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Immunoprecipitation of ribosome-bound mRNAs from astrocytic perisynaptic processes of the mouse hippocampus.

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Summary

RNA compartmentalization and local translation is an evolutionary mechanism occurring in polarized cells. It has been observed in astrocytes, whose processes contact blood vessels and synapses. Here, we describe a protocol for the purification of the entire pool of ribosome-bound mRNAs in perisynaptic astrocytic processes (PAPs). Our procedure combines the preparation of synaptogliosomes with a refined translating ribosome affinity purification (TRAP) technique. This approach can be developed in any brain region to probe the physiological relevance of local translation in PAPs.

For complete details on the use and execution of this protocol, please refer to (Mazaré et al., 2020).

Graphical Abstract

Before You Begin

Day 1

Timing: 2-3 hours

Make sure all equipment are clean and RNase-free before use: clean bench and pipettes with RNase ZAP™, bake all glass materials at more than 200°C for a few hours in a dry oven.

1. Prepare 10% NP-40 (Igepal CA-630) by dissolving 2 mL of stock solution in 18 mL DEPC-treated H₂O for a total volume of 20 mL.

Note: Keep in mind that NP40 is very viscous, which does not dilute easily into water. We thus recommend to prepare it well in advance and store it at 4°C.

2. Prepare buffer solutions (see Material and Equipment section).
3. Prepare pre-clean and immunoprecipitation (IP) tubes (**Fig. 1**)

Note: we have added two pre-cleaning steps to the initial translating ribosome affinity purification (TRAP) protocol (Heiman et al., 2014) in order to remove non-specific binding of mRNAs. Therefore, in addition to the IP tube, it is necessary to prepare a tube with no antibody (pre-clean tube 1) and a tube with non-specific mouse IgG (pre-clean tube 2). The IP tube also contains yeast tRNA and BSA in order to saturate non-specific binding sites. **We advise to prepare the tubes one day before the immunoprecipitation** and incubate with antibodies overnight at 4°C, but this can also be performed right before the synaptosome extraction by incubating the tubes for 2 h at room temperature. We recommend the use of protein G beads for the IP because mouse antibodies' binding strength to protein G is higher than protein A.

- a. Place 600 µl Protein G beads per sample in a tube on a magnetic holder, wait 30 seconds for the solution to clear and remove supernatant, making sure not to disrupt the beads with the micropipette tip.
- b. Add 1 mL 0.15 M KCl Wash Buffer to the tube and invert several times. If necessary, perform a short-pulse spin to remove drops from the wall and lid of the tube. Place the tube back in the magnetic holder, wait 30 seconds and remove supernatant. Repeat twice more for a total of three washes.
- c. Resuspend beads in 600 µl 0.15 M KCl after the last wash. Aliquot 200 µL of resuspended Protein G beads into three individual tubes.
- d. Place the first tube aside (pre-clean tube 1) and keep it at 4°C. It will be used for the first pre-cleaning step.
- e. Place the second tube (pre-clean tube 2) in the magnetic holder, remove the supernatant and add 25 µg mouse IgG (3 mg/mL) to 175 µl 0.15 M KCl Wash buffer. Resuspend the beads and **incubate overnight at 4°C** or 2 h at room temperature on slow tilt rotation. This will be used for the second pre-cleaning step.
- f. Place the third tube (IP tube) in the magnetic holder, wait 30 seconds and remove the supernatant, then proceed as follows:
 - i. Add 0.2 mg yeast tRNA (0.1 mg/100 µl beads) and 2% BSA to the IP tube in 1 mL 0.15 M KCl Wash Buffer (final volume) and **incubate 1 h at 4°C** on slow tilt rotation.
 - ii. After 1 h, place the tube in the magnetic holder, wait 30 seconds for the solution to clear and remove supernatant. Add 1 mL 0.15 M KCl Wash Buffer to the tube and invert several times. If necessary, perform a short pulse spin to

remove drops from the wall and lid of the tube. Place the tube back in the magnetic holder, wait 30 seconds and remove supernatant. Repeat twice more for a total of three washes.

- iii. Add 50 μ g mouse anti-eGFP 19C8 and 50 μ g mouse anti-eGFP 19F7 in 175 μ l final volume of 0.15 M KCl Wash Buffer to IP tube and **incubate overnight at 4°C** or 2 h at room temperature on slow tilt rotation.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GFP 19C8 and 19F7 bioreactor supernatant	Sloan Kettering Institute, New York	n/a
Chicken polyclonal anti-GFP	Aves	Cat#1020
Rabbit polyclonal anti-Homer1	Synaptic Systems	Cat#160003
Mouse monoclonal anti-VGluT1	Synaptic Systems	Cat#135511
qPCR probes		
Cacna1a	ThermoFisher	Mm00432190_m1
Slc12a5	ThermoFisher	Mm00803929_m1
Grin1	ThermoFisher	Mm00433790_m1
Bsn	ThermoFisher	Mm00464452_m1
Slc1a2	ThermoFisher	<u>Mm01275814_m1</u>
RNA45S (18S)	ThermoFisher	Mm04277571_s1
Chemicals, Peptides, and Recombinant Proteins		
RNase ZAP™	Invitrogen	AM9782
UltraPure™ DEPC-treated water	ThermoFisher	750024
Cycloheximide (CHX)	Sigma-Aldrich	C7698-5G
Dynabeads™ Protein G for immunoprecipitation	ThermoFisher	10004D
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	4693159001
1,2-diheptanoyl-sn-glycero-3-phosphocholine (07:0 PC DHPC)	Avanti	850306P-500MG
Mouse IgG Isotype control	Invitrogen	10400C
tRNA from brewer's yeast	Roche	10 109 517 001
Hepes Buffer solution (1M)	Gibco	15630056
KCl (2M), Rnase-free	Invitrogen	AM9640G
MgCl ₂ (2M)	Invitrogen	AM9530G
DL-Dithiothreitol (DTT)	Sigma-Aldrich	43815-1G
Igepal® CA-630 (NP40)	Sigma-Aldrich	I8896-50ML
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A4503-100G
Sucrose	Euromedex	200-301
Ribonuclease inhibitor recombinant 4x2500u (40u/ μ L)	Euromedex	09-0312
RNeasy Lysis Buffer (Buffer RLT)	Qiagen	79216
Experimental Models: Organisms/Strains		

Mouse: Tg(Aldh1l1-eGFP/Rpl10a) JD130Htz	Nathaniel Heintz's laboratory (Rockefeller University, New York City, NY)	MGI:5496674 www.bactrap.org
Other		
Magnetic holder (Pureproteome™ Protein A Magnetic Bead System)	Merck Millipore	11710353
Rotoflex Tube Rotator	Argos Technologies	FV-04397-33
1mL tissue Grinder, Dounce, with Tight Pestle	Wheaton	357538
Tissue Grinder, Potter Elvehjem, with PTFE Pestle	DWK Life Sciences	SCERSP886000-0019
ES Overhead stirrer (driver unit for the tissue grinder)	Velp Scientifica	F201A0152
Microcentrifuge	Eppendorf	5415R
1.5 mL Eppendorf tubes	Eppendorf	0030120086
Nanodrop	LabTech	ND-1000
Capillary electrophoresis 2100 Bioanalyzer instrument	Agilent	G2939BA
Agilent RNA 6000 Pico Kit	Bioanalyser Agilent	5067-1513

Note: Any other tube rotator and magnetic tube holder will be suitable for this protocol. Any benchtop centrifuge which goes up to 20,000 x g can be used to perform this protocol.

Materials and Equipment

0.15 M KCl Wash Buffer

Reagent	Final Concentration (mM or μ M)	Amount
Hepes-KOH (1 M, pH 7.2-7.5)	10 mM	250 μ L
MgCl ₂ (1 M)	5 mM	125 μ L
KCl (2 M)	150 mM	1875 μ L
NP-40 (10%)	1%	2.5 mL
DEPC-treated water	n/a	20.25 mL
Total	n/a	25 mL

Synaptosome Buffer (SB)

Reagent	Final Concentration (mM or μ M)	Amount
Sucrose	0.32 M	1.1 g
Hepes-KOH (1 M, pH 7.2-7.5)	10 mM	100 μ L
DEPC-treated water	n/a	9.9 mL
Total	n/a	10 mL

TRAP Homogenization Buffer

Reagent	Final Concentration (mM or μ M)	Amount
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Hepes-KOH (1 M, pH 7.2-7.5)	10 mM	100 μ L
MgCl ₂ (1 M)	5 mM	50 μ L
KCl (2 M)	150 mM	750 μ L
DEPC-treated water	n/a	9.1 mL
Total	n/a	10 mL

0.35 M KCl Wash Buffer

Reagent	Final Concentration (mM or μ M)	Amount
Hepes-KOH (1M, pH 7.2-7.5)	10 mM	200 μ L
MgCl ₂ (1M)	5 mM	100 μ L
KCl (2M)	350 mM	3.5 mL
NP-40 (10%)	1%	2 mL
DEPC-treated water	n/a	14.2 mL
Total	n/a	20 mL

Step-by-Step Method Details

Synaptogliosome extraction

DAY 2**Timing: 1 - 2 hours**

This step aims at purifying synaptogliosomes (**Fig. 2A**). Synaptogliosomes are composed of pre- and postsynaptic compartments along with the PAP, which surrounds the synapse. This protocol enables the conservation of these compartments' cellular contents, including mRNAs and ribosomes. The following protocol has been developed on **hippocampal tissue** from **adult (P60 and older) mice**. Performing this protocol on younger brain tissue and on different brain regions might require adjustments in homogenization volume and sucrose concentrations.

Note: All steps are performed on ice, in RNase-free conditions.

1. Prepare CHX at stock concentration 100 mg/mL in MetOH; Prepare DTT at stock concentration 100 mM in DEPC-treated water.
2. Set centrifuge to 4°C.

Note: DTT stock solution can be prepared beforehand and stored as aliquots at -20°C for several months. Once defrosted, aliquots cannot be re-used. CHX should be prepared right before use.

3. Add the following to 10 mL Synaptogliosome buffer (SB) right before use:
 - a. 1 tablet of cComplete™, Mini, EDTA-free Protease Inhibitor Cocktail
 - b. 10 μ L CHX (100 μ g/mL)

- c. 5 μ l 1 M DTT (0.5 mM)
 - d. 10 μ l Ribonuclease inhibitor (1 μ l/mL)
4. Add 800 μ l SB in a 1 mL tissue Grinder kept on ice.
 5. Extract mouse brain and dissect hippocampus. Place one hippocampus directly in the tissue grinder, place the other one in a small cell culture dish filled with SB.
 6. Homogenize one hippocampus by applying 20-30 strokes with a tight glass pestle.

Critical: Make sure the pestle does not rise above solution to avoid forming air bubbles.

7. Transfer the homogenate to 1.5 mL tubes and keep on ice.
8. Repeat step 5 with the second hippocampus.
9. Spin the two tubes at 900 x g for 15 min at 4°C in microcentrifuge.
10. Collect supernatant (S1) fractions and transfer to new tubes. Discard pellet (P1).
11. Spin S1 at 16,000 x g for 15 min at 4°C.
12. Discard supernatants and resuspend pellets (P2) with 600 μ l synaptogliosomes buffer (SB).
13. Spin again at 16,000 x g for 15 min at 4°C.
14. Meanwhile, add Ribonuclease inhibitor (1 μ l/mL final), CHX (100 μ g/mL final) and DTT (0.5 mM final) to TRAP homogenization buffer.
15. At the end of the centrifugation, discard supernatants. The pellets (P3) contain the synaptogliosomes fraction.
16. Resuspend the first pellet in 500 μ l of TRAP homogenization buffer and take this homogenate to resuspend the second pellet. The preparations from both hippocampi are now pooled (500 μ l total volume).

Note: It is necessary to test the quality of the synaptosome. It can be done by Western blot (Fig. 2B). Compared with the first supernatant obtained from hippocampal homogenate (S1), the synaptogliosome fraction (P3) should contain a lower level of GFAP and higher levels of the postsynaptic protein PSD95 and the cytosolic PAP protein Ezrin (Derouiche and Frotscher, 2001; Lavalie et al., 2011). Rpl10a-eGFP should also be detected in this fraction, indicating that the P3 fraction comprises astrocytic ribosomes (Fig. 2B). The presence of astrocyte ribosomes and synapses in the P3 fraction can also be visualized via the immunofluorescent detection of eGFP, VGlut1 (pre-synapse) and Homer1 (post-synapse) (Fig. 2C). Histone 3, a nuclear marker, is present in the total tissue fraction but should be absent from both S1 and P3 fractions (not shown).

TRAP

Timing: 3 hours

This step consists in extracting astroglial ribosome-bound mRNAs from the previously isolated synaptogliosomes from Tg(Aldh1l1-eGFP/Rpl10a) JD130Htz mice (Fig. 3A).

17. Get the pre-clean 1, pre-clean 2 and IP tubes.

18. Place the tubes in the magnetic holder, wait 30 seconds and remove supernatants. Add 1 mL 0.15 M KCl Wash Buffer to the tube and invert several times. If necessary, perform a short-pulse spin to remove drops from the wall and lid of the tube. Place the tubes back in the magnetic holder, wait 30 seconds and remove supernatants. Repeat twice more for a total of three washes. Store the tubes on ice in 0.15 M KCl Wash Buffer.

Critical: Make sure you leave at least 200 μ L 0.15 M KCl Wash Buffer on tubes to avoid the beads from drying.

19. Add Ribonuclease inhibitor (1 μ L/mL final), CHX (100 μ g/mL final) and DTT (0.5 mM final) to 0.15 M Wash KCl Wash Buffer and 0.35 M KCl Wash Buffer.
20. Transfer the homogenate to the 1 mL tissue grinder potter-elvehjem kept on ice.
21. Connect the pestle to the ES overhead stirrer, preferably installed in a cold room, and insert it into glass tube. Gently rise the speed to 900 rpm and perform 20 strokes. Transfer to a 1.5 mL tube on ice.

Critical: Make sure the pestle does not rise above solution to avoid forming air bubbles.

Critical: Ensure that the homogenate stays at 4°C during the whole homogenization procedure.

22. Spin at 2,000 \times g for 10 min at 4°C.
23. Transfer the supernatant to a new tube and add NP-40 to 1% final concentration and DHPC to 30 mM final concentration.
24. Mix by inversion and incubate for 5 min on ice.
25. Spin at 20,000 \times g for 15 min at 4°C.
26. Place pre-clean 1 tube on the magnetic holder, wait 30 seconds for the solution to clear and discard extra 0.15 M KCl Wash buffer.
27. Transfer the supernatant to the pre-clean tube 1 and incubate for 30 min at 4°C on slow tilt rotation using the Rotoflex Tube Rotator.
28. Place the pre-clean tube 2 on the magnetic holder, wait 30 seconds for the solution to clear and discard extra 0.15 M KCl Wash buffer.
29. Place the pre-clean tube 1 on the magnetic holder, wait 30 seconds for the solution to clear and transfer the supernatant from the pre-clean tube 1 to the pre-clean tube 2. Incubate for 30 min at 4°C on slow tilt rotation using the Rotoflex Tube Rotator.
30. Place the IP tube on the magnetic holder, wait 30 seconds for the solution to clear and discard extra 0.15 M KCl Wash buffer.
31. Place pre-clean 2 tube on the magnetic holder, wait 30 seconds for the solution to clear and transfer the supernatant to the IP tube. Add 20 μ L of 300 mM DHPC, resuspend and incubate for 30 min at 4°C on slow tilt rotation using the Rotoflex Tube Rotator.
32. Collect IP beads with magnetic holder, wait 30 seconds until the solution clears, then place the supernatant, which contains the unbound fraction, in a new tube.
33. Store the unbound fraction at -80°C.

Note: Astroglial ribosome-bound mRNAs are now bound to the anti-GFP-coated magnetic beads.

Note: After immunoprecipitation, the 0.35 M KCl Wash Buffer is used for a more stringent wash.

34. Add 1 mL 0.35 M KCl Wash Buffer to the IP beads and invert several times. If necessary, perform a short pulse spin to remove drops from the wall and lid of the tube. Place the IP tube back in the magnetic holder, wait 30 seconds and remove supernatants. Repeat twice more for a total of three washes.
35. Place the IP tube back in the magnetic holder, wait for the solution to clear and discard extra 0.35 M KCl Wash buffer, making sure all liquid is removed.
36. Add 300 μ L RLT (RNeasy lysis buffer), remove the magnetic holder, resuspend beads and incubate for 5 min at room temperature.

Note: the ribosome-bound mRNAs are now detached from the beads and free in the supernatant.

37. Collect beads with the magnetic holder, wait until the solution clears and transfer the supernatant to a new 1.5 mL tube.

Critical: Make sure no beads are transferred with the RLT supernatant.

Pause point: Ribosome-bound mRNAs can be store at -80°C in RLT for several months as well as the unbound fraction.

38. Proceed to mRNA extraction following the RNeasy Mini kit standard protocol. Elute RNA in 30 μ L of RNase-free water.

Note: Yeast tRNAs are about 70 bp length and are therefore lost during the RNA purification step. It is possible to test the purity of the ribosome-bound PAP mRNA extraction with regards to neuronal transcripts by qPCR, comparing in the unbound vs. TRAP fraction the level of mRNAs such as Grin1, Slc12a5, Cacna1a or Bsn, which have previously been detected in the synapses or in the neuropil of the hippocampus (Cajigas et al., 2012; Hafner et al., 2019) (**Fig. 3B**). The level of mRNAs from other neural cell types, including oligodendrocytes, vascular cells (endothelial cells, and mural cells (pericytes and vascular smooth muscle cells)) and microglia, were also verified by RNA-Seq and found to be below the threshold of detection (**Fig. 3C**).

Expected Outcomes

RNA yield from TRAP performed on synaptogliosomes from two mouse hippocampi is compatible with RNASeq and qPCR techniques. Expected Nanodrop RNA concentration for Unbound samples range from 10 to 25 ng/ μ L for synaptogliosomes from two hippocampi. TRAP samples present RNA concentrations around 2 ng/ μ L. Ribosomal RNAs bands at 2 kb (RNA 18S) and 4 kb (RNA 28S) should be visible by capillary electrophoresis (**Fig. 3D**).

See **Figure 2B-C** for expected outcomes on synaptogliosome protein content (**Fig. 2B**) and immunostaining (**Fig.2C**).

See **Figure 3B-C** for expected outcomes on TRAP purity.

Limitations

This protocol was optimized for isolation of synaptogliosomes from two dorsal or whole hippocampi. It works in the same conditions for entire hippocampi for the extraction of polysomes from whole astrocytes. If you choose another part of the brain, the yield and purity of the synaptogliosomes extraction may change. You may need to adjust the volumes for extraction to the quantity of tissue, as well as the number of strokes for homogenization.

Troubleshooting

Problem 1:

No RNA is detected, suggesting a degradation of mRNAs

Potential Solution:

1. Make sure you perform all experiments in clean, RNase free conditions. Autoclave tissue grinder before use, disinfect pipettes and lab benches with RNase ZAP™. Change your gloves regularly over the course of the experiment.
2. Do not increase incubation times. mRNAs are very fragile, unstable structures and the longer the experiment, the more mRNA you will lose. 30 min for each pre-clean step and immunoprecipitation step is enough to obtain a good, uncontaminated RNA content.

Problem 2:

Contamination by neuronal transcripts is observed.

Potential Solution:

1. Make sure that all pre-clean steps have been properly performed. Neuronal contamination can be assessed by qPCR or RNAseq.

Problem 3

RNA yield below Nanodrop threshold of detection.

Potential Solution:

The RNA yield for synaptogliosomes is usually lower than for a whole tissue RNA extraction, because PAP RNAs represent a small fraction of all astroglial RNAs. Our IP conditions have been set with highly specific mouse monoclonal anti-GFP antibodies (Heiman et al., 2014), **which should not be replaced by other antibodies**. In addition, it is very important to perform all experiments with fresh CHX, which guarantees the integrity of the polysomes.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martine Cohen-Salmon, martine.cohen-salmon@college-de-france.fr

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Author Contributions

Conceptualization, M.C.-S.; Methodology, M.C.-S., N.M., G.C. and A.C.B.; Investigation: A.C.B., N.M., M.O., G.C.; Writing – Original Draft, N. M.; Funding Acquisition, M.C.-S.; Supervision, M.C.-S.

Declaration of Interests

We declare no conflict of interest.

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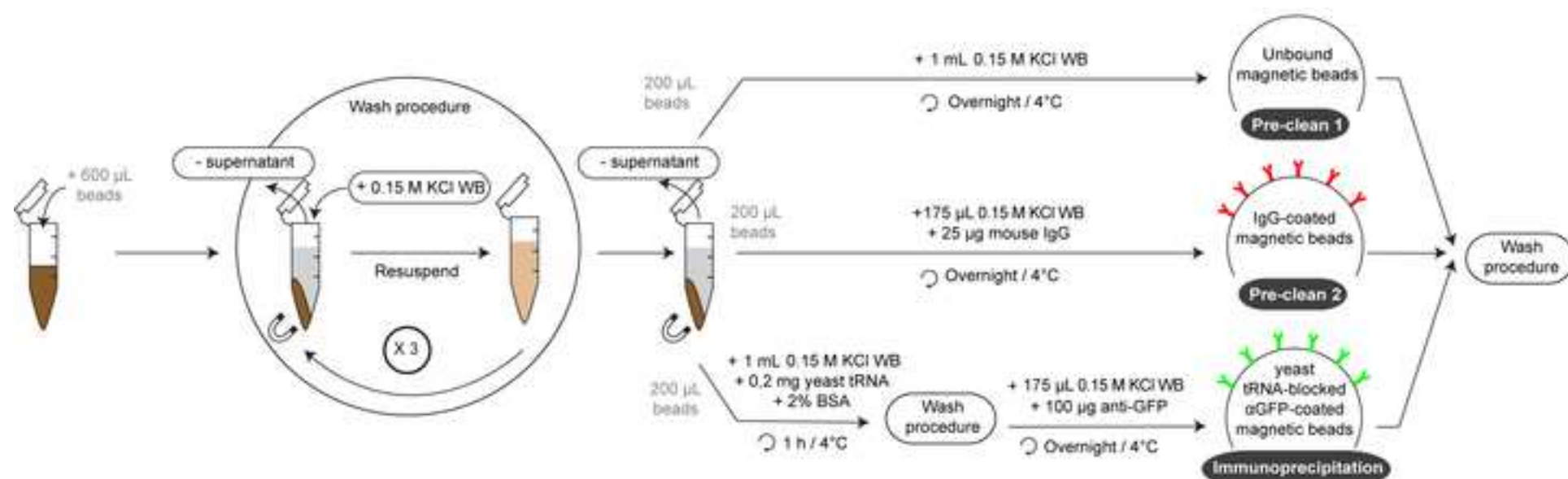
Figure Legends

Figure 1. Preparation of the pre-clean and immunoprecipitation (IP) tubes.

Figure 2. Synaptogliosomes preparation. **A.** Flowchart of the synaptogliosome preparations from the hippocampus of *Aldh1l1:L10a-eGFP* transgenic mice. **B.** Western blot comparison of S1 and P3 fractions. * $p=0.05$, ** $p=0.01$, and ns (not significant) in a two-tailed paired *t* test. The data are presented as mean \pm SEM ($n = 4$). **C.** A confocal immunofluorescence microscopy image of synaptogliosomes from an *Aldh1l1:L10a-eGFP* mouse. The ribosomes in PAPs were immunolabeled with eGFP (green). Pre- and postsynaptic areas were immunolabeled for VGluT1 (red) and Homer1 (blue), respectively. Scale bar: 2 μm .

Figure 3. Optimized Translating Ribosome Affinity Purification (TRAP). **A.** Flowchart for TRAP of ribosome-bound mRNAs extracted from *Aldh1l1:L10a-eGFP* transgenic mice. **B.** qPCR comparison of a set of synapse-enriched mRNAs between the Unbound vs. TRAP fraction of hippocampal synaptogliosomes. * $p=0.05$ in a one-tailed Mann-Whitney test. From Mazaré et al. 2020 **C.** Purity heat map of RNA-seq data for TRAP on synaptogliosomes for a selection of mRNAs specific for each type of brain cell. The centered value represents mRNAs with \log_2 (500 normalized reads), which corresponds to our threshold for the presence of an mRNA. Each column represents an independent cDNA library ($n = 3$ libraries). From Mazaré et al. 2020. **D.** Capillary electrophoresis of RNAs extracted from synaptogliosomes from dorsal hippocampi (PAP-TRAP). As a comparison, capillary electrophoresis of RNAs extracted from whole dorsal hippocampi (Astro-TRAP) is also shown.

Figure 1



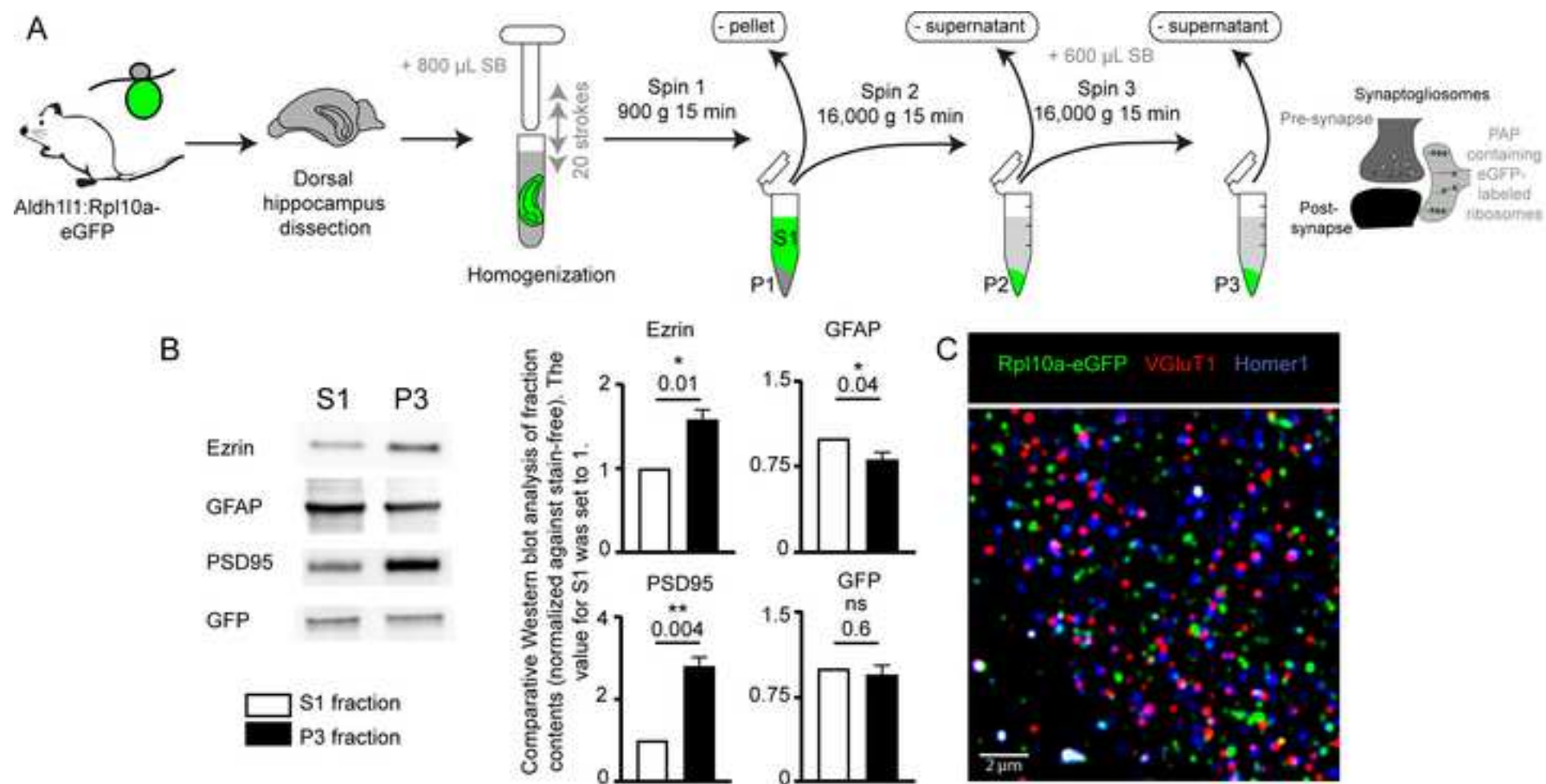
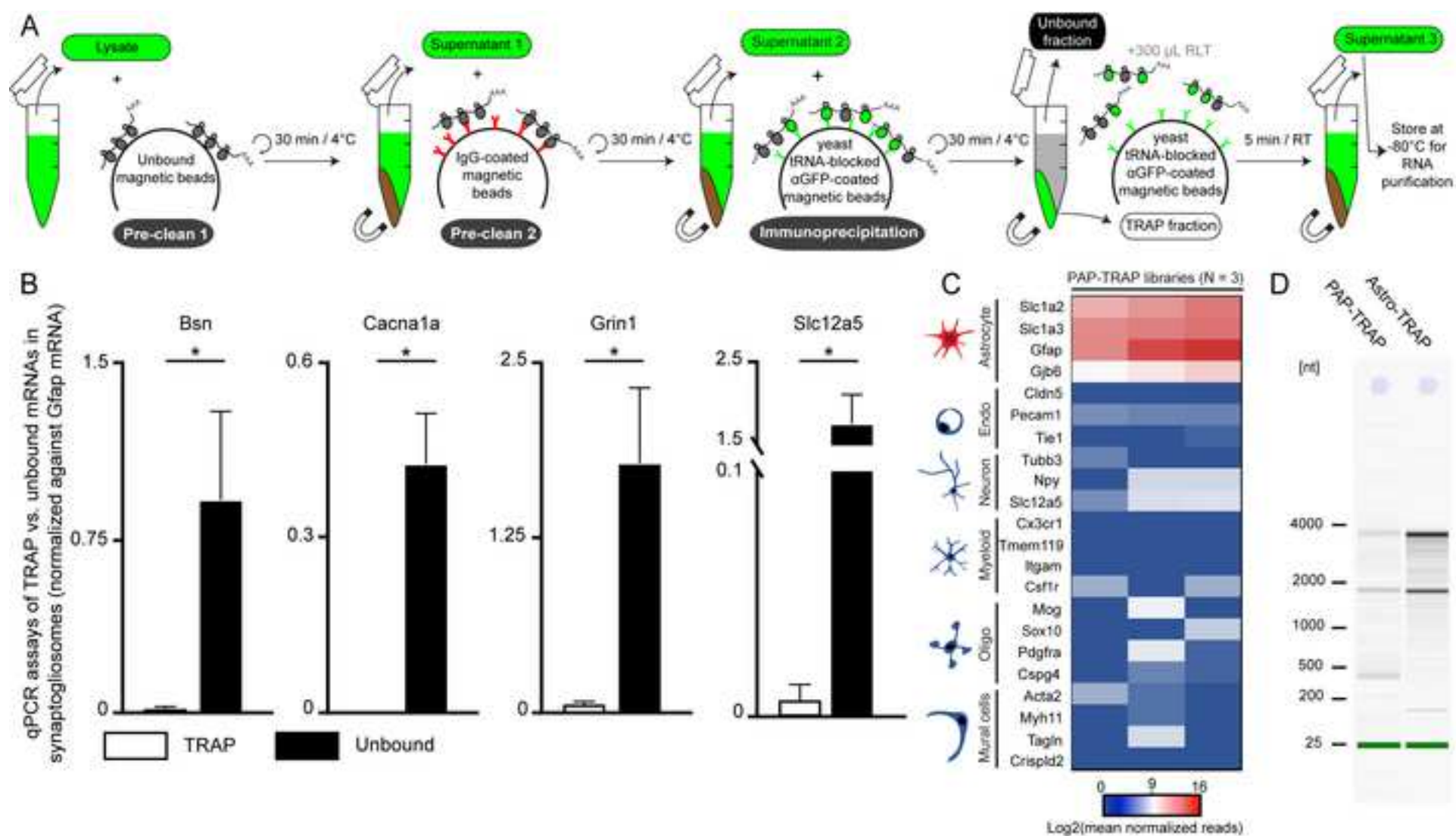
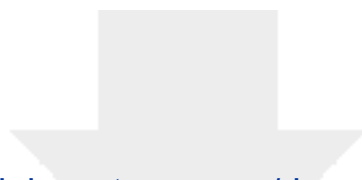


Figure 3

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