HUMAN THALAMIC ORGANOID PROTOCOL GUIDE

Integrating: Xiang et al. (2019) Cell Stem Cell / Xiang et al. (2020) STAR Protocols

1. Developmental Context: What Is the Thalamus?

The thalamus is the brain's central relay station. It routes sensory, motor, and limbic information between cortical and subcortical regions, and almost all sensory input—except olfaction—passes through thalamic nuclei before reaching the cortex. Thalamic neurons are mostly glutamatergic and project to specific cortical targets in organized, point-to-point maps. They fire in rhythmic bursts that gate sensory signals, direct attention, drive sleep oscillations, and sustain consciousness.[1,2]

Developmental origin: The thalamus derives from the diencephalon (prosomere 2), specifically from the caudal portion of the prosencephalon—a fundamentally different anteroposterior position than the midbrain (mesencephalon).[3,4] It sits between the pretectum (prosomere 1, caudal) and the prethalamus (prosomere 3, rostral), and its development depends on a precise combination of signals distinct from both cortical and midbrain protocols.

Key thalamic identity transcription factors include:

- **GBX2** — a homeobox gene that defines the thalamic progenitor domain, mutually repressive with OTX2 (which marks midbrain/forebrain).[5,6]
- **TCF7L2** — marks the thalamic mantle zone and differentiating thalamic neurons.[7,8]
- **OLIG3** — a bHLH transcription factor expressed in thalamic progenitors, essential for glutamatergic thalamic neuron specification.[9,10]
- **IRX1/3** — Iroquois family genes that define the ZLI (zona limitans intrathalamica) boundary and the thalamic compartment.[4]

Midbrain comparison: In the midbrain protocol, the target transcription factors are FOXA2, LMX1A, EN1/2 — floor plate/dopaminergic markers. The thalamic protocol targets a completely different transcriptional program reflecting its distinct embryonic origin.

2. Base Media: Composition and Rationale

2.1 mTeSR — Pluripotency Maintenance (Day −1)

mTeSR is a defined, feeder-free iPSC maintenance medium. Its high concentrations of bFGF and TGF-β1 activate the SMAD2/3 and MAPK/ERK pathways, which maintain the core pluripotency network (OCT4, SOX2, NANOG). [11] The iPSCs are cultured in mTeSR the day before aggregation to ensure they are in an optimal pluripotent state before induction begins.

2.2 KSR-Based Induction Media (Days 0–5)

This protocol uses DMEM/F12 + 15% KSR (KnockOut Serum Replacement) as the base for the induction phase, rather than the N2B27 base used in some midbrain protocols. This is a significant difference with specific developmental logic.

Mechanism: KSR media has a critical signaling property: its lipid-rich albumin sequesters hydrophobic signaling molecules, providing a low-level "default neuralizing" environment. [12] This makes it a favorable base for dual-SMAD inhibition—the KSR background cooperates with added SB/LDN rather than fighting against them, as undefined serum would.

Midbrain comparison: The midbrain protocol (Smits/Nickels) uses N2B27 + small molecules from Day 1. The thalamic protocol instead uses KSR-based media during induction (Days 0–5), then transitions to N2B27 starting at Day 6—mirroring the original Chambers & Studer (2009) dual-SMAD protocol more closely. [12]

β-Mercaptoethanol (0.055 mM)

β-Mercaptoethanol is a thiol-containing reducing agent that maintains a reducing intracellular environment and serves as a precursor for cysteine/glutathione synthesis, boosting the cell's antioxidant defenses. iPSCs and early neural progenitors are particularly susceptible to oxidative damage from supraphysiological O_2 levels in culture (~20% vs. ~3–5% in the embryo).

Note: The protocol note says to omit β-mercaptoethanol once switching to full BrainPhys. BrainPhys is designed for physiological conditions where β-ME's reducing activity can interfere with redox-sensitive synaptic signaling—for example, NMDA receptors have redox-sensitive cysteine residues. [13] Ascorbic acid replaces β-ME as the antioxidant at that stage.

NEAA (Non-Essential Amino Acids, 1%)

NEAA provides L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine at defined concentrations. L-proline supports collagen synthesis for extracellular matrix deposition, and L-serine is a precursor for sphingolipids (critical for neural membrane biogenesis) and phosphatidylserine.

2.3 N2B27 Media — Transition at Day 6

At Day 6, the protocol switches from KSR-based to N2B27 base (DMEM/F12 + Neurobasal 1:1, N2 1%, B27 w/o Vit A 2%). The logic mirrors the midbrain protocol: DMEM/F12 supports proliferating progenitors while Neurobasal supports differentiating neurons. B27 lacks vitamin A to avoid retinoic acid's caudalizing influence during patterning.

2.4 BrainPhys Transition — The Maturation Switch

Starting around Day 19–24, the protocol begins replacing DMEM/F12 with BrainPhys in a 50:50 BrainPhys:Neurobasal mix, then transitions to full BrainPhys by Day 34+. [13]

Mechanism: BrainPhys provides physiological ionic concentrations—lower glucose, appropriate K^+ levels, CSF-like osmolarity—that allow maturing thalamic neurons to fire action potentials properly. Standard DMEM tonically depolarizes neurons and suppresses electrophysiological activity. [13] The gradual transition (50:50 first, then full BrainPhys) ensures cells have expressed sufficient ion channels before they encounter the full physiological ionic environment.

3. Induction Phase (Days 0–5): Dual-SMAD Inhibition + Thalamic Patterning

This phase converts pluripotent iPSCs into neural progenitors and begins specifying thalamic (diencephalic) identity. The small molecule cocktail differs strategically from the midbrain protocol. [5,6]

Molecule	Conc.	Primary Role
SB-431542	5 μM	ALK4/5/7 inhibitor → blocks TGF-β/Activin/Nodal → prevents mesendoderm
LDN-193189	0.1 μM	BMP type I receptor inhibitor → blocks SMAD1/5/8 → neural induction
CEPT	1×	Multi-target survival cocktail → replaces simple ROCK inhibitor
CHIR99021	1 μM (D5)	Low Wnt activation for diencephalic (not midbrain) A-P positioning

3.1 SB-431542 (5 μM) — Blocking TGF-β/Activin/Nodal

SB-431542 inhibits ALK4, ALK5, and ALK7, preventing phosphorylation of SMAD2/3 and blocking mesendoderm specification. [12,14] Without this inhibition, pluripotent cells would readily differentiate toward mesoderm (*BRACHYURY/T*) and endoderm (*SOX17, FOXA2*).

Why this concentration: The protocol uses 5 μM here vs. 10 μM in the midbrain (Nickels) induction phase. The IC50 for ALK5 is ~94 nM, [14] so 5 μM far exceeds the threshold for effective pathway blockade. The Xiang/Nowakowski protocol uses this lower dose because KSR-based media already partially suppresses the TGF-β pathway through its albumin component. [5,6]

3.2 LDN-193189 (100 nM) — Blocking BMP/SMAD1/5/8

LDN-193189 inhibits BMP type I receptors ALK2 and ALK3, blocking SMAD1/5/8-mediated transcription. Together with SB-431542, this constitutes dual-SMAD inhibition for neural induction. [12]

Why this concentration: 100 nM matches the concentration used in midbrain expansion media (Smits) and is slightly lower than the 150 nM in some midbrain induction protocols. Since LDN is extremely potent (IC50 ~5 nM for ALK2), 100 nM provides strong but not maximal BMP blockade. Some residual BMP signaling may support appropriate diencephalic patterning—eliminating BMP completely can bias cells too strongly toward telencephalic (cortical) fates. [12,15]

Mechanism: Dual-SMAD inhibition at these concentrations converts iPSCs to neuroectoderm within 5–6 days. The cells downregulate pluripotency markers (OCT4, NANOG) and upregulate pan-neural markers (PAX6, SOX1, SOX2 maintained). [12] The specific regional identity is then determined by the Wnt/SHH signals layered on top.

3.3 CEPT (1×, Days 0–3; Removed Day 4)

CEPT is a survival cocktail that replaces the single-agent ROCK inhibitor (Y-27632) used in the midbrain protocol. CEPT consists of four components: [16]

- **Chroman 1 (C):** A highly selective ROCK inhibitor (IC50 ~0.9 nM), approximately 100-fold more potent than Y-27632. [16]
- **Emricasan (E):** A pan-caspase inhibitor that directly blocks caspase-3, -7, -8, and -9, providing a second line of defense against apoptosis downstream of ROCK. [16]

- **Polyamines (P):** Trans-ISRIB, a small molecule that rescues the integrated stress response (ISR) by preventing eIF2α-mediated translational arrest. [16]
- **Thiazovivin (T):** Another ROCK inhibitor with additional activity on TGF-β signaling, providing further anti-apoptotic support. [16]

3.4 CHIR99021 (1 µM, Day 5 Only) — The Critical Wnt Pulse

This is the most strategically important difference from the midbrain protocol. CHIR99021 inhibits GSK3β, stabilizing β-catenin and activating canonical Wnt signaling. The critical parameters are:

1. Concentration: 1 µM (thalamic) vs. 3 µM (midbrain)

The 1 µM concentration produces a level of Wnt activation appropriate for diencephalic identity—more posterior than cortex but more anterior than midbrain. [5,6,7]

2. Timing: Day 5 only (thalamic) vs. continuous from Day 1 (midbrain)

The late, brief Wnt pulse is deliberately timed to act on cells that have already committed to neuroectoderm (via 5 days of dual-SMAD inhibition) but have not yet irreversibly adopted a specific A-P identity. [5,6]

Mechanism: At the transcriptional level, this brief 1 µM CHIR pulse activates GBX2 (the key thalamic identity gene) while maintaining lower levels of OTX2 expression. [5,9] Moderate Wnt combined with the preceding dual-SMAD inhibition positions cells in the diencephalic domain where GBX2 and TCF7L2 are expressed. Sustained 3 µM CHIR would push past this domain into EN1/EN2-expressing midbrain territory.

Why this concentration: 1 µM CHIR is below the threshold for robust midbrain specification (~2–3 µM) and above the near-zero Wnt level that produces cortex. This narrow concentration window distinguishes thalamic from cortical organoid protocols—many cortical protocols use no CHIR at all. [5,6,15]

4. Maintenance Phase 1 (Days 6–33): Progressive Thalamic Patterning

After induction, the protocol switches to N2B27-based media and introduces a carefully timed sequence of patterning factors. This is where thalamic identity is consolidated and refined through signals that mimic the zona limitans intrathalamica (ZLI) and dorsal diencephalic signaling environment. [4,17,18]

4.1 Days 6–8: CHIR99021 Only (1 μM)

Low-level Wnt activation continues through the transition to N2B27 media. SB and LDN have been withdrawn, allowing endogenous BMP and TGF-β signaling to begin driving differentiation while Wnt maintains diencephalic A-P identity. [5,6]

4.2 Days 9–11: CHIR99021 (1 μM) + SAG (100 nM)

SAG is introduced for the first time, at a very different concentration than the midbrain protocol. At 100 nM (vs. 500 nM in the midbrain protocol), SAG activates the Sonic Hedgehog pathway through Smoothened but at a much lower level. In the embryo, the zona limitans intrathalamica (ZLI)—the boundary between thalamus and prethalamus—expresses SHH and acts as a local signaling center. [17,18,19]

Mechanism: At 100 nM, SAG activates GLI transcription factors less strongly than at 500 nM. In the diencephalon, moderate SHH promotes expression of OLIG3 (a thalamic progenitor marker that drives glutamatergic neuron specification) and GBX2. [9,10,18] Low Wnt combined with moderate SHH creates the specific signaling environment of the thalamic primordium.

Why this concentration: At 100 nM, SAG is 5-fold lower than the midbrain's 500 nM, preventing the floor plate induction that high SHH would cause. [17,18] The goal is ZLI-level SHH signaling—which specifies glutamatergic relay neurons—not floor plate-level signaling, which specifies dopaminergic neurons in the midbrain. [9,10]

Note: SAG is present only during Days 9–14, then removed. This brief window mimics the transient nature of ZLI SHH signaling—the ZLI provides a positional cue during a specific developmental window, then cells consolidate their identity through intrinsic transcriptional networks. [17,18]

4.3 Days 12–18: CHIR (1 μM) + SAG (100 nM) + BMP7 (30 ng/mL)

BMP7 is the signature thalamic patterning signal—the molecule that most clearly distinguishes the thalamic protocol from midbrain, cortical, or any other organoid protocol. [5,6,7,8]

In the developing diencephalon, BMP7 (along with BMP5 and BMP6) is expressed in the dorsal midline of the thalamic primordium—the roof plate and adjacent alar plate. [7,8,20] BMP7 signals through BMP type I receptors ALK2/ALK3/ALK6 and type II receptors BMPRII/ActRIIA, activating SMAD1/5/8 to drive a transcriptional program specific to thalamic neurogenesis.

Mechanism: In committed diencephalic progenitors, BMP7 signals through SMAD1/5/8 to upregulate TCF7L2 and GBX2, consolidating thalamic identity; dorsalize the diencephalon, pushing cells away from ventral (hypothalamic/prethalamic) fates; and activate OLIG3-dependent transcriptional cascades that drive glutamatergic neuron specification. [7,8,9,10,20]

Why this concentration: Shiraishi et al. (2017) established 30 ng/mL BMP7 in their mouse ESC thalamic differentiation system, where it significantly increased Tcf7l2+/Gbx2+ and Tcf7l2+/Olig3+ cell populations. [7] This moderate concentration activates SMAD1/5/8 in the diencephalic context without causing non-neural fate specification (which would only occur in undifferentiated cells). The timing is critical: BMP7 is introduced AFTER 12 days of neural induction and diencephalic patterning. [5,6,7]

Midbrain comparison: The midbrain protocol actively blocks BMP signaling during patterning to prevent dorsalization and maintain ventral (floor plate) identity. The thalamic protocol actively supplies BMP7 to drive dorsalization within the diencephalon. [5,6,7,20] This opposition reflects the structures' embryonic origins: the thalamus is a dorsal structure (alar plate derivative) while midbrain dopaminergic neurons are ventral (floor plate derivative).

SAG removal after Day 14: From Day 15 onward, SAG is withdrawn but BMP7 + CHIR continue. The SHH signal has served its purpose as a ZLI-like positional cue. [17,18] Continued BMP7 drives thalamic progenitors toward their dorsal character without the ventral counterbalance.

4.4 Days 19–23: BDNF (20 ng/mL) + DAPT (2.5 µM) — Differentiation Initiation

At this stage, all patterning factors (CHIR, SAG, BMP7) are withdrawn and replaced with maturation and differentiation signals. The preceding 19 days have established thalamic identity. [5,6]

BDNF (20 ng/mL)

Brain-derived neurotrophic factor binds TrkB (NTRK2), activating Ras/MAPK/ERK, PI3K/Akt, and PLCγ/Ca^{2+}/CaMKII cascades. [21] In thalamic neurons, BDNF promotes survival of glutamatergic projection neurons, drives dendritic arborization, and upregulates ion channels (HCN channels for Ih current, T-type Ca^{2+} channels for burst firing) that underlie thalamic neurons' characteristic electrophysiological signatures.

Why this concentration: 20 ng/mL is double the 10 ng/mL used in some midbrain maturation media. The higher concentration may reflect greater TrkB expression in thalamic neurons or the need for stronger survival signals during this earlier differentiation window. [5,6,21]

DAPT (2.5 µM)

DAPT blocks γ-secretase, preventing Notch signaling. Notch cleavage normally releases the NICD, which activates HES/HEY transcription factors that repress proneural genes (ASCL1, NEUROG1/2) and maintain progenitor self-renewal. [22]

Mechanism: Notch inhibition forces remaining progenitors to synchronously exit the cell cycle and differentiate into neurons. In the thalamic context, this drives NEUROG2 expression (critical for glutamatergic thalamic neuron specification) and subsequent NEUROD1-mediated cell cycle exit. [9,10,22]

Why this concentration: 2.5 µM is lower than the 10 µM used in the midbrain protocol. This less aggressive Notch inhibition allows a more gradual differentiation that may better suit the thalamic progenitor context. [5,6]

5. Maturation Phase (Day 24+): Terminal Differentiation

By Day 24, patterning factors and DAPT are withdrawn. The maturation media transitions through BrainPhys. [13]

5.1 B27 WITH Vitamin A (Day 24+)

After Day 24, the protocol switches from B27 without vitamin A to B27 with vitamin A. Earlier, retinoic acid's caudalizing influence had to be avoided during patterning. Now that regional identity is locked in, retinoic acid serves beneficial maturation functions: it activates RAR/RXR nuclear receptors that drive expression of synaptic proteins, ion channels, and vesicular glutamate transporters (VGLUT1/VGLUT2 (SLC17A7/SLC17A6)) that define thalamic projection neuron identity. [5,6]

> **Midbrain comparison:** The midbrain maturation media (D16+) does NOT switch to B27 with vitamin A, possibly because dopaminergic neurons are more sensitive to RA-induced changes in regional character.

5.2 Ascorbic Acid (200 µM)

Ascorbic acid appears explicitly in the Day 34+ media. At this stage it serves as a TET enzyme cofactor for ongoing epigenetic maturation, an antioxidant replacing β-mercaptoethanol (which is omitted from BrainPhys), and a cofactor for histone demethylases that support chromatin remodeling at maturation gene loci.

5.3 Notably Absent: GDNF, TGF-β3, ActivinA, Dibutyryl cAMP

The thalamic maturation media is remarkably simpler than the midbrain's D16+ cocktail. The midbrain protocol uses BDNF + GDNF + dcAMP + TGF-β3 + ActivinA + DAPT. The thalamic protocol uses only BDNF + DAPT briefly, then no exogenous growth factors at all. [5,6]

- **GDNF** is specifically a dopaminergic neuron survival factor (via GFRα1/RET). Thalamic glutamatergic neurons don't express GFRα1 at high levels, so GDNF would have minimal effect.
- **TGF-β3 and ActivinA** support dopaminergic identity through NURR1 cooperation. These are midbrain-specific maturation signals.
- **dcAMP** drives PKA/CREB-mediated maturation. The thalamic protocol may rely on endogenous cAMP signaling rather than pharmacological elevation.

6. Alternate Protocol Track: EGF/FGF-2 Expansion Method

The spreadsheet contains an alternate protocol track that uses a different approach for the proliferation and maturation phases:

6.1 Days 8–24: EGF (20 ng/mL) + FGF-2 (20 ng/mL)

EGF + FGF-2 is the classic neurosphere-generating cocktail.[23] These growth factors maintain neural progenitors in a proliferative, undifferentiated state (sustaining SOX2 and Nestin expression) while driving symmetric cell division. The rationale is to expand the progenitor pool before differentiation to produce larger organoids.

6.2 Days 25–42: BDNF (20 ng/mL) + NT3 (20 ng/mL)

NT3 (Neurotrophin-3) binds TrkC (NTRK3). In the thalamus, NT3/TrkC signaling is particularly important for thalamocortical projection neuron survival.[21] BDNF and NT3 together provide two complementary neurotrophic pathways: BDNF/TrkB for general neuronal maturation and NT3/TrkC for thalamocortical projection neuron-specific support.

7. Integrated Signaling Timeline

This summary shows how each change in the cocktail maps to a specific developmental transition in thalamic specification.

Days	SB/LDN	CHIR	SAG	BMP7	Other
0–4	5/0.1 μM	—	—	—	CEPT
5	5/0.1 μM	1 μM	—	—	—
6–8	—	1 μM	—	—	N2B27
9–11	—	1 μM	100 nM	—	—
12–14	—	1 μM	100 nM	30 ng/mL	—
15–18	—	1 μM	—	30 ng/mL	—
19–23	—	—	—	—	BDNF+DAPT
24–33	—	—	—	—	BrainPhys
34+	—	—	—	—	Full BP+AA

8. References

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