish, arrange the brain slices on one side of the peak-folded filter paper in a sequence from anterior to posterior direction (Figure 2A).
15. To obtain brain slices from ataxin-1 KO mouse, repeat steps 9–14. Ice-cold aCSF in the vibratome should be changed. When transferring the ataxin-1 KO slices to temporal incubation dishes, place them on the other side on the filter paper (Figure 2B).

Note: One or two brain slices from WT and ataxin-1 KO mice are used to measure initial electrophysiological activity in hippocampal CA1 before the start of slice culture (steps 21–28). Use the remaining aCSF for perfusion solution.

Brain slice culture to analyze mRNA stability: Actinomycin D treatment

Timing: 0.5–1 h for Act-D treatment; 48 h for culture incubation

This section describes how to apply aCSF solution containing either Act-D or vehicle to brain slices and maintain the treated slice cultures up to 2 days.

⚠ CRITICAL: To minimize variation in culture condition, brain slices from two mice (i.e., WT and ataxin-1 KO) to be compared are maintained side-by-side throughout the incubation within same culture dishes.

16. For each brain slices kept in temporal dish (prepared at steps 14 and 15), from anterior to posterior, assign numbers (e.g., WT1, WT2, …, KO1, KO2, …).
17. Transfer brain slices of WT mice to incubation culture dishes.
    a. Transfer the odd-numbered brain slices on to the left side of filter paper barrier (peak) in the long-term slice incubation culture dish that contains vehicle control.
    b. Transfer the remaining even-numbered brain slices on to the left side of filter paper in the other long-term slice incubation culture dish that contains Act-D (Figure 2B).

Note: The two sets of coronally-sectioned brain slices from one mouse, divided half by this way (even- or odd-numbered; 4–6 slices each), are very comparable to each other in their structural composition. This approach reduces experimental variations that are generated from different mice used in examining the effects of pharmacological agents (vehicle vs. Act-D) on cultured brain slices.

18. Transfer brain slices of ataxin-1 KO mice to incubation culture dishes.
    a. Transfer the odd-numbered brain slices on to the right side of filter paper barrier (peak) in the long-term slice incubation culture dish that contain vehicle control.
    b. Transfer the remaining even-numbered brain slices on to the right side of filter paper in the other long-term slice incubation culture dish that contains Act-D.

Note: Brain slices from WT and ataxin-1 KO mice should not be mixed or confused. The separation of the two brain slice sets within a same culture dish can be maintained by a filter paper that has a folded barrier (peak) in the middle and by stones that hold down the filter paper (Figures 1D and 2B). Substantial physical impact on the dish or sudden move of the dish, which can cause detachment of the brain slices from filter papers and floating and mixing of the slices in the dish, should be avoided.

19. Cover the dishes with corresponding plastic lids to avoid excess evaporation. Cover the entire culture dishes with dark fabric and/or aluminum foil to block light as Act-D is sensitive to light. The slice cultures are incubated at room temperature (20°C–22°C).

⚠ CRITICAL: Maintaining continued oxygenation of the slice cultures throughout incubation period is essential. Make sure 95% O_2/5% CO_2 tank has plenty of gas for the entire incubation period.

20. 24 h after the start of incubation, replace 75% of the culture media with fresh prepared room temperature aCSF containing either vehicle or Act-D.

a. To avoid the floating and mixing of brain slices within culture dish, carefully and slowly take out the used media and add the new media with a Pasteur pipet from the edge of culture dish.
b. Slice culture is incubated for 24 or 48 h before harvesting them for electrophysiological recordings or total RNA isolation.

Electrophysiological setup and recordings

Timing: 1–2 h per mouse

This section describes electrophysiological extracellular field recordings in individual slices (Dzhala et al., 2012), before and after the slice culture. One of the purposes of this procedure is to validate the neuronal action potential activity and suitability of the brain slices for *ex vivo* pharmacological experiments.

21. Prepare setup for electrophysiological recordings.
    a. Use a flexible tubing to connect a leveling laboratory bottle (e.g., 1,000 mL Duran Aspirator bottle) outlet with a 1-way polycarbonate on/off stop valve for solution flow.
    b. Fill up DURAN Aspirator (leveling) bottle with 500 mL aCSF. Continuously oxygenate the aCSF solution with 95% O_2/5% CO_2 gas.
    c. Use a flexible tubing to connect the leveling bottle with a single in-line solution heater (e.g., SF-27B, SF-28) and recording submersion-type chamber.
    d. Setup solution flow by gravity, flow rate control and temperature control at 32°C.
22. For electrophysiological recordings, transfer individual slice to a conventional submersion-type chamber (Figures 3A and 3B) and continuously superfuse with oxygenated aCSF at 32°C at a rate of 2 mL/min. Stabilize tissue brain slice using appropriate U-shape slice anchor made by platinum bar.
23. Use commercial or custom-made tungsten microelectrodes for extracellular field potential recordings of multiple-unit activity (MUA) and population field potential activity.

Note: Use of microelectrodes made from coated tungsten wire 50 μm in diameter enables simultaneous recordings of multiple-unit activity (MUA, 400 Hz high-pass filter) and population field activity (EEG band, 1–100 Hz) from tens to hundreds of neurons near each electrode. Root-mean-square noise level with an electrode placed in the perfusion solution is typically 4–5 μV, whereas the amplitude of action potentials recorded from the stratum pyramidale ranges from this noise level up to 200 μV.

24. Attach the electrodes to the electrode holders of the amplifier head-stages and turn into position (Figure 3A).
25. Use fine micromanipulators to position the recording electrodes to the area of interest within the brain slice (Figure 3B).
26. Once electrode is in contact with healthy pyramidal cell layer, monitor extracellular field potential activity using computer-controlled software.
27. Perform extracellular field potential recordings of MUA using computer-controlled software (pCLAMP version 10.3 or higher, Molecular Devices) and data acquisition (analog-to-digital converter Digidata 1440, Molecular Devices).
28. Perform data analysis and statistical comparison of the data. Optional, use Mini Analysis Program (Synaptosoft), and Origin (OriginLab Corporation) programs for data analysis. Determine the amplitude and frequency of MUA from high-pass filtered (400 Hz) raw data with a spike detection algorithm (Mini Analysis Program) and verify visually (Figure 4).

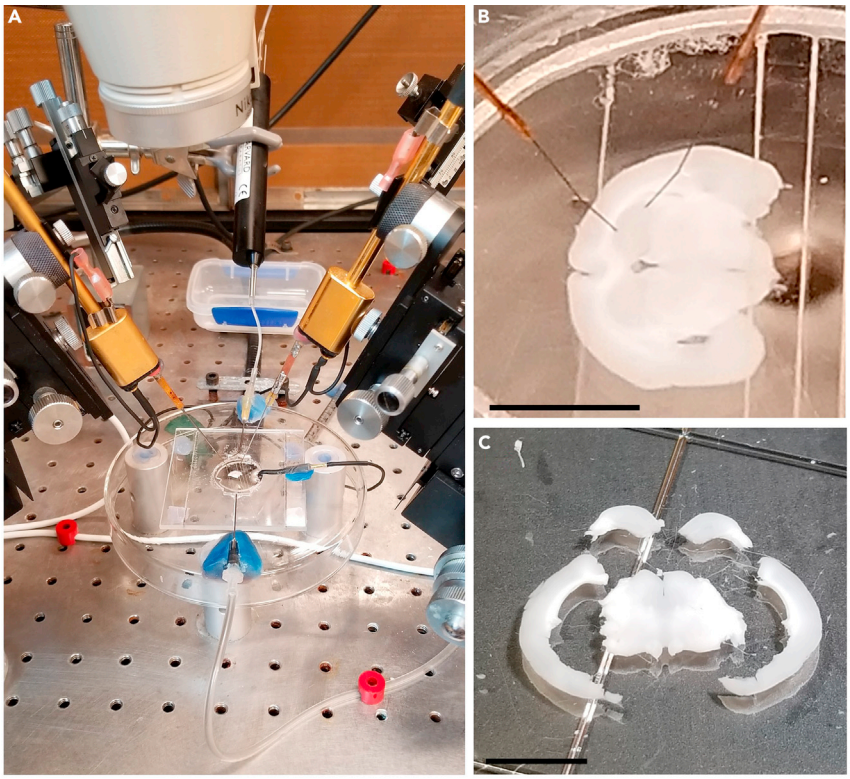

Figure 3. Electrophysiological recording and brain slice dissection

(A) Settings for electrophysiological recording of brain slices *in vitro* superfused with oxygenated aCSF.

(B) Extracellular field potential recordings are performed in CA1 pyramidal cell layer using tungsten microelectrodes. Size bar = 0.5 cm.

(C) Dissected cortex and hippocampus from brain slice.

Dissection of brain regions after slice incubation

Timing: 1–2 h per mouse

This section describes how to dissect out individual brain regions (e.g., cortex) after slice incubation.

29. Prepare forceps and dissection microscope.
30. Transfer cultured brain slices to Petri dishes that are filled with fresh ice-cold aCSF solution. During the slice transfer and dissection, aCSF does not need to be oxygenated.
    a. To avoid mixing the slices from different mice, transfer WT and ataxin-1 KO slices to separate dishes.
    b. Transfer vehicle- and Act-D treated slices to different dishes. So, the brain slices from two incubation dishes are now divided into four for dissection.
    c. Keep the dishes containing the brain slice on ice during the dissection.
31. Using forceps, cut out cortex from brain slices under dissection microscope (Figure 3C).

Note: Here, the cortex is dissected out for further mRNA analysis as it exhibits, among several brain regions, the most distinct difference in steady-state *Bace1* mRNA level between WT and ataxin-1 KO mice. Other brain regions (e.g., hippocampus or striatum) can be dissected if needed.

32. Remove aCSF droplets from dissected cortex with Kimwipe tissue paper.

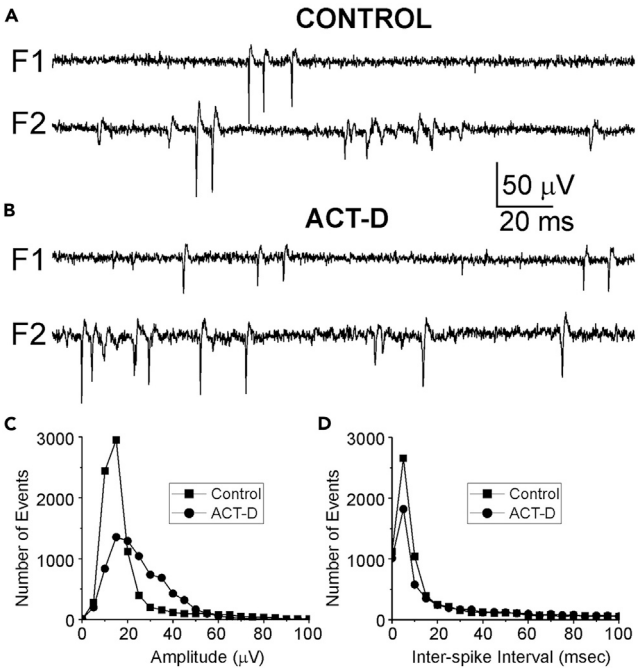

Figure 4. Spontaneous neuronal activity of CA1 and CA3 cells in incubated slices

(A and B) Simultaneous extracellular field potential records of multiple unit activity in the CA1 and CA3 areas in control (A) and Actinomycin D-treated (B) brain slices 48 h after incubation. Wide-band recordings at 0.1–10 kHz.

(C and D) Histograms representing spike amplitude distribution (C) and inter-event interval (D) in control and Actinomycin D-treated slices.

33. Put cortex pieces into 1.5 mL Eppendorf tubes and keep on dry ice to freeze.
34. Repeat step from steps 31 to 33 until all brain slices are dissected.

Pause point: Dissected brain slice piece samples (e.g., cortex) can be stored at −80°C until use.

Isolation of total RNA

Timing: 4–6 h for 20 brain slices

This section describes how to isolate total RNA with Trizol reagent from mouse brain slice pieces (e.g., cortex) for subsequent RT-qPCR analysis. Total RNA can be isolated by other methods, such as utilizing RNeasy Kits (QIAGEN).

35. Add 1 mL ice-cold Trizol reagent into 1 mL-size Dounce homogenizer.
36. Put pieces of cortex from one brain slice into the Trizol reagent.
37. Grind the tissue pieces with a tight pestle for 20 strokes up and down with twist.
38. Follow Trizol reagent manufacturer's instruction to isolate total RNA (Invitrogen, www.thermofisher.com/us/en/home/brands/product-brand/trizol.html). DNase treatment is not needed. Briefly,
    a. Incubate for 5 min to allow complete dissociation of the tissue lysates.
    b. Add 0.2 mL chloroform and vortex for 30 s.
    c. Incubate for 5 min at room temperature and centrifuge for 10 min at 13,000 × *g* at 4°C.
    d. Transfer the upper clear aqueous phase (400 μL) that contains RNA to a new tube.
    e. Add equal volume (400 μL) of isopropanol to the aqueous phase and vortex for 10 s.
    f. Incubate for 30 min on ice and centrifuge for 10 min at 13,000 × *g* at 4°C.

Note: Incubation on ice for longer time helps the precipitation of the relatively small amount of RNA from brain slice pieces. After centrifugation, total RNA precipitate forms a white pellet at the bottom of the tube.

g. Carefully discard the supernatant without disturbing the pellet and add 1 mL 75% ethanol to the remaining pellet.
h. Rinse the pellet with the 75% ethanol solution by inverting the tube several times.
i. Centrifuge for 5 min at 13,000 × *g* at 4°C and carefully discard the supernatant.
j. Air-dry RNA pellet on 70°C heat block until no remaining solution is visible in the tube.

Note: Avoid over-drying (> 3 min) the RNA pellet. Over-drying may impede the resuspension of RNA pellet in nuclease-free water in the subsequent step. It may also cause poor RNA quality.

k. Resuspend RNA pellet (invisible) with 15 μL nuclease-free water.

Pause point: Isolated RNA samples can be stored at −80°C until use.

39. RNA integrity can be assessed by the detection of distinct 28S and 18S rRNA bands in 1% agarose gel running.

Note: The weight of all cortical piece from one coronally sectioned brain slice is ~10 mg. Total RNA amount isolated from the cortical piece is ~4–8 μg.

RT-qPCR analysis of *Bace1* mRNA

Timing: 4–6 h for 20 RNA samples

40. Dilute each RNA samples to 250 ng/μL concentration in nuclease-free distilled water.
41. Set up reverse transcription (RT) reaction in a 96-well PCR plate as indicated in the table below.
    a. To run RT reaction for multiple RNA samples, make a master mix that includes all reagents except RNA.
    b. For RT reaction, iScript Advanced cDNA Synthesis Kits is used (refer to "key resources table").
    c. To assess any genomic DNA or PCR product contamination, control RT reaction (no RT) that does not contain reverse transcriptase should be included.

Reverse transcription reaction using total RNA

Reagent	Volume per RT reaction
Nuclease free distilled water	11 μL
5× iScript advanced reaction mix	4 μL
iScript advanced reverse transcriptase	1 μL
RNA (250 ng/μL)	4 μL
Total	20 μL

42. Run the RT reaction for 20 min at 46°C followed by 10°C holding in a PCR machine (CFX96 Touch Real-Time PCR Detection System).
43. Transfer all the RT products into new Eppendorf tubes.

Pause point: RT product samples can be stored at −80°C until use.

44. For each RT product, set up *Bace1* cDNA qPCR reaction in a 96-well PCR plate as indicated in table below.
    a. To run qPCR reaction for multiple RT products, make a master mix that includes all reagents except RT products.
    b. The PCR reactions should be performed in duplicate or triplicate.

qPCR reaction for Bace1 cDNA

Reagent	Volume per qPCR reaction
SsoAdvanced Universal Probes Supermix (2×)	10 μL
Bace1 (or Gapdh) TaqMan gene expression assays (20×)	1 μL
Nuclease free water	7 μL
Reverse transcription product	2 μL
Total	20 μL

45. Within same 96-well PCR plate, set up *Gapdh* cDNA qPCR reactions as described for *Bace1* cDNA qPCR (step 44). Gapdh expression level is used as control to calculate ΔCt (threshold cycle) values.

    Note: In this protocol, qPCR reactions for *Bace1* and *Gapdh* are run in separate PCR reaction wells. However, both qPCR reactions for one RT product can be run within same well by adding 1 μl each of TaqMan assay solution that includes forward and reverse PCR primers of corresponding genes and fluorescent probes. For this multiplex qPCR reaction, the amount of nuclease-free water is reduced from 7 to 6 μl for each qPCR reaction.

46. Run qPCR reaction as indicated in table below.

qPCR cycling conditions (TaqMan probe)

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	40
Annealing/extension	60°C	30 s	
Plate reading	60°C	n/a	
Hold	10°C	forever	

47. Determine Ct values for *Bace1* and *Gapdh*, based on the earliest cycles of exponential phase of the PCR reactions. Get an average of Ct value for each target gene (e.g., *Bace1*) from duplicate or triplicate PCR reactions.
48. Calculate ΔCt value of each target mRNA. For example, ΔCt(Bace1) = Ct(Bace1) – Ct(Gapdh). Calculate the average of ΔCt values for each treatment group (e.g., 0 h_WT). Details of qPCR result analysis can also be found at https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center.html.
49. Calculate ΔΔCt values of each sample. ΔΔCt value is calculated based on the control experimental group (e.g., 0 h_WT). For example, ΔΔCt (Bace1_24 h_KO_VH) = average of ΔCt (0 h_WT) – ΔCt (Bace1_24 h_KO_VH).
50. Calculate $100 \times 2^{\Delta\Delta Ct}$ value of each sample and analyze the results.
    a. Average of $100 \times 2^{\Delta\Delta Ct\ (0\ h\_WT)}$ will be assign as 100 as control group (Figure 5A).
    b. The relative amount of *Bace1* mRNA in each sample will be $100 \times 2^{\Delta\Delta Ct}$. For example, the relative amount of Bace1 mRNA in 24 h_KO_VH sample will be $100 \times 2^{\Delta\Delta Ct\ (24\ h\_KO\_VH)}$.

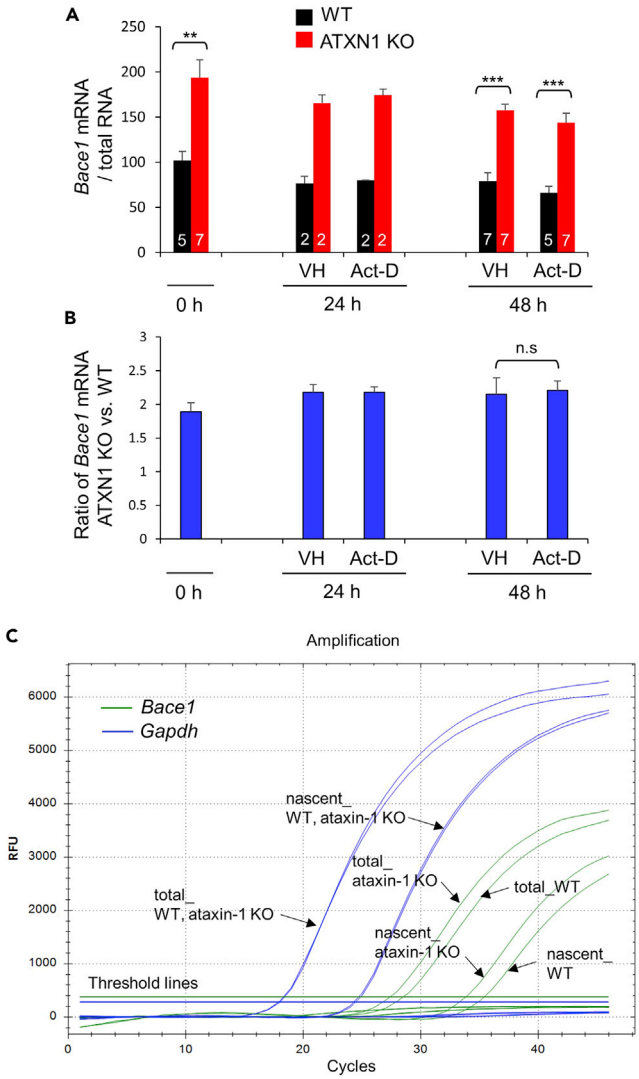

Figure 5. RT-qPCR analysis of brain slices to assess the stability and transcription of Bace1 mRNA

(A) Actinomycin D or vehicle (DMSO) was treated for 24 and 48 h in acute brain slice cultures. In bar, number of brain slice is analyzed. Data are pooled from 3 mice per each genotype. Data are represented as mean ± SEM. **p < 0.01, ***p < 0.001, *t* test.

(B) Ratio of *Bace1* mRNA levels in ataxin-1 KO and WT brain slices. n.s., not significant.

(C) Example of real-time qPCR amplification curves of steady-state (total) and nascent *Bace1* mRNA (green). *Gapdh* mRNA (blue) levels were measured as control. For the analysis of nascent mRNA, brain slices were incubated for 3 h in aCSF containing 5-ethynyl uridine.

c. Analyze $100 \times 2^{\Delta\Delta Ct}$ values of each treatment group at each time point (Figure 5A).

d. Analyze ratios of *Bace1* mRNA levels between ataxin-1 KO and WT in vehicle or Act-D treatment group at each time point (Figure 5B).

Brain slice culture to analyze mRNA synthesis: 5 ethynyl uridine (5EU) treatment

Timing: 1 day

This section describes how to apply aCSF solution containing either 5EU or vehicle to brain slices to measure newly synthesized mRNA. For the 5EU treatment and subsequent nascent mRNA isolation (Steps from 51 to 69), we utilized Click-iT Nascent RNA Capture Kit (Refer to "key resources table").

⚠ CRITICAL: To minimize variation in culture conditions (e.g., exposure to 5EU), brain slices from two mice (i.e., WT and ataxin-1 KO) to be compared are maintained side-by-side within same culture dishes throughout the incubation.

51. Prepare 5-ethynyl uridine (5EU) working solution by adding 50 μL of the 5EU stock solution to 50 mL aCSF for a final concentration of 0.2 mM. Prepare vehicle control solution by adding 50 μL DMSO to 50 mL aCSF for a final concentration to 0.1%.
52. Prepare mouse brain slices from ataxin-1 KO and WT mice as described in steps 1–15 at "preparation of acute mouse brain slices from adult mice," except for step 6. Instead of Act-D, use 5EU solution and corresponding vehicle control solution made in step 51.
53. Transfer WT mouse brain slices to culture dishes as similar to what is described for Act-D treatment ("brain slice culture to analyze mRNA stability: Actinomycin D treatment").
    a. Transfer odd-numbered brain slices of WT mice on to the left side of filter paper peak in one culture dish that contains vehicle control.
    b. Transfer the remaining even-numbered brain slices on to the left side of the filter paper peak in the other culture dish that contains 5EU.
54. Transfer ataxin-1 KO mouse brain slices to culture dishes.
    a. Transfer even-numbered brain slices of ataxin-1 KO mice on to the right side of filter paper peak in the culture dish that contains vehicle control.
    b. Transfer the remaining even-numbered brain slices on to the right side of filter paper peak in the other culture dish that contains 5EU.
55. Cover the dishes with corresponding plastic lids to avoid excess evaporation. Cover the entire dishes with dark fabric and/or aluminum foil to block light at room temperature (20°C–22°C).

⚠ CRITICAL: Maintaining continued oxygenation of the slice cultures throughout incubation period is essential.

56. Harvest brain slices at 3 and 16 h after incubation and dissect brain regions (e.g., cortex) as described in steps 29–34 at "dissection of brain regions after slice incubation."

Note: After the incubation, one or two brain slices can be used to measure the electrophysiological activity of hippocampal CA1 neurons, as described in steps 21–28 at "electrophysiological recordings of brain slices."

Biotinylation of 5EU containing nascent RNA

Timing: 1 day for 6 RNA samples

This section describes how to biotinylate 5EU containing nascent RNA by utilizing Click-iT reaction. We utilized Click-iT Nascent RNA Capture Kit (key resources table) for this procedure. Refer to manufacturer's instruction for details (www.thermofisher.com/order/catalog/product/C10365).

57. Isolate total RNA from dissected brain slices containing 5EU (step 56) with Trizol reagent as described in steps 35–39 at "isolation of total RNA."

Note: Total RNA concentration for Click-iT reaction should be 1 μg/μL. To achieve this concentration, put 4–6 cortex pieces of same treatment group (e.g., 16 h-WT) in 1 mL Trizol reagent when isolating total RNA and resuspend the resulting RNA pellet in 15 μL distilled nuclease-free water. Approximately 20–40 μg of total RNA could be obtained from this preparation.

58. Dilute the RNA into 1 μg/μL and use 10 μg RNA for one Click-iT reaction.
59. Prepare Click-iT reaction cocktail (50 μL per reaction) according to Table below. Add the reaction components in the order listed.

Click-iT reaction cocktail (50 μL total reaction volume/sample)

Order of addition	Reaction component	Final concentration	Amount or volume
1	Nuclease-free water	n/a	3.25 μL
2	Click-iT EU buffer	1×	25 μL
3	$CuSO_4$	2 mM	4 μL
4	Biotin azide	1 mM	5 μL
5	EU-RNA (1 μg/ul)	n/a	10 μL
6	Click-iT reaction buffer additive 1	10 mM	1.25 μL
7	Click-iT reaction buffer additive 2	12 mM	1.5 μL
	Total	n/a	50 μL

60. Incubate the Click-iT reaction mix for 30 min by gently mixing it on a laboratory vertical rotating mixer.
61. Precipitate the RNA with glycogen following the manufacturer's protocol (https://www.thermofisher.com/order/catalog/product/C10365). Briefly,
    a. Add 1 μL of UltraPure Glycogen (key resources table), 50 μL of 7.5 M ammonium acetate, and 700 μL of ice-cold 100% ethanol to the click reaction. Mix the tube contents by inverting the tube several times.
    b. Incubate the tube overnight at –70°C and centrifuge at 13,000 × *g* for 20 min at 4°C.
    c. Carefully discard the supernatant without disturbing the pellet and add 1 mL 75% ethanol to the remaining pellet.
    d. Rinse the pellet with the 75% ethanol solution by inverting the tube several times.
    e. Centrifuge for 5 min at 13,000 × *g* at 4°C and carefully discard the supernatant.
    f. Air-dry RNA pellet on 70°C heat block until no remaining solution are visible in the tube.

    Note: Avoid over-drying (> 3 min) the RNA pellet.

    g. Resuspend RNA pellet (invisible) in 50 μL distilled nuclease-free water.

    Note: The RNA yield after Click-iT reaction is ~ 80%–100% of the starting material. The resuspended RNA can be stored at −80°C until further use.

Isolation of biotinylated RNA by streptavidin magnetic beads

Timing: 2–3 h for 6 RNA samples

This section describes how to bind biotinylated nascent RNA to streptavidin magnetic beads. The binding reaction and the isolation of nascent RNA transcript are carried out with Click-iT Nascent RNA Capture Kit following manufacturer's instruction (https://www.thermofisher.com/order/catalog/product/C10365). Below are brief steps for one RNA sample from brain slice cultures. For more reactions, scale up the volumes accordingly.

62. Using a magnetic separation rack, wash 50 μL streptavidin-conjugated bead 3 times with 500 μL Click-iT reaction wash buffer 2.
63. Dilute RNA from steps 61 to 100 ng/μL with distilled nuclease free water. Use 10 μL of the diluted RNA (total 1 μg) for "biotinylated nascent RNA:streptavidin magnetic beads" binding reaction.
64. Prepare RNA:bead binding reaction mix according to Table below and heat the mix at 70°C for 5 min.

RNA binding reaction mix (250 μL total reaction volume/reaction)	
Reaction component	Amount or volume
Click-iT RNA binding buffer, 2×	125 μL
RNAseOUT Recombinant Ribonuclease Inhibitor	2 μL
RNA (from step 9.2; 100 ng/μL)	10 μL
UltraPure DNase/RNase-free distilled water (to 250 μL)	113 μL

65. Add 50 μL of bead suspension (washed in step 62) into each of heated RNA binding reaction mixes (step 64).
66. Incubate the microcentrifuge tubes containing the RNA binding reaction mix on a laboratory vertical rotating mixer at room temperature (20°C–22°C) for 30 min to prevent the beads from settling.
67. Using a magnetic separation rack, wash the beads 5 times with 500 μL Click-iT reaction wash buffer 1 at room temperature.
68. Wash the beads additional 5 times with 500 μL Click-iT reaction wash buffer 2.
69. Resuspend the beads in 50 μL of Click-iT reaction wash buffer 2 and proceed immediately to cDNA synthesis using the RNA captured on beads as a template.

Note: At this point, the beads are ready to be used in a cDNA synthesis reaction. Do not store the RNA for future use.

cDNA synthesis of the nascent RNA and qPCR

Timing: 3–4 h

This section describes how to synthesize cDNA from the RNA bound to beads (from step 69) as a template. For the reverse transcription reaction to generate first-strand cDNA, we utilized SuperScript VILO cDNA synthesis kit (key resources table). This cDNA synthesis kit utilizes random primers for reverse transcription. Below are the brief steps for one RNA sample from brain slice culture. For more RT reactions, scale up the volumes accordingly.

70. Prepare reverse transcription reaction from the bead-bound RNA. Table below lists the reaction components and their recommended volumes for a 100 μL cDNA synthesis reaction.

Reverse transcription reaction using biotinylated RNA on beads	
Reagent	Volume
RNA, captured on beads	50 μL
5× VILO Reaction Mix	20 μL
Nuclease free water	20 μL
10× SuperScript Enzyme Mix	10 μL
Total reaction volume	100 μL

a. Heat the RNA-bound bead suspension (step 69) from at 70°C for 5 min.
b. Immediately add 20 μL of 5× VILO reaction mix into each bead suspension tube and mix it by pipetting up and down.
c. Bring the reaction mixture to room temperature (20°C–22°C) and gently mix for 5 min on a laboratory vertical rotating mixer.
d. Add 20 μL of nuclease free water to the reaction mixture.
e. Add 10 μL of 10× SuperScript Enzyme mix to the reaction mixture. Mix the reaction well by pipetting it up and down.

71. Incubate the reaction at 46°C for 1 h. Mix gently the reaction tube to prevent the beads from settling during the incubation.
72. Heat the reaction mixture at 85°C for 5 min to terminate the RT reaction and to release the cDNA from the beads.
73. Use magnetic separation rack to immobilize the beads and transfer the supernatant containing the cDNA to a new tube.

Note: The RT product can be stored at –80°C until further use.

74. Proceed to qPCR for BACE1 and GAPDH as described in steps 44–50 at "RT-qPCR analysis of Bace1 mRNA." qPCR for ATXN1 can be used as control for ataxin-1 KO and WT samples. To assess any genomic DNA or PCR product contamination, control RT reaction (no RT) that does not contain reverse transcriptase may be included.

EXPECTED OUTCOMES

From one adult mouse brain, 8–12 coronally sectioned slices (400-μm thick) containing substantial portions of cortical regions (Bregma – 3.5 to 1 per coronal position) can be obtained. Extracellular field potential recordings from the pyramidal cell layer of hippocampal CA1 and CA3 regions invariably detected spontaneous multiple unit activity in control and transcription inhibitor Actinomycin D-treated brain slices 48 h after incubation (Figure 4). The amplitude and frequency distribution of detected action potentials were similar in control and pharmacologically treated brain slice cultures (Figures 4C and 4D). These data demonstrate the preservation of the intrinsic neuronal properties and suitability of the adult brain slice cultures for *ex vivo* electrophysiological and pharmacological experiments, and support that the experimental system could be applied to various studies that investigate gene expression mechanisms in adult brain.

From the cortex of one brain slice, several μg of total RNA can be isolated, which is enough for RT-qPCR analysis of mRNA. The ratio of cortical *Bace1* mRNA levels between ataxin-1 KO and WT mice was ~2, and this ratio was not changed when transcription was blocked by Act-D for 48 h (Figures 5A and 5B). This suggests that loss of ataxin-1 does not affect the stability of *Bace1* mRNA in the brain.

Newly synthesized mRNA in cultured brain slices comprises some portion of total mRNA. Thus, in RT-qPCR analysis, the Ct values of nascent *Bace1* mRNA that harbor 5EU were bigger than those of total *Bace1* mRNA (Figure 5C). Similar to their steady-state levels, the amount of newly synthesized *Bace1* mRNA in ataxin-1 KO cortex was higher than that in WT. This result suggests that loss of ataxin-1 potentiates *Bace1* transcription in the brain.

LIMITATIONS

Acute brain slices derived from adult mice provide a unique opportunity to study various aspects of the nervous system. The brain slices *ex vivo* enables examination of electrical behavior of specific neurons and assessment of biochemical changes in the brain (e.g., cell surface biotinylation), which are otherwise challenging to evaluate with live animals or cultured neural cells (Kim et al., 2007; Thomas-Crusells et al., 2003).

In the protocol above, we utilized acute brain slice culture to examine the effects of ataxin-1 on the stability and synthesis of *Bace1* mRNA. Previous experiments with ataxin-1 KO mice revealed that the levels of *Bace1* mRNA increase selectively in the cerebrum of adult mice; the increase was not observed in other brain regions (e.g., cerebellum), embryonic brains, or cultured neurons (Suh et al., 2019). For this reason, it was essential to use cerebrum from adult mice in the investigation to uncover the underlying mechanism of BACE1 increase in ataxin-1 KO brains. For the mechanistic study, first, it must be determined whether the increase of *Bace1* mRNA is due to either increased mRNA stability or enhanced transcription. With live mice, testing these two possibilities is not readily

feasible as the modulation and measurement of transcriptional processes is technically challenging. However, with acute brain slice cultures, those tests are feasible with *in vitro* application of transcription inhibitors (e.g., Act-D) and nucleotide analogs (5EU) into artificial cerebrospinal fluid.

While the experiments described here are limited to *Bace1* mRNA expression analysis of cortex, the protocol can be modified and used for mRNA expression of other genes and other brain regions. We believe this protocol can also be readily adapted for the analysis of protein metabolism.

Acute brain slices that are cultured as described above maintain structural integrity and biological activity 48 h after the incubation. However, in some case, we found brain slices that are incubated more than 44 h start to exhibit loss of tissue quality and were gradually deteriorating. The loss of viability and tissue integrity can be caused by either external (e.g., bacterial infection) or internal factors (e.g., tissue damage during the slice preparation and subsequent release of toxic materials) (Buskila et al., 2014). To avoid bacterial infection, experimental tools and area should be sterilized and maintained clean. To ensure viability, hippocampal neurons of cultured slices can be tested for electrophysiological activity (Ting et al., 2018). If applicable, shorter culture time (< 24 h) of brain slices is desired to avoid deterioration of tissue quality.

TROUBLESHOOTING

Problem 1

Cultured brain slices lose viability and tissue integrity (steps 1–20 in the preparation of acute mouse brain slices from adult mice sub-section).

Potential solution

Good handling of equipment and tools are desired for healthy brain slices preparation. Use 70% ethanol to clean up the slice culture areas and tools before procedures are strongly recommended to avoid bacterial contamination. Always move slices slowly and smoothly and avoid strong vibrations. Make sure not to touch slices with any surgical or sharp tools. Shorter time of slice preparation, transferring and incubation can reduce the chance of viability issues. During prolonged incubation time, non-optimal preparation and/or condition can cause brain slices to become progressively fragile and lose their structural integrity. Those slices may look wrinkled and pale/white, as compared to healthy ones, and should not be used for experiments. Electrophysiological recordings in deteriorated slices will reveal progressive and rapid decrease in the amplitude and frequency of extracellular multiple unit activity (MUA) or lack of spontaneous neuronal activity.

Problem 2

Brain tissue tears off while sectioning with vibratome (steps 7 and 12 in the preparation of acute mouse brain slices from adult mice sub-section).

Potential solution

Sectioning speed and blade frequency may be not optimal. Practice working with the control settings without a blade holder inserted. Only insert the blade holder when you are completely familiar with all control functions of vibratome. For sectioning, perform blade feed to approach the brain surface at speed 2.5 mm/s. When blade edge is 2–3 mm close to the brain surface, stop the feed, set up a start limit position and reduce sectioning speed to ~0.25 mm/s. Perform one complete section cut and stop the feed for setting the limit stop of the sectioning window. Practice working with controls for optimization of sectioning speed and blade frequency.

Problem 3

Brain sections (slices) are not being cut evenly (steps 11–13 in the preparation of acute mouse brain slices from adult mice sub-section).

Potential solution

Practice dissecting the brain perpendicularly along the rostral (anterior)-caudal (posterior) axis of the brain. Before slicing, fix a piece of agar with adhesive glue (super glue) on the specimen to provide additional support to place the brain for slicing (Figures 1E and 1F). Gently apply optimal amount of the fast adhesive glue to the specimen disc and fix the brain for coronal slicing. Always transfer brain slowly and smoothly to avoid any potential damage.

Problem 4

Sections remain attached at the ventral side of the brain after cutting (steps 11–13 in the preparation of acute mouse brain slices from adult mice sub-section).

Potential solution

First, make sure that the blade is completely passing through the length of the tissue being cut according to any programmed settings for automatic cutting on the vibratome. Second, make sure that the agar support block is of sufficient height and width to support the tissue. The blade can even be programmed to cut through the tissue and into the agar block. If the blade is stopped after cutting and some tissue remains intact, a small plastic spatula can be run along the blades edge to cut through any remaining connected pieces of tissue.

Problem 5

Too low or no mRNA expression (high Ct value; step 47 in the RT-qPCR analysis of Bace1 mRNA sub-section).

Potential solution

One possibility is that the isolated RNA from brain slices is degraded. To check this possibility, run an RNA gel with an RNA sample from whole brains as a control. 1% agarose or 1% bleach-agarose that contains sodium hypochlorite bleach can be used to make an RNA gel. Total RNA generally exhibits two clear bands for 28S and 18S rRNA. If the two bands are not clear and look smeared, it indicates the RNA is degraded. Alternatively, RNA integrity can be determined by Qubit RNA IQ assay (Thermo Fisher Scientific, cat# Q33221). Another possibility of no mRNA expression is that RNA pellet may be lost during the RNA isolation procedures. The RNA pellet from a cortical piece of one brain slice is very small and barely visible at the bottom of Eppendorf tube after alcohol precipitation (step 38). Be careful not to throw away the RNA pellet during subsequent pellet washing steps. If the pellet is too small or almost invisible, to facilitate the alcohol precipitation, RNAase-free glycogen (10 μg) can be added to the aqueous phase before adding isopropanol (step 38).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jaehong Suh (suh.jaehong@mgh.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.S.; Methodology, V.D., A.J.F., B.A.D., and J.S.; Investigation, V.D. and J.S.; Writing – Original Draft, V.D. and J.S.; Writing – Review & Editing, V.D., A.J.F., B.A.D., and J.S.; Funding Acquisition, J.S.; Supervision, K.J.S. and J.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Buskila, Y., Breen, P.P., Tapson, J., van Schaik, A., Barton, M., and Morley, J.W. (2014). Extending the viability of acute brain slices. Sci. Rep. *4*, 5309.

Dzhala, V., Valeeva, G., Glykys, J., Khazipov, R., and Staley, K. (2012). Traumatic alterations in GABA signaling disrupt hippocampal network activity in the developing brain. J. Neurosci. *32*, 4017–4031.

Kim, D.Y., Carey, B.W., Wang, H., Ingano, L.A., Binshtok, A.M., Wertz, M.H., Pettingell, W.H., He, P., Lee, V.M., Woolf, C.J., et al. (2007). BACE1 regulates voltage-gated sodium channels and neuronal activity. Nat. Cell Biol. *9*, 755–764.

Matilla, A., Roberson, E.D., Banfi, S., Morales, J., Armstrong, D.L., Burright, E.N., Orr, H.T., Sweatt, J.D., Zoghbi, H.Y., and Matzuk, M.M. (1998). Mice lacking ataxin-1 display learning deficits and decreased hippocampal paired-pulse facilitation. J. Neurosci. *18*, 5508–5516.

Suh, J., Romano, D.M., Nitschke, L., Herrick, S.P., DiMarzio, B.A., Dzhala, V., Bae, J.S., Oram, M.K., Zheng, Y., Hooli, B., et al. (2019). Loss of ataxin-1 potentiates Alzheimer's pathogenesis by elevating cerebral BACE1 transcription. Cell *178*, 1159–1175.

Thomas-Crusells, J., Vieira, A., Saarma, M., and Rivera, C. (2003). A novel method for monitoring surface membrane trafficking on hippocampal acute slice preparation. J. Neurosci. Methods *125*, 159–166.

Ting, J.T., Lee, B.R., Chong, P., Soler-Llavina, G., Cobbs, C., Koch, C., Zeng, H., and Lein, E. (2018). Preparation of acute brain slices using an optimized N-Methyl-D-glucamine protective recovery method. J. Vis. Exp. *132*, e53825.