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HIGHLIGHTS
LA-N-2 and LA-N-5 are female and male neuron-like cell lines
They can be differentiated to a cholinergic phenotype using neurokines
This protocol describes cell culture, differentiation process, and post-processing

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Establishing Human Male and Female Models of Cholinergic Neurons via Neurokine-Mediated Differentiation of LA-N-2 and LA-N-5 Neuroblastoma Cells

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SUMMARY
Cholinergic neurons control numerous primate-specific and sexually dimorphic brain functions. Here, we present our differentiation protocol for the closely related human female and male neuroblastoma-originated cell lines LA-N-2 and LA-N-5. Pro-cholinergic differentiation (with upregulation of choline acetyltransferase) of both lines can be achieved using neurokines such as ciliary neurotrophic factor (CNTF). Comparative RNA sequencing and mass spectrometry analyses between those two cell lines, supported by experimental intervention, will deepen our understanding of cholinergic systems in human psychiatric and neurologic disease.

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

BEFORE YOU BEGIN

Note: Abbreviations: CHAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; IL-6, interleukin 6; LIF, leukemia inhibiting factor; PBS, phosphate-buffered saline; RPMI 1640, Roswell Park Memorial Institute (Medium); SLC18A3, solute carrier, vesicular transporter of acetylcholine.

1. Initially: Establish a stable culture of LA-N-2, LA-N-5, or both. Experiments should only be performed after a minimum of two passages after thawing to enable equilibration, and inside a low range of total passages to avoid genetic drift. Acetylcholine production declines during continuous culture, and a limit of 25 passages has been previously suggested (Crosland, 1996). LA-N-2 and LA-N-5 cells can be passaged approximately once a week.
2. Initially: Prepare stock solutions and store aliquots at –20°C.
3. On each day of experiment: Warm media and other experimental substances to 37°C, as needed.
4. All procedures are performed in a cell culture laboratory using a laminar flow hood and a humidified incubator at 37°C with 8% CO₂.
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| CNTF, human, recombinant | Thermo Fisher Scientific | PHC7015 |
| Glycogen, RNA-grade | Thermo Fisher Scientific | R0551 |
| Phenol/guanidinium thiocyanate solution (TRizol) | Thermo Fisher Scientific | 15596018 |
| Roswell Park Memorial Institute (RPMI 1640) medium | Thermo Fisher Scientific | 11530586 |
| Dulbecco’s modified Eagle’s medium (DMEM) | Thermo Fisher Scientific | 41965039 |
| Fetal calf serum (FCS) | Thermo Fisher Scientific | 26140079 |
| Penicillin/streptomycin (100x) | Thermo Fisher Scientific | 10378016 |
| Trypan blue solution | Thermo Fisher Scientific | 15250061 |
| Dulbecco’s phosphate-buffered saline (PBS) | Thermo Fisher Scientific | 14190250 |
| **Experimental Models: Cell Lines** | | |
| LA-N-2 | DSMZ | ACC 671 |
| LA-N-5 | DSMZ | ACC 673 |
| **Oligonucleotides** | | |
| CHAT primer | This paper and Lobentanzer et al., 2019 | n/a |
| FW: CAC TTG GTG TCT GAG CA, RV: AGT TTC TGC TGC AGG GTC TC | | |
| **Other** | | |
| Aspirating pipettes | n/a | n/a |
| Centrifuge | n/a | n/a |
| Centrifuge tubes | n/a | n/a |
| Microcentrifuge tubes (2 mL) | n/a | n/a |
| Hemocytometer (for cell counting) | n/a | n/a |
| Vortex machine | n/a | n/a |
| Heating block for microcentrifuge tubes | n/a | n/a |
| Nanodrop 2000 | Thermo Fisher Scientific | ND-2000 |

**Note:** Also required are standard cell culture laboratory equipment and consumables such as pipetting equipment (for volumes 5 mL, 10 mL, 25 mL, 50 mL), micro-pipettors and tips (1–10 μL, 10–100 μL, 100–1,000 μL). Electric pipettors can be used to reduce handling time and potential errors.

**Note:** For RNA isolation, an RNase-free workspace with separate micro-pipettors, tubes, a vortex machine (not RNase-free), and a heating block for microcentrifuge tubes (not RNase-free) are recommended; for measuring of RNA concentrations, a Nanodrop 2000 (Thermo Fisher Scientific) or equivalent.

**Alternatives:** All described materials can be purchased from any available source, so long as they are equivalent to the ones given in the KRT.

## MATERIALS AND EQUIPMENT

### Prepare CNTF Stock Solution

© Timing: 30 min
CNTF (and other peptides of the neurokine family, such as LIF and IL-6) can be purchased in freeze-dried form, which can be easily resuspended and is stable at –20°C to –80°C for months. Please refer to your vendor’s instructions for more information on aliquotation and storage.

Add water to lyophilized CNTF. Gently pipette up and down five times. Let the suspension rest for approximately 5 min at 15°C–25°C. Gently pipette up and down five more times. Store in aliquots at –20°C or –80°C.

### Prepare Culture Medium

**Timing:** 30 min

LA-N cells grow best at a very high serum content of 20% and incubated at 8% CO₂. The medium can contain antibacterial substances (usually, penicillin/streptomycin), but should not need to, given perfectly sterile conditions. Their use and the amount added are at the discretion of the researcher (generally, 50–100 units of penicillin/streptomycin per mL of medium are recommended, please refer to your supplier’s instructions).

| Reagent                  | Final Concentration | Amount |
|--------------------------|---------------------|--------|
| RPMI 1640                | 40%                 | 200 mL |
| DMEM                     | 40%                 | 200 mL |
| FCS                      | 20%                 | 100 mL |
| Penicillin/streptomycin  | n/a                 | q.s.   |
| Total                    | 100%                | 500 mL |

- Mix components in any order.
- Store for limited time at 4°C. Reconstituted medium should be stable for up to six months, but should be used up as quickly as possible due to potential contamination.

### STEP-BY-STEP METHOD DETAILS

The LA-N cell lines were developed by R.C. Seeger around 1980 (Seeger et al., 1977, 1982). They are immortalized biopsies of neuroblastoma, from a 3-year-old girl (LA-N-2, Seeger et al., 1977) and a 4-months-old boy (LA-N-5, Seeger et al., 1982). Most studies describing these cell lines had been performed in the 1980s and 1990s, and there is substantially more literature on LA-N-2 than on LA-N-5. Our own study (Lobentanzer et al., 2019) is the first take on LA-N cells using modern methods of molecular biology, most importantly, RNA sequencing. To our knowledge, these cell lines are the closest immortalized semblance of human cholinergic neurons that are currently available.

### Day 0: Seeding

**Timing:** 1–2 h

This step prepares single wells of LA-N cells for experimentation on the following day. Depending on the duration of the experiment, choose a starting number of cells/well that enables near confluency
at the end of the experiment. A good starting point are 200,000 cells in 1 mL of medium in the well of a 12-well plate (surface area 3.5 cm²); this amount will be used exemplarily in the following protocol and should be adjusted to the needs of the experiment. In this context, note that differentiation agents tend to slow the cells’ replication. Differentiation agents are defined substances – usually small molecules or peptides – that coerce cultured cells to undergo phenotypic changes. Well-known and widely used differentiation agents include retinoic acid, 5-azacytidine, and bro-mo-de-oxy-uri-dine (Crosland, 1996). Thus, the more differentiation agent is introduced, the higher the number of seeded cells should be. In this step, make sure that enough culture flasks are available for the total number of cells needed for seeding.

**Note:** Before starting, prepare warm medium in sufficient quantity, hemocytometer, aspiration pipette, 12-well plates.

1. Check the viability and confluency of cells in the culture flasks in an inverted microscope.
2. Collect the cells from their culture flasks.

**Note:** LA-N cells do not adhere very tightly to standard culture flasks, so they can be detached by pipetting along the wall of the flask with a standard 10 mL pipette (electric pipettor).

3. Centrifuge cell suspension at 1,000 $\times$ $g$ for 5 min. Discard supernatant. Resuspend cells in an appropriate amount of fresh warm medium (e.g., 10 mL).
4. Count cells and optionally assess viability.

**Note:** Cells can be counted traditionally in an inverse microscope, using a hemocytometer, and using Trypan Blue, which allows assessment of viability. Modern, digital cell counter solutions can assess viability automatically.

5. Dilute cell suspension to yield your desired cell concentration (e.g., 200,000 cells/mL) using fresh warm medium.
6. Seed cells into 12-well plates using 1 mL of cell-containing medium (i.e., in our example, 200,000 cells/well).

⚠ CRITICAL: LA-N cells are sensitive to ambient changes, resulting in reactions to extended periods outside of the incubator, potentially impacting the results (growth slowdown due to stress, apoptosis). We recommend filling plates only with the amount of samples to be lysed in one sitting. After lysing a number of wells of a plate, do not replace the remaining cells into the incubator to be lysed at a later time. In our experiments, we kept the number of occupied wells per plate at four. That way, all occupied wells can be lysed with adequate speed (see the section on lysis for the exact procedure).

7. Transfer to incubator and incubate 24 h at 37°C, 8% CO₂.

**Day 1: Add Differentiation Agent**

© **Timing:** 5 min

LA-N cells undergo pro-cholinergic differentiation upon stimulation with neurokines such as CNTF, LIF, and IL-6, which can be measured by the elevation of CHAT and SLC18A3 mRNA. The individual responses of the different LA-N lines to the different neurokines can diverge, so a time-dose estimation is recommended when starting experimentation. In our study (Lobentanzer et al., 2019), we used CNTF, of which we used 100 ng/mL for LA-N-2 and 10 ng/mL for LA-N-5, informed by our time-dose experiments. For ease of reading, the following description will assume LA-N-2 conditions.
Note: Before starting, pre-warm stock solution of differentiation agent (CNTF).

8. Visually assert the condition of the culture in an inverted microscope.

Note: For comparison, please refer to the cell culture images by the original creator (Seeger et al., 1977, 1982) and the cell culture vendor (e.g., by the DSMZ at https://www.dsmz.de/collection/catalogue/details/culture/ACC-671 and https://www.dsmz.de/collection/catalogue/details/culture/ACC-673).

9. With minimal disturbance, add pre-warmed aqueous CNTF stock solution to each well to yield a final concentration of 100 ng/mL. In our example with 1 mL of medium in the well and a stock solution of 25 μg/mL of CNTF, this equates to 4 μL of stock solution per well.

10. Replace plates into the incubator and minimally disturb for the duration of the experiment.

△ CRITICAL: LA-N cells appear to react sensitively to changes in ambient temperature and/or CO₂ content. Thus, it is recommended to keep handling time at an absolute minimum through thorough preparation and speedy execution of the steps. For this reason, it is also recommended to supply enough medium at the start of the experiment to enable survival until the end of experimentation. Medium can be replaced (optimally by partial replacement) during the course of the experiment, but the cells handle best if they are not disturbed at all during differentiation. Additionally, medium replacement requires the re-addition of differentiation agent to the fresh medium, introducing another experimental variable. For instance, a part of the CNTF peptide may have been taken up by the cells or degraded, and thus, re-introduction of an amount of fresh CNTF may increase its concentration. However, this may have unforeseeable consequences for the differentiation process and should be done in a reproducible fashion (quantity of replaced medium, exact time point).

Day 5: Cell Lysis

© Timing: 15 min per plate

This step aims to accomplish a quick and thorough lysis of cells to enable accurate measurements of biochemical states. As LA-N cells grow in monolayer, lysis is most easily performed in situ by adding phenol/guanidinium thiocyanate solution (TRIzol) to the cell layer after removal of the supernatant. Depending on the quantitative requirements of subsequent analyses (protein, DNA, or RNA determinations), wells can be combined, taking care that the total amount of TRIzol per sample is 1 mL. For example: when combining two wells into one sample use 500 μL TRIzol per well, for combining four wells into one sample use 250 μL TRIzol per well, and so on. The following exemplary protocol assumes combination of four wells into one sample (i.e., 250 μL of TRIzol per well).

The day of lysis can be modified to adjust for the experimental needs. To ensure minimal handling time and equal treatment of all samples, we adopted a cyclic washing/incubation/transfer protocol, using four wells of a 12-well plate. Each of the following steps is performed on each of the four wells in quick succession, going back to the first well after finishing the step at the last well.

Note: Before starting, prepare warm PBS for washing, TRIzol, aspiration pipette, micro-pipettor and tips, sterile microcentrifuge tubes, ice box.

11. Aspirate cell supernatant (almost) completely and wash with 250–500 μL of warm PBS. Keep PBS in well until the other wells have been similarly treated.

12. Repeat washing step (11) as often as desired (between 1 and 3 times), always keeping cells covered to avoid drying out.
13. Lysis step:
   a. Aspirate as much of the supernatant as possible.
   b. Quickly add TRIzol (250 μL) to the well using a micro-pipettor with a slightly larger tip than
      needed, pipetting up and down until all cells are detached and largely dissolved.
   c. Set a timer to 5 min.

   △ CRITICAL: This is the time-critical step, as the incubation with TRIzol at 15°C–25°C should
   be consistent between all samples/subsamples. The cyclic protocol using four wells was
   devised to allow step 13 to be performed on wells #2–4 in the incubation period of
   5 min for well #1 (100 s to perform step 13 for each of the wells #2–4). Timers can be
   set separately for each well or cumulatively/alternating, as long as the incubation period
   can be adequately monitored.

14. For each well, after the lapse of 5 min of 15°C–25°C incubation (timer), transfer TRIzol containing
    the cell lysate to a 2 mL microcentrifuge tube and place on ice. Combine all wells belonging to
    one sample in the same tube.

15. Store at −20°C for few days or directly proceed to RNA/protein isolation.

   □ Pause Point: TRIzol lysate of cells can be stored at 4°C up to 12 h or at −20°C for up to a year.

Optional: RNA Isolation

© Timing: 4 h

The TRIzol cell lysate can be a source of many soluble biologic materials of the cell, DNA, RNA, and
protein. Exemplarily, we will describe RNA isolation as performed in Lobentanzer et al. (Lobentanzer
et al., 2019). This inexpensive “low-tech” isolation yields high amounts of high-quality RNA and can
be tailored to small RNA processing. However, it can be substituted by modern kit-based solutions,
if so desired. This protocol is modified from the official instructions (http://tools.thermofisher.com/
content/sfs/manuals/trizol_reagent.pdf) for TRIzol issued by Thermo Fisher.

The amount of samples for parallel processing is primarily limited by centrifuge capacity. More
importantly, the air-drying step requires high acuity to avoid over-drying of the pellet, and thus is
a secondary limiting factor for isolation of lots of samples.

Note: Before starting the procedure, prepare an ice box, chloroform, pure ethanol (ice cold),
70% ethanol (ice cold) in autoclaved Milli-Q H₂O, microcentrifuge tubes for phase separation,
fresh RNase-free tubes for storage of aqueous RNA solution, RNA workspace (RNase-free)
including micro-pipettors, preferably inside a laminar flow hood. Set centrifuge to 4°C.

Optional: Phase separation can be enhanced by the use of specialized phase separation
tubes.

Optional: The formation of a visible pellet at small amounts of RNA can be enhanced by the
addition of 5–10 μg of RNase-free glycogen to the supernatant of the first centrifugation step.
Glycogen does not interfere with most subsequent RNA analysis methods. It is recommended
to check suitability for the actual planned analysis.

Optional (if samples are frozen): Retrieve TRIzol lysate from freezer and allow to thaw on ice.

16. Add chloroform to TRIzol lysate at 20% of TRIzol volume (200 μL for 1 mL of sample). Shake
    manually and vigorously (do not vortex) for 15 s.

17. Allow each sample to rest for 2–3 min at 15°C–25°C.
18. First centrifugation: 12,000 × g, 15 min, at 4°C.
19. Retrieve the top, aqueous layer, containing RNA, and transfer to fresh tube. 1 mL of TRIzol sample should yield approximately 500 μL aqueous phase.

*Note:* At this point, the optional glycogen can be added.

20. Add to the 500 μL of RNA-containing supernatant 1–1.25 mL of pure ethanol (ice cold). Mix by inverting and incubate 10–15 min on ice to allow precipitation of RNA.

*Note:* The isopropyl alcohol of the original TRIzol instructions has been replaced by pure ethanol in our workflow, facilitating the air-drying step due to its higher volatility. Final ethanol concentration in the precipitation solution should reach about 70%. See also the section on Troubleshooting for details regarding small RNA yield.

21. Second centrifugation: 12,000 × g, 10 min, at 4°C.
22. Washing the pellet: discard the supernatant of the previous centrifugation step and wash the pellet, using 1 mL of 70% ethanol and a vortex apparatus, for approximately 10 s.

*Note:* Addition of glycogen to the supernatant of step 19 can help with handling of the pellet, making it more visible at low amounts of RNA.

23. Third centrifugation: 7,500 × g, 5 min, at 4°C. Transfer on ice to RNase-free section of laminar flow hood, avoiding all percussion that may disturb the pellet.

24. Air-drying the pellet:
   a. Using a suitable RNase-free micro-pipettor (or multiple), remove as much of the ethanolic supernatant as possible.
   b. Keep the microcentrifuge tube open inside the laminar flow hood, allowing the pellet to air-dry under close supervision.

⚠️ **CRITICAL:** The pellet must not be allowed to dry completely, making it insoluble. Allow most of the residual water to evaporate without letting the pellet dry out. A suitable state for resuspension is recognizable via the “turning translucent” of the edges of the (formerly white) pellet. Due to this time-critical process, it is not recommended to dry too many pellets at the same time, necessitating a continuous or batch-wise process.
   c. As soon as the pellet is quite – but not completely – dry, resuspend it in an adequate amount of RNase-free water (commercial or autoclaved Milli-Q), such that the concentration is in the range needed for subsequent analyses. For example, we usually resuspend using 30–50 μL of RNase-free water. Support resuspension and dissolution of the RNA by gently pipetting up and down.

25. Heat the re-dissolved RNA inside the tube on a heating block, 10 min at 55°C–60°C.
26. Measure RNA concentration at a Nanodrop 2000 or a similar device, optionally aliquot and freeze the samples or directly proceed to further analysis such as RT-qPCR or next-generation sequencing.

★★ **Pause Point:** Aqueous RNA in RNase-free water is stable for months to years at −80°C.

**EXPECTED OUTCOMES**

**Technical Outcome**

For the amounts, duration, and combination of wells described above, we expect an RNA yield of several micrograms per sample (i.e., four wells of approximately 200,000 cells each should yield
approximately 10 μg total RNA). If applied correctly, the in situ TRIzol lysis, ethanol precipitation and air-drying RNA isolation yields high-quality RNA (Bioanalyzer RIN > 9), also for small RNA species.

**Biological Outcome**

For the long-term differentiation using CNTF, we expect a pro-cholinergic differentiation of LA-N cells, measurable by the elevation of the core cholinergic transcript, CHAT (Figure 1). Its expression can be measured by RT-qPCR using the primers given in the Key Resources Table. Additionally, the vesicular transporter SLC18A3 can be assessed.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Primary yield quantification of resuspended RNA is performed by Nanodrop 2000 measurement.

Primary outcome quantification is performed via RT-qPCR via the main marker of cholinergic differentiation, CHAT. Samples from differentiated LA-N cells are compared to non-differentiated controls in technical duplicate and biological quadruplicate. Quantification is performed using the ΔΔC_T method (Schmittgen and Livak, 2008), correcting for technical replicates (using the mean),
and applying a statistical test (e.g., ANOVA or Student’s t-test) to the biological replicates of differentiated vs. non-differentiated cells (Figure 1).

LIMITATIONS
Since the LA-N cells appear sensitive to environmental influences, outcomes may vary depending on humidity, air composition, temperature, etc. at the location of experimentation. We recommend a thorough check of the cells’ behavior upon taking up experimentation with any of the cell lines, and to generally be mindful when it comes to observing the cells and reacting to nuisance factors in their treatment.

TROUBLESHOOTING
The FCS content used in experimentation is quite high, which may interfere with the use of peptide mediators such as the neurokines. It thus is probable that the CNTF concentrations presented in this protocol are also quite high and could be reduced by experimenting in a serum-free medium. Due to the sensitivity of the cell lines to change, we did not exhaust all possible options of serum-free culture, and thus it is possible that there is a medium composition that enables a significant decrease in the neurokine concentration without inhibiting cell viability. Future experimentation can guide the use of serum-free media, which potentially have the combined benefit of presenting less confounding of molecular processes and enabling a more accurate understanding of the biology while being more animal-friendly.

Problem 1
The cells show low viability at lysis, after the differentiation period (step 11).

Potential Solution
This may occur if the cells have been disturbed during the differentiation period or run out of nutrition due to overcrowding. Make sure that the cells are in a constant environment in a humidified incubator with 8% CO₂. Also, make sure that the amounts of seeded cells and medium are adequate for the duration of differentiation and the amount of differentiation agent.

Problem 2
The phases are not separated well after initial centrifugation of phenol/chloroform extraction; the RNA quality as measured by Nanodrop 260/280 ratio is low (step 19 and step 26).

Potential Solution
The top aqueous phase containing RNA may have been contaminated by phenol, protein, and/or DNA. Adjust the handling of the separated solution to avoid disturbing the middle or lower phases, and/or use a phase separation tube.

Problem 3
The yield of RNA is very low and there is no visible pellet in the air-drying step (step 24).

Potential Solution
The pellet was potentially lost in the washing step (step 22). Use glycogen to enhance visibility of the pellet, and perform the washing step after precipitation more carefully. To enhance precipitation, particularly of small RNA fragments, precipitation time can be extended (e.g., to 1 h), and/or 0.01 M MgCl₂ (final concentration) can be added to the precipitation solution.

Problem 4
The quality of the RNA is low, the air-dried pellet was difficult to resuspend and fully translucent (step 24c).
Potential Solution
The pellet may have been allowed to dry completely, rendering it partially or fully insoluble. This may be ameliorated by extended heating of the resuspended pellet. Better is to carefully observe the drying pellet to avoid over-drying.

RESOURCE AVAILABILITY
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contacts, Jochen Klein, klein@em.uni-frankfurt.de, and Hermona Soreq, hermona.soreq@mail.huji.ac.il.

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The published article includes all datasets and code generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS
S.L. developed the protocol and wrote the initial draft of the manuscript. J.K. and H.S. supervised the work, secured funding, and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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