Perspective: Molecular-Action Pathways for Autism Drug Discovery: Organoids, Circadian Dynamics, and Genetic Engineering

Peter De Ceuster

Abstract

Since in this Perspective, we outline an integrated molecular-action framework that combines human patient-derived cerebral organoids, circadian biology, and targeted genetic, we will cover chemical interventions to identify translational drug leads. We explicitly distinguish between experimentally supported mechanisms and *hypothetical* links, adding falsifiable endpoints for each proposed mechanism. We prioritize experimental strategies based on feasibility, resource demands, and translational potential. The aim is to serve as a blueprint for mechanistic ASD drug discovery, while inviting the community to critically evaluate, replicate, and refine each proposed step. Autism spectrum disorder (ASD) is genetically heterogeneous and mechanistically complex. We move beyond associative descriptions to provide explicit biochemical sequences for core nodes: clock transcription factors (BMAL1, CLOCK, REV-ERBα), synaptic scaffold proteins (SHANK3), ion channel regulators (SCN2A), and key signaling effectors (mTORC1, NF-κB). For each node we map the molecular cascade (DNA binding, post-translational modification, subcellular localization, proteasomal turnover, and downstream signaling). We then present a fully specified experimental pipeline — organoid generation, CRISPRa/i manipulation, PER2::luc rhythmic reporters, affinity-matured peptide ligands, and multi-omic readouts (time-series RNA-seq, proteomics, ChIP-seq, ATAC-seq, phospho-proteomics) — designed to validate causal chains and test therapeutic hypotheses. Finally, we discuss translational strategies including chronopharmacology and allosteric modulators targeted by directed evolution. This manuscript is not intended as a ready-to-implement blueprint for mechanistic ASD drug discovery, and should be seen as developing science.

Keywords

Autism spectrum disorder; cerebral organoids; circadian clock; BMAL1; SHANK3; CRISPRa/i; directed evolution; mTOR; chronopharmacology.

1 Introduction

Autism spectrum disorder (ASD) encompasses a set of neurodevelopmental conditions characterized by deficits in social communication and restricted, repetitive behaviors. The genetic architecture is complex and highly polygenic with numerous high-penetrance risk genes that converge on synaptic, transcriptional, and chromatin regulation pathways [De Rubeis et al., 2014].

This Perspective reworks current conceptual descriptions into explicit molecular chains and integrates them into a single experimental pipeline leveraging human cerebral organoids and modern engineering tools (CRISPRa/i, directed evolution). Our aim: (i) to provide concrete mechanistic sequences for each key molecular node implicated in ASD, (ii) to propose assays and readouts that measure those molecular events, and (iii) to describe intervention strategies that reverse the chain at defined mechanistic steps.

2 Background: core molecular-action frameworks

This section provides the molecular sequences for the principal nodes in our framework. Each subsection explicitly tracks the flow: gene → mRNA → protein → post-translational modification (PTM) → interaction → pathway output → cellular phenotype.

2.1 Circadian transcriptional machinery (BMAL1/CLOCK/REV-ERBα)

The mammalian circadian oscillator is organized as a transcriptional–translational feedback loop (TTFL). CLOCK and BMAL1 heterodimerize via PAS domains, bind canonical E-box motifs (CACGTG) in promoters of Period (PER1/2/3) and Cryptochrome (CRY1/2) genes and recruit RNA polymerase II and histone acetyltransferases (HAT activity associated with CLOCK) to initiate transcription. Newly translated PER and CRY proteins form complexes stabilized by casein kinase I (CKI/) phosphorylation at defined serine/threonine residues; phosphorylation regulates nuclear import by unmasking nuclear localization signals and also primes PER/CRY for ubiquitination (e.g., recogni-

tion by the FBXL3/FBXL21 E3 ligases) leading to proteasomal degradation. Nuclear PER/CRY complexes bind and inhibit CLOCK–BMAL1, closing the negative limb.

BMAL1 also regulates metabolism and growth pathways: BMAL1 drives transcription of REDD1 (DDIT4), which inhibits Rheb via activation of TSC1/2 complex; loss of BMAL1 reduces REDD1 expression, disinhibiting Rheb and hyperactivating mTORC1. Additionally, BMAL1 function is modulated by acetylation (CLOCK-mediated) and deacetylation (SIRT1), coupling clock amplitude to NAD^+ metabolism. Pharmacologically, REV-ERBα agonists suppress BMAL1 transcription through recruitment of the NCoR/HDAC3 repressor complex to RORE elements; antagonists disinhibit BMAL1. Thus, targeted modulation at the level of transcription (CRISPRa/i), ligand binding (REV-ERB pocket), or PTM (SIRT1 activators) have distinct and testable molecular outcomes.

2.2 Synaptic scaffold SHANK3 → clock coupling (casestudy)

SHANK3 encodes a postsynaptic density (PSD) scaffold coordinating glutamatergic receptors (AMPA/NMDA), cytoskeletal regulators (Homer, cortactin), and signaling molecules (CaMKII). Loss-of-function reduces PSD integrity, lowering NMDA receptor trafficking and calcium influx. The molecular chain proceeds: SHANK3 loss → decreased actin polymerization at the spine (via lower Rac1/CDC42 activation) → reduced NMDA-mediated Ca^{2+} transients → attenuated CaMKII autophosphorylation → decreased CREB phosphorylation at Ser133 → reduced transcription of activity-dependent genes including BHLHE41 and PER3. Thus, synaptic scaffold disruption directly alters clock gene expression by perturbing activity-dependent transcriptional regulators.However, several compensatory pathways may partially buffer this effect. Activity-dependent upregulation of BDNF and MEF2C, for instance, can maintain a baseline of PER and BHLHE gene expression independent of CaMKII–CREB signaling. Failure to account for these redundancies could lead to overestimation of direct SHANK3–clock coupling strength, underscoring the need for perturbation experiments that selectively block these compensatory arms. Rescue strategies can target (a) restoration of receptor trafficking, (b) enhancement of actin dynamics, or (c) direct reactivation of clock gene promoters via CRISPRa.

2.3 mTORC1 as integrator of clock and synaptic signaling

mTORC1 senses growth and energy state through inputs from Rheb, TSC1/2, AMPK and REDD1. At synapses, mTORC1 controls local translation of synaptic proteins via phosphorylation of S6 kinase (S6K) and 4E-BP1, which regulate ribosomal activity and

cap-dependent translation. BMAL1 insufficiency reduces REDD1 transcription, releasing TSC1/2 inhibition and hyperactivating Rheb-mTORC1 signaling → increased local protein synthesis → dendritic spine overgrowth and aberrant synapse numbers. Conversely, mTORC1 inhibition (rapamycin analogs) reduces S6K phosphorylation, normalizes translation rates, and may correct spine density abnormalities. Mapping phosphorylation sites (phospho-S6 Ser235/236, phospho-4E-BP1 Thr37/46) and ribosome profiling provides mechanistic readouts for mTOR activity.

2.4 Neuroimmune molecular actions: perspective.

Clock genes modulate innate immune gene expression rhythmically: BMAL1 represses NF-κB target genes via direct and indirect mechanisms (e.g., BMAL1 influences acetylation state of NF-κB subunits). Chronic circadian disruption elevates basal IL-6 and TNF-α production via sustained NF-κB activation and microglial priming. Molecular interventions that restore BMAL1 amplitude or that selectively inhibit NF-κB (IKK inhibitors) can be assayed at the level of canonical phosphorylation (IκBα Ser32/36) and nuclear translocation of p65.For experimental rigor, NF-B modulation assays could include phospho-p65 ELISA, chromatin immunoprecipitation for NF-B promoter occupancy, and cytokine rhythm mapping under both acute and chronic inflammatory challenges. These endpoints would enable finer-grained resolution of clock–immune coupling in the ASD context. Recent advances (2021–2024) further reinforce the integration of circadian and synaptic mechanisms in ASD. New organoid models incorporating vascularization and long-term network maturation have improved synaptic fidelity and clock gene stability in vitro . Multi-omic profiling in patient-derived SHANK3 organoids has revealed unexpected oscillatory resilience, suggesting compensatory transcriptional programs (e.g., MEF2C, BDNF) that modulate PER gene expression despite synaptic deficits . At the same time, conflicting results exist: certain CRISPR-based BMAL1 rescues have yielded only partial electrophysiological normalization, with variability linked to donor genetic background.

3 Aims and strategic hypotheses

Hypothesis 1: Restoring BMAL1 transcriptional amplitude in ASD patient-derived organoids will normalize REDD1 expression and downstream mTORC1 activity, reducing synaptic overproduction and normalizing network electrophysiology. Unconfirmed.

Hypothesis 2: Restoring SHANK3 function (by genetic rescue or synapse-stabilizing peptides) will reestablish NMDA-Ca^{2+} signaling, normalizing activity-dependent clock

gene transcription and improving PER2 rhythmicity. Recent animal research around SHANK3 function is promising.

Hypothesis 3: Small molecules selected by directed evolution to bind BMAL1 PAS domains or REV-ERB ligand pockets can shift oscillator properties and produce durable synaptic and immune improvements when applied at optimal circadian phases (chronotherapeutic window).

4 Methods

Please note all protocols described below should follow institutional biosafety and human subject regulations for iPSC work and genetic modification.

4.1 Patient samples and iPSC reprogramming

Obtain fibroblasts or PBMCs from consenting ASD subjects carrying validated pathogenic mutations (e.g., SHANK3 microdeletion, BMAL1 haploinsufficiency, PER3 variants) and matched controls. Reprogram with Sendai virus vectors (OCT4, SOX2, KLF4, c-MYC). Validate pluripotency (OCT4/NANOG immunostaining) and genomic integrity (karyotype or low-coverage WGS).

4.2 Cerebral organoid differentiation

Follow guided dorsal forebrain protocol with dual-SMAD inhibition (Noggin + SB431542), Matrigel embedding, and spinner cultures. Grow to 8–16 weeks for network maturation. Optionally incorporate microglia (iPSC-derived) to recapitulate neuroimmune interactions.

4.3 Genetic manipulation — CRISPRa/i and knock-in reporters

Construct CRISPRa (dCas9-VP64-p65-Rta or dCas9-p300) and CRISPRi (dCas9-KRAB) systems. For BMAL1 activation use 3–4 gRNAs targeting promoter proximal E-box/RORE flanking regions; for REV-ERB inhibition target RORE proximal elements with dCas9-KRAB. Validate gRNA activity by RT-qPCR and ChIP for histone acetylation (H3K27ac) and recruitment (dCas9 ChIP).

Introduce a PER2::luciferase reporter into the AAVS1 safe harbor locus for longitudinal bioluminescent monitoring. Use lentiviral transduction or electroporation for organoid delivery; track expression stability and mosaicism.

4.4 Directed evolution and small-molecule ligand development

Generate peptide libraries (mRNA display, phage display) targeting BMAL1 PAS-B and REV-ERB ligand-binding pockets. Select using immobilized recombinant domains, iterate affinity maturation, and convert lead peptides into stabilized, cell-penetrant analogs (stapled peptides, peptidomimetics). For small molecules, use DNA-encoded libraries where practical.

4.5 Assays and readouts — molecular to network scale

Time-series PER2::luc bioluminescence (continuous measurement, 10-min resolution) to quantify period, amplitude, and damping. Sample organoids at 4-hour intervals across at least 48 hours for:

- RNA-seq (bulk and single-cell) for rhythmic transcriptomes (JTK_CYCLE or MetaCycle analysis).
- ChIP-seq for BMAL1, CLOCK, H3K27ac, and H3K4me3 to map promoter/enhancer occupancy.
- ATAC-seq for chromatin accessibility at circadian and synaptic loci.
- Tandem mass tag (TMT) quantitative proteomics and phospho-proteomics (include phospho-S6, phospho-4E-BP1, phospho-CaMKII, phospho-CREB).
- Electrophysiology: multi-electrode array (MEA) for network burst dynamics and phase locking; whole-cell patch clamp for E/I balance (mEPSC/mIPSC frequency and amplitude).
- Immunocytochemistry for synaptic proteins (PSD95, SHANK3, GluA1/GluN2B) and spine morphology quantification (confocal + 3D reconstructions).
- Cytokine multiplex (Luminex) for IL-1β, IL-6, TNF-α, IL-10 across circadian sampling points.

4.6 Data analysis and causal chain testing

Define primary mechanistic metrics: BMAL1 transcript/protein amplitude, REDD1 mRNA levels, phospho-S6 abundance, mEPSC frequency, PER2::luc amplitude. Use causal path models (structural equation modeling) to test whether BMAL1 manipulation changes REDD1 $\rightarrow$ mTORC1 $\rightarrow$ synaptic phenotype and whether these changes mediate network electrophysiology rescue. We would like to recommend AI-augmented SEM.

5 Potential experiments and decision tree

Phase 1 — Baseline phenotyping: generate organoid panels from multiple ASD genotypes + controls; measure PER2 rhythms, synaptic proteome, and baseline electrophysiology.

Phase 2 — Genetic perturbations: CRISPRa BMAL1 and CRISPRi REV-ERB in parallel. Primary endpoints: restoration of PER2 amplitude, REDD1 expression, phospho-S6 normalization, mEPSC frequency correction.

Phase 3 — Chemical screens: test directed evolution leads and small-molecule libraries for PER2 rhythmic rescue and downstream normalization. Use tiered screening: high-throughput PER2::luc primary assay; secondary MEA and proteomics.

Phase 4 — Integration: combine genetic rescue (BMAL1) with synaptic stabilizers (SHANK3 mimetic peptides) and test for synergistic effects on molecular chains and network behavior.

6 Expected results (preliminary)

We expect that BMAL1 CRISPRa will increase BMAL1 mRNA protein amplitude (measured by RT-qPCR and Western blot), upregulate REDD1 mRNA, decrease phospho-S6 levels, reduce excessive spine density, and normalize network burst synchrony. SHANK3 rescue is predicted to restore NMDA receptor trafficking, increase CaMKII and CREB phosphorylation, and re-establish rhythmic induction of PER3/BHLHE41.

7 Discussion

7.1 Translational strategies

Chronopharmacology: deliver candidate drugs at times corresponding to maximum receptor expression or clock phase predicted to maximize efficacy; for example, administer REV-ERB antagonists when BMAL1 expression is naturally low to amplify amplitude.

Allosteric modulators and peptide mimetics: directed evolution can yield highly specific ligands that modulate clock protein conformation (alter DNA affinity or cofactor recruitment) without global transcriptional disruption.

Combination approaches: combined synaptic (SHANK3 rescue) and clock (BMAL1 activation) therapies may have supra-additive effects by correcting both the upstream synaptic trigger and the downstream circadian amplifier.

7.2 Limitations and contingencies

Organoid heterogeneity and variable maturation are practical challenges; include large donor numbers and multiple differentiation batches. CRISPR mosaicism can be mitigated by optimized delivery (AAV or integrating lentiviral systems) or by generating stable edited iPSC lines prior to differentiation. Small-molecule penetration and stability must be validated in organoids before claiming translational potential.Ethical considerations extend beyond biosafety and consent. The use of advanced genetic editing in patient-derived organoids—especially from individuals with neurodevelopmental disorders—raises questions about donor privacy, data governance, and potential stigmatization. Transparent communication with participants, clear governance over genomic data sharing, and oversight by multidisciplinary ethics boards are critical to ensure responsible deployment of these technologies. More research is needed to finetune potential protocols. Beyond biological variability, the proposed framework is resource-intensive, requiring multi-modal omics, live-cell imaging, and high-throughput screening infrastructure. To broaden accessibility, a minimum viable pipeline could prioritize: (i) core circadian reporters (PER2::luc), (ii) targeted RT-qPCR for clock and synaptic transcripts, and (iii) basic MEA assays for network activity. These steps can yield actionable mechanistic insights without full-scale proteomics or directed evolution facilities. Furthermore, organoid-to-human translation remains a major bottleneck, with reported concordance rates between organoid phenotypes and clinical outcomes varying from 40–60 percent.

8 Conclusion

We provide a suggestive molecular-action blueprint linking clock genes, synaptic scaffolds, mTOR signaling, and neuroimmune activity in ASD. The proposed organoid-centric experimental pipeline specifies interventions and the precise molecular readouts needed to establish causality and evaluate therapeutics. Implementing this plan could enable discovery of chronomodulators and synapse-stabilizing agents with a clear mechanistic basis for clinical translation.

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