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Understanding the Molecular Interactions of Ketamine in Neuronal Cell Models

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U.S. DEPARTMENT
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Abstract

Ketamine produces rapid antidepressant effects, yet its molecular interactions and mechanisms in human neurons remain poorly understood. Traditional tissue-based assays lack the spatial, temporal, and biological context needed to capture dynamic drug responses. This project has developed a framework for characterizing drug interactions using ketamine as a model by defining molecular targets in human iPSC-derived neurons, mapping multi-omics responses to physiologically relevant exposure, and applying chemical probes and multimodal imaging to visualize intracellular dynamics and identify non-canonical targets.

Summary

This project has developed a framework for characterizing drug interactions using ketamine as a model by defining molecular targets in human iPSC-derived neurons, mapping multi-omics responses to physiologically relevant exposure, and applying chemical probes and multimodal imaging to visualize intracellular dynamics and identify non-canonical targets.

Acknowledgments

This research was supported by the Open Call LDRD, under the Laboratory Directed Research and Development (LDRD) Program at Pacific Northwest National Laboratory (PNNL). PNNL is a multi-program national laboratory operated for the U.S. Department of Energy (DOE) by Battelle Memorial Institute under Contract No. DE-AC05-76RL01830.

Acronyms and Abbreviations

Cer	Ceramide
DAPI	4',6-Diamidino-2-phenylindole
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DMSO	Dimethyl sulfoxide
DOE	U.S. Department of Energy
ESCRT	Endosomal sorting complexes required for transport
GTPase	Guanosine triphosphatase
HC	Hydrocortisone
HCK	Hydrocortisone followed by ketamine
iPSC	Induced pluripotent stem cell
K	Ketamine
LDRD	Laboratory Directed Research and Development
MPLEx	Metabolite, Protein, and Lipid Extraction
mRNA	Messenger ribonucleic acid
NMDA	N-Methyl-D-aspartate
PDB	Protein Data Bank
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PI	Phosphatidylinositol
PKN	Protein kinase N
PNNL	Pacific Northwest National Laboratory
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SRP	Signal recognition particle
SRS	Stimulated Raman scattering
TAMRA	Tetramethylrhodamine
TG	Triglyceride
TRD	Treatment-resistant depression
UV	Ultraviolet

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1.0 Introduction

Treatment-resistant depression (TRD) remains a significant clinical challenge, with approximately 30% of individuals treated for major depressive disorder failing to respond adequately to conventional therapies (Zhdanova et al. 2021). Ketamine is unusual among antidepressant treatments because it can produce rapid therapeutic effects within hours, with benefits that can persist beyond the period of drug exposure (Shiroma et al. 2020). Despite its clinical efficacy, the molecular mechanisms underlying these effects remain incompletely understood. In particular, the cellular targets and molecular pathways that connect acute ketamine exposure to sustained changes in neuronal function have not been fully defined (Zanos and Gould 2018).

Ketamine is best known as an antagonist of the N-methyl-D-aspartate (NMDA) receptor, but increasing evidence suggests that its antidepressant activity involves broader molecular and cellular processes beyond direct NMDA receptor inhibition (Zanos and Gould 2018). Defining these interactions is important for understanding how a relatively short pharmacological exposure can initiate longer-lived changes in neuronal signaling, metabolism, and plasticity. Human induced pluripotent stem cell (iPSC)-derived neuronal systems provide an experimentally tractable approach for examining these mechanisms directly in human neuronal cells and have emerged as useful models for investigating neuropsychiatric disease and ketamine response (Marcatili et al. 2020; Autar et al. 2022).

The objective of this LDRD project was to develop an experimental framework for characterizing ketamine interactions and responses across molecular and cellular scales using human iPSC-derived cortical neurons. The approach combined transcriptomics, proteomics, metabolomics, and lipidomics with chemical-probe labeling and advanced imaging. Multi-omics measurements were used to characterize time-dependent responses to physiologically relevant ketamine exposure, while the Metabolite, Protein, and Lipid Extraction (MPLEx) workflow enabled complementary molecular measurements from common biological samples (Nakayasu et al. 2016). Together, these measurements were designed to distinguish changes in molecular abundance from changes associated with cellular state and drug exposure.

Chemical probes provided an additional means of investigating molecular engagement and intracellular behavior. Photoaffinity labeling can covalently capture transient small-molecule–protein interactions in intact biological systems, enabling subsequent enrichment and identification of candidate binding proteins by mass spectrometry (Smith and Collins 2015). Clickable ketamine analogs have previously been shown to retain relevant biological activity and accumulate in neuronal cells, supporting the use of reporter-enabled ketamine mimics to investigate intracellular interactions (Emnett et al. 2016). The photoaffinity-probe used in this study was obtained from a previous PNNL project. The probe contained a reporter alkyne and a diazirine photo reactive group. Raman-active chemical tags offer a complementary approach for following small molecules and associated chemical changes in living cells while requiring only small structural modifications to the parent compound (Zhao et al. 2017). The project also incorporated cysteine-reactive chemical probes to examine treatment-associated changes in the reactive thiol proteome and cellular redox state (Sadler et al. 2014; Fu et al. 2020).

Through this work, we established and validated a 21-day-matured human iPSC-derived cortical neuronal platform integrating molecular profiling, chemical-probe labeling, fluorescence microscopy, calcium measurements, and stimulated Raman scattering imaging. Ketamine exposure produced time-dependent molecular remodeling across multiple molecular layers, including changes in the proteome, lipidome, and glutamate–glutamine metabolism. Competitive

chemical-probe labeling in whole cell showed the probe effectively competed with ketamine and in protein profiling approach revealed associated biological pathways, while stress-response studies demonstrated that cellular state influenced the molecular response to ketamine. A computationally guided alkyne-tagged ketamine analog was also synthesized and applied to live-cell Raman imaging. Collectively, these results establish a human neuronal framework for investigating neuromodulator interactions and provide candidate molecular targets and pathways for subsequent mechanistic validation.

2.0 Results and discussion

2.1 Establishment and Validation of Human iPSC-Derived Cortical Neurons

A critical first step in this project was to establish a reproducible human neuronal model suitable for investigating ketamine-induced molecular responses. The study used human iPSC-derived cortical neurons because they provide a human-relevant cellular system in which molecular targets, signaling responses, and intracellular drug interactions can be examined under controlled conditions. The neuronal identity and functional maturation were evaluated before proceeding with ketamine perturbation and multi-omics measurements.

RNA sequencing demonstrated expression of genes associated with neuronal differentiation, cortical identity, excitatory neuronal phenotype, and synaptic function (Figure 1A). These included general neuronal markers such as TUBB3, MAP2, and RBFOX3; cortical lineage-associated markers including FOXG1, TBR1, and SATB2; glutamatergic neuronal markers SLC17A7 and SLC17A6; and genes associated with neuronal signaling and synaptic machinery, including CAMK2A, GRIA2, GRIN1, SNAP25, DLG4, and GRIN2B. The combined expression profile was consistent with the intended cortical glutamatergic neuronal phenotype.

Neuronal identity was further evaluated by immunocytochemistry (Figure 1B). Cells displayed Synapsin 1, a presynaptic neuronal marker, together with NMDAR2B/GRIN2B, a subunit of the NMDA receptor relevant to glutamatergic signaling and ketamine pharmacology. DAPI staining was used to visualize cell nuclei. These measurements provided protein-level confirmation of the neuronal and glutamatergic characteristics indicated by the transcriptomic data.

Functional activity was assessed using calcium imaging. Time-resolved imaging demonstrated intracellular calcium dynamics in the matured neuronal cultures, including representative measurements following ketamine exposure (Figure 1C). Together, transcriptomic marker expression, immunocytochemical characterization, and calcium imaging provided complementary molecular and functional evidence supporting use of the 21-day-matured cultures for subsequent ketamine exposure experiments.

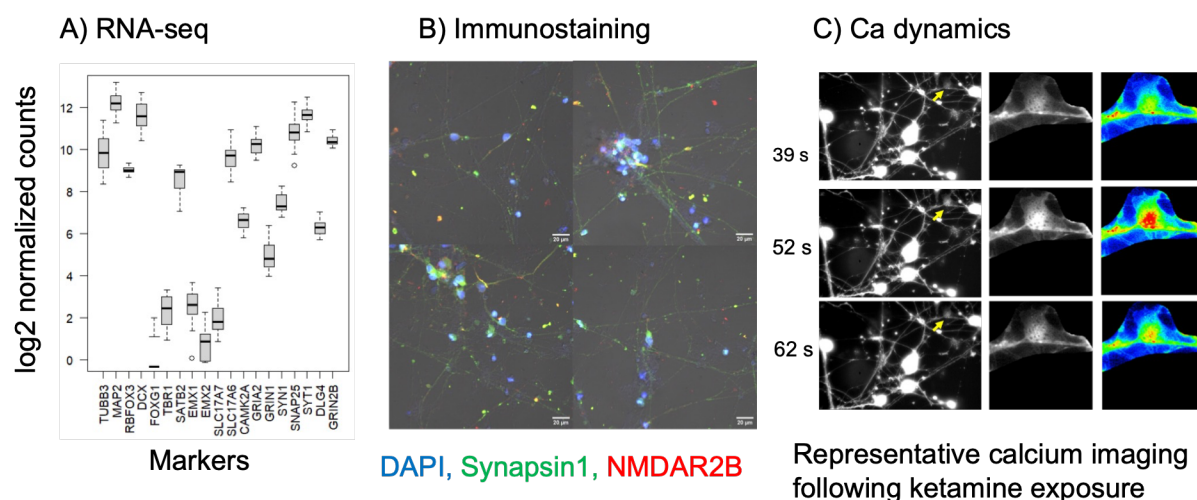


Figure 1. Establishment and validation of human iPSC-derived cortical neurons. Human iPSC-derived glutamatergic neurons were matured for 21 days and characterized using complementary molecular and functional measurements. **(A)** RNA-seq expression of markers associated with neuronal differentiation, cortical identity, glutamatergic phenotype, and synaptic function. **(B)** Representative immunostaining for nuclei (DAPI), Synapsin 1, and NMDAR2B. **(C)** Representative time-resolved calcium imaging following ketamine exposure, demonstrating measurable calcium dynamics in the neuronal cultures.

2.2 Ketamine Induces Time-Dependent Molecular Remodeling in Human Cortical Neurons

Following establishment of the cortical neuronal cultures, we examined the molecular response to ketamine exposure using complementary proteomic, lipidomic, and metabolomic measurements. The temporal profiling is important because the rapid pharmacological effects of ketamine may initiate molecular responses that evolve after the initial exposure. Accordingly, 21-day-matured human iPSC-derived glutamatergic neurons were treated with 1 μ M ketamine and analyzed after 6 and 24 hours. The 1 μ M concentration was selected to imitate the a physiologically relevant concentration of ketamine observed after ketamine infusion for the treatment of TRD. The two time points are used to characterize early molecular responses. The multi-omics strategy was intended to determine whether ketamine produced a persistent molecular signature or a dynamic transition between cellular states.

Proteomic analysis demonstrated extensive remodeling following ketamine treatment (Figure 2A). Importantly, substantial differences were also observed between the 6- and 24-hour vehicle controls, emphasizing the need for time-matched comparisons when evaluating neuronal responses. Relative to the corresponding controls, ketamine treatment was associated with changes in hundreds of proteins at both time points. At 6 hours, 405 proteins were lower and 89 were higher in abundance based on the criteria represented in the analysis, whereas at 24 hours, 233 proteins were lower and 188 were higher. Direct comparison of the 6- and 24-hour ketamine-treated samples identified an additional strong temporal shift, with 61 proteins lower and 289 higher at 24 hours relative to the earlier treatment state. These results indicate that the proteomic response to ketamine was not static but underwent substantial reorganization between the early and later exposure periods.

The lipidome displayed a similar temporal pattern (Figure 2B). At the lipid-class level, the 6-hour response was characterized by negative enrichment of several classes, most prominently ceramides (Cer), phosphatidylethanolamines (PE), and phosphatidylinositols (PI). By 24 hours, this pattern shifted toward positive enrichment, including increased enrichment of triglycerides (TG), ceramides, and PE, while PI approached the control state. The reversal in the direction of several lipid-class responses between 6 and 24 hours further supports a model in which ketamine induces a dynamic cellular transition rather than a uniform sustained perturbation.

Metabolomic measurements from MPlex of extraction of upto 500k cells still produced limited metabolomics coverage. However, target extraction of metabolites of interest relevant to neuronal signaling was possible and we were able to identify a pronounced temporal effect on glutamate–glutamine metabolism (Figure 2C). Glutamate increased modestly at 6 hours, by approximately 16% relative to the untreated control, and returned to near-control levels by 24 hours. In contrast, glutamine showed a much larger early response, decreasing by approximately 77% at 6 hours before recovering substantially by 24 hours to approximately 84%

of the control level. Thus, the most pronounced metabolic response was a transient depletion of glutamine rather than a sustained change in glutamate abundance. The coordinated but distinct trajectories of these two metabolites suggest that ketamine perturbs glutamate–glutamine homeostasis during the early neuronal response, followed by substantial recovery over the subsequent 18 hours.

Taken together, the proteomic, lipidomic, and metabolomic datasets indicate that ketamine induces time-dependent molecular remodeling in human cortical neurons. The 6-hour state was distinct from both the untreated state and the 24-hour response, while many molecular features either recovered or transitioned in a different direction by 24 hours. This temporal behavior is important because it suggests that measurements obtained at a single endpoint would capture only a portion of the ketamine response. Rather than producing a single persistent molecular signature, ketamine appears to initiate a sequence of molecular changes involving protein abundance, membrane-associated lipid composition, and glutamate–glutamine metabolism. These observations provided the basis for subsequent analyses focused on identifying the proteins directly engaged by ketamine and determining how the cellular response is altered under stress conditions.

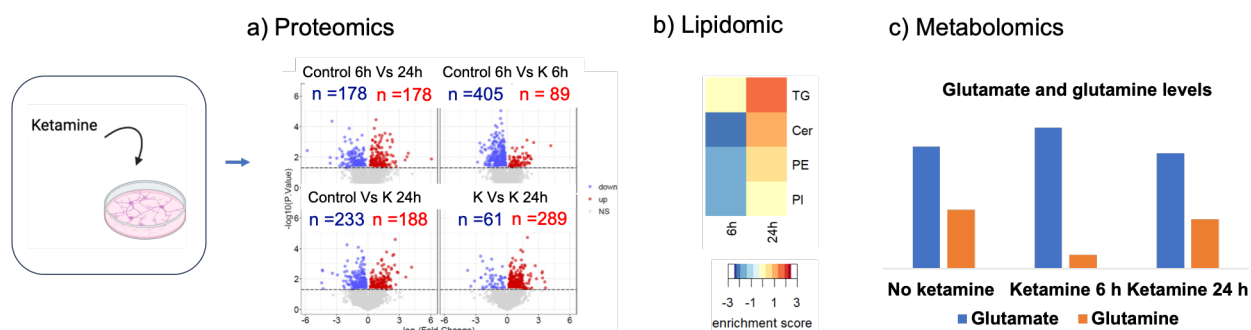


Figure 2. Ketamine induces time-dependent molecular remodeling in human iPSC-derived cortical neurons. 21-days matured human iPSC-derived glutamatergic neurons were treated with 1 μ M ketamine for 6 and 24 hours. **(A)** Proteomic comparisons between time-matched controls and ketamine-treated neurons and between 6- and 24-hour treatment states demonstrate extensive and temporally distinct changes in protein abundance. **(B)** Lipid-class enrichment profiles show differential responses of triglycerides (TG), ceramides (Cer), phosphatidylethanolamines (PE), and phosphatidylinositols (PI) at 6 and 24 hours. **(C)** Glutamate and glutamine measurements reveal a transient increase in glutamate and a pronounced early decrease in glutamine, followed by substantial recovery at 24 hours.

2.3 Cellular Stress Reshapes the Molecular Response to Ketamine

To determine whether the molecular response to ketamine depends on neuronal state, we first introduced a defined cellular stress and then examined the response to ketamine. This design allowed us to assess how prior stress alters ketamine-responsive pathways compared with baseline conditions.

To examine this state dependence, cortical neurons were treated with ketamine (K), hydrocortisone (HC), or hydrocortisone followed by ketamine (HCK), proteomic was extracted and analyzed by LCMS. The pathways enriched in these proteomics results was evaluated at 6

and 24 hours relative to time-matched DMSO controls (Figure 3A). The analysis revealed coordinated changes across pathways associated with mitochondrial function, membrane trafficking, cell-cycle regulation, DARPP-32 signaling, translation and RNA processing, and cellular stress responses. Importantly, the magnitude and direction of these responses depended on both treatment condition and time.

The strongest distinction between the hydrocortisone and hydrocortisone-plus-ketamine conditions occurred at the early 6-hour time point. For several pathways, HCK produced an enrichment pattern opposite to, or substantially different from, hydrocortisone alone. For example, ESCRT, DNA-damage checkpoint, G2/M checkpoint, Rho GTPase–PKN signaling, translation-associated processes, and SRP-dependent protein targeting shifted toward positive enrichment following HCK at 6 hours despite neutral or negative responses following hydrocortisone alone. Conversely, DARPP-32 signaling and several mRNA-processing pathways showed negative enrichment following HCK despite neutral or positive responses under hydrocortisone alone. These patterns indicate that ketamine did not simply add to the hydrocortisone-induced response; rather, it redirected multiple components of the early stress-associated molecular program.

By 24 hours, the hydrocortisone and HCK conditions showed substantially greater convergence. Both treatments displayed negative enrichment across aerobic respiration, ESCRT, several cell-cycle pathways, Rho GTPase signaling, global translation and translation elongation, protein targeting, mRNA processing, nonsense-mediated decay, and stress-response pathways. DARPP-32 signaling represented a notable exception, remaining positively enriched under both HC and HCK conditions at 24 hours. The transition from divergent early responses to more similar later profiles suggests that ketamine most strongly modifies the early trajectory of the stress response rather than establishing a completely independent long-term molecular state.

These results demonstrate that the molecular effects of ketamine are strongly influenced by the pre-existing cellular state. The ketamine response observed under baseline conditions therefore cannot be assumed to represent its behavior in a stressed neuronal system. Instead, ketamine appears capable of redirecting the early molecular response to hydrocortisone exposure across pathways involving mitochondrial activity, intracellular trafficking, cell-cycle regulation, signaling, translation, and RNA processing. The subsequent convergence of HC and HCK profiles by 24 hours further emphasizes the temporal nature of this interaction and supports the broader conclusion of the project that cellular stress state shapes the molecular response to ketamine.

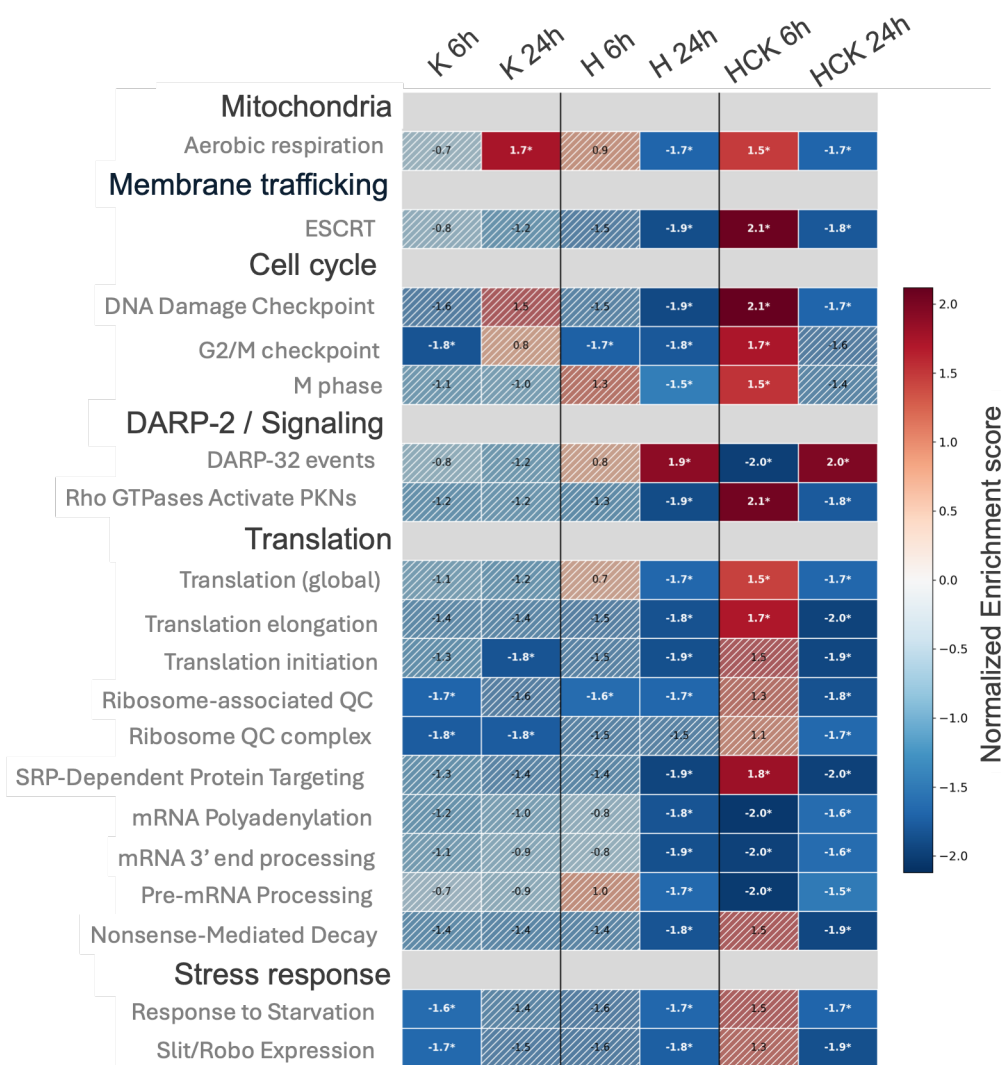


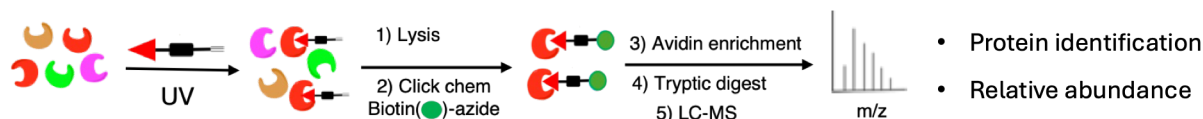
Figure 3. **Cellular stress reshapes the molecular response to ketamine.** Pathway enrichment in human iPSC-derived cortical neurons treated with ketamine (K), hydrocortisone (HC), or hydrocortisone followed by ketamine (HCK) at 6 and 24 hours relative to time-matched DMSO controls. Selected pathways represent mitochondrial function, membrane trafficking, cell-cycle regulation, DARPP-32 signaling, translation and RNA processing, and stress responses. Hydrocortisone and HCK produced divergent early pathway responses that became more similar by 24 hours. Adjusted $p < 0.05$; hatched cells indicate pathways tested but not statistically significant.

2.4. Redox-Probe Profiling Reveals Treatment-Dependent Remodeling of Redox Homeostasis

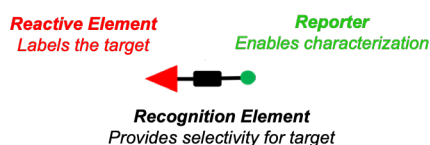
Because oxidative state can influence protein function independently of protein abundance, we complemented the global molecular measurements with a cysteine-reactive chemical-probe approach. We used redox probes to label accessible protein thiols in intact cells, followed by enrichment and mass spectrometry to identify proteins whose cysteine reactivity changed (cysteinome) with treatment (Figure 4). Because probe labeling depends on the availability of

reactive cysteine thiols, differences in labeling provide a readout of changes in the redox-sensitive proteome (cysteinome) rather than protein abundance alone (Sadler et al. 2014; Duan et al. 2017; Fu et al. 2020).

A) Reactivity/Photoaffinity-based protein profiling



B) Reactivity/Photoaffinity-based probes



C) Cysteine reactive probe used in this work

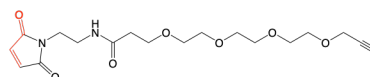


Figure 4. Reactivity- and photoaffinity-based protein profiling strategy used to interrogate protein function and molecular interactions. (A) General workflow in which reactive or photoaffinity probes label proteins in cells or lysates, followed by reporter installation, enrichment, and mass spectrometry-based protein identification. (B) Modular probe design consisting of a target-directed reactive or photoaffinity element and a reporter handle that enables downstream visualization or enrichment. (C) Cysteine-reactive probe used in this study to profile changes in accessible protein thiols associated with cellular redox state.

Redox-sensitive proteins identified following ketamine (K), hydrocortisone (HC), and hydrocortisone followed by ketamine (HCK) treatment were organized into major redox-homeostasis systems, including the glutathione system, peroxiredoxins, catalase, oxidative protein folding, and redox signaling (Figure 5B). The treatment conditions produced distinct patterns of cysteine-probe labeling, demonstrating that the redox state of these proteins depended strongly on the cellular perturbation.

Hydrocortisone alone was associated with increased probe labeling for several proteins involved in antioxidant defense and oxidative protein folding. Among the strongest responses shown in the figure were GSTP1 (+1.9 log₂ fold change), PRDX2 (+1.5), PRDX5 (+1.5), PDIA3 (+1.3), and PDIA4 (+2.7). In contrast, the HCK condition produced marked decreases in probe labeling across multiple components of the same redox systems. Within the glutathione-associated group, GSTP1 decreased by 2.8 log₂ fold, while IDH1 and IDH2 decreased by 4.0 and 4.3 log₂ fold, respectively. Peroxiredoxin labeling was similarly reduced, including PRDX1 (−2.6), PRDX2 (−4.0), and PRDX5 (−3.6). Catalase decreased by 2.0 log₂ fold, and several proteins involved in oxidative protein folding also showed pronounced reductions, including P4HB (−5.8), PDIA3 (−2.6), and PDIA4 (−1.3). NNT, which contributes to cellular redox balance, decreased by 2.3 log₂ fold in the HCK condition. Several additional targets were significant but did not have defined fold-change values because they were not detected in one of the comparison conditions.

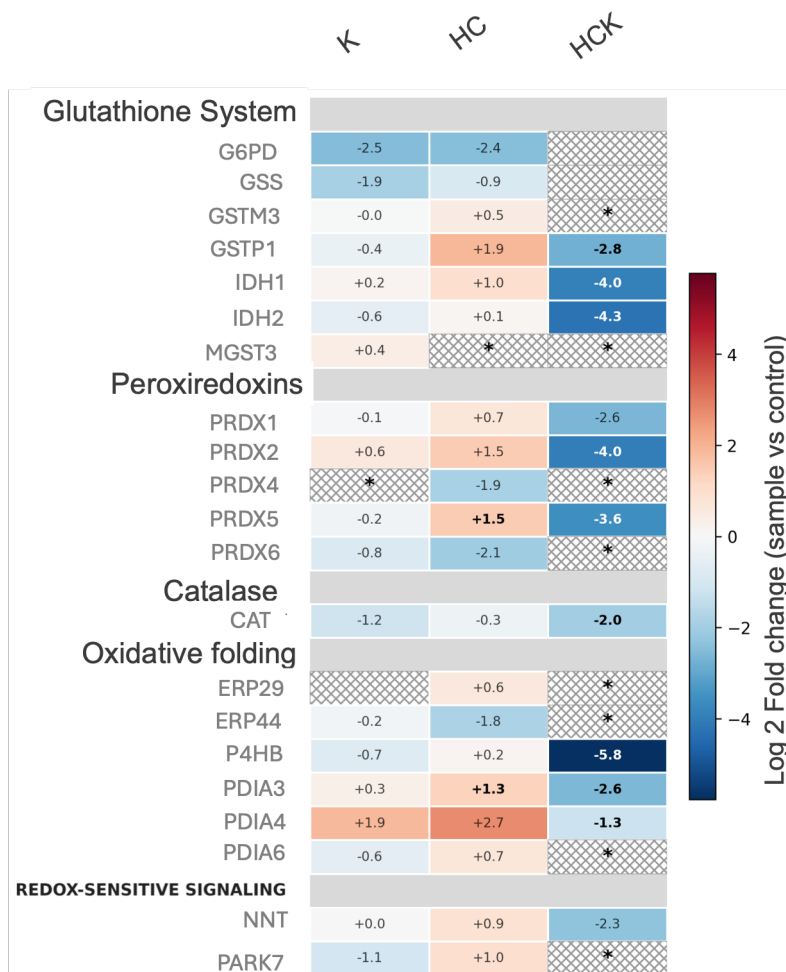


Figure 5. Treatment-dependent remodeling of redox-sensitive proteins in human cortical neurons. Cysteine-reactive maleimide-probe profiling of proteins involved in redox homeostasis following ketamine (K), hydrocortisone (HC), or hydrocortisone followed by ketamine (HCK) treatment relative to control. Selected proteins are grouped according to the glutathione system, peroxiredoxins, catalase, oxidative protein folding, and redox signaling. Values indicate log2 fold change in probe labeling relative to control. Bold values indicate statistically significant changes; asterisks indicate significant changes for which a fold change could not be defined, and hatched cells indicate proteins not detected in the corresponding condition. The HCK condition produced pronounced changes across multiple antioxidant and redox-regulatory systems, demonstrating that ketamine modifies the redox-sensitive proteome in a state-dependent manner.

The contrast between HC and HCK was particularly notable. Several proteins that showed increased cysteine-reactive labeling following hydrocortisone alone shifted toward strongly decreased labeling when ketamine was introduced after hydrocortisone exposure. PRDX2, PRDX5, PDIA3, and PDIA4 illustrate this pattern. This behavior parallels the pathway-level results in Figure 5A, where ketamine redirected the early molecular response to hydrocortisone rather than simply adding an independent response. Together, the two analyses indicate that the influence of ketamine on the stressed neuronal state extends to proteins directly involved in maintaining cellular redox homeostasis.

Ketamine alone produced a more moderate and heterogeneous redox-probe profile than the HCK condition. Changes were observed across glutathione-associated proteins, peroxiredoxins, catalase, protein-disulfide isomerases, and redox-signaling proteins, but the broad coordinated decrease observed following HCK was not reproduced by ketamine treatment alone. This distinction reinforces the conclusion that the molecular response to ketamine depends on the pre-existing cellular state.

Importantly, decreased maleimide-probe labeling should not be interpreted directly as decreased protein abundance. Because the probe preferentially reacts with accessible reduced cysteine thiols, lower labeling may reflect changes in cysteine oxidation, accessibility, or other modifications that alter thiol reactivity, in addition to potential changes in protein abundance. Thus, these measurements identify redox-sensitive proteins and pathways whose functional chemical state changes with treatment, providing information complementary to the abundance-based proteomic measurements. The strong treatment dependence observed in the HCK condition supports the broader conclusion that ketamine modifies the biochemical state of stressed neurons at both the pathway and protein-reactivity levels.

2.5 Photoaffinity Profiling Identifies Candidate Ketamine-Interacting Proteins

The multi-omics and redox-profiling experiments demonstrated that ketamine alters the molecular state of cortical neurons but do not distinguish proteins that may be directly engaged by ketamine from downstream responses. A major objective of the project was therefore to use ketamine-mimicking photoaffinity probes to capture transient small-molecule–protein interactions in intact neuronal cells. Photoaffinity labeling provides a complementary approach to conventional proteomics because UV activation converts reversible probe–protein interactions into covalent adducts that can subsequently be visualized or enriched for mass spectrometry analysis (Smith and Collins 2015). The clickable reporter handle of the ketamine probe allowed the same labeling strategy to be coupled either to a fluorescent dye for cellular imaging or to biotin for affinity enrichment and protein identification.

We first evaluated labeling of the ketamine-mimicking photoaffinity probe in 21-day-matured cortical neurons. Cells were treated with 20 μ M probe, followed by UV irradiation to covalently capture probe-associated proteins. The alkyne reporter was subsequently coupled to a fluorescent TAMRA reporter by click chemistry, allowing probe labeling to be visualized by fluorescence microscopy (Figure 6A). To assess whether the observed labeling represented interactions that could also be occupied by ketamine, neurons were preincubated with 20 μ M ketamine before addition of the probe. Ketamine competition reduced cellular probe fluorescence relative to probe-only treatment (Figure 6B–C), indicating that a portion of the photoaffinity-probe labeling was competitively displaced by the parent compound. The competition experiment was then extended to chemoproteomic analysis. For these experiments, cortical neurons were treated with the ketamine probe with or without ketamine competition, followed by UV crosslinking. Probe-labeled proteins were conjugated to azido-PEG3-biotin, enriched using streptavidin affinity chromatography, digested, and analyzed by mass spectrometry. Four biological replicates were used for the proteomic labeling experiments. Proteins enriched by the probe and reduced under ketamine competition were prioritized as candidate ketamine-interacting proteins.

The resulting protein set encompassed multiple biological functions and pathways (Figure 6D), demonstrating that the ketamine-mimicking probe sampled a broader interaction landscape than would be expected from consideration of the NMDA receptor alone. The probe enabled

proteomics was limited because of the technical quality of the probe only dataset and because of resource and time constraints. Thus, we could not fully obtain the non-conical targets from this dataset. However, the remaining dataset was used to obtain ketamine associated biological pathways. Repeating these experiments and obtaining quality data will provide high-quality non-canonical ketamine targets for furthermore validation.

These results complement the preceding multi-omics and redox-profiling studies. Whereas global proteomics described changes in protein abundance and cysteine-reactive profiling measured treatment-dependent changes in protein chemical state, photoaffinity profiling provided a route to identify proteins associated with the ketamine-like molecular scaffold itself. Together, the approaches begin to separate three interconnected components of the neuronal response—molecular remodeling, functional protein state, and candidate drug–protein engagement—and generated a prioritized target set for subsequent mechanistic studies.

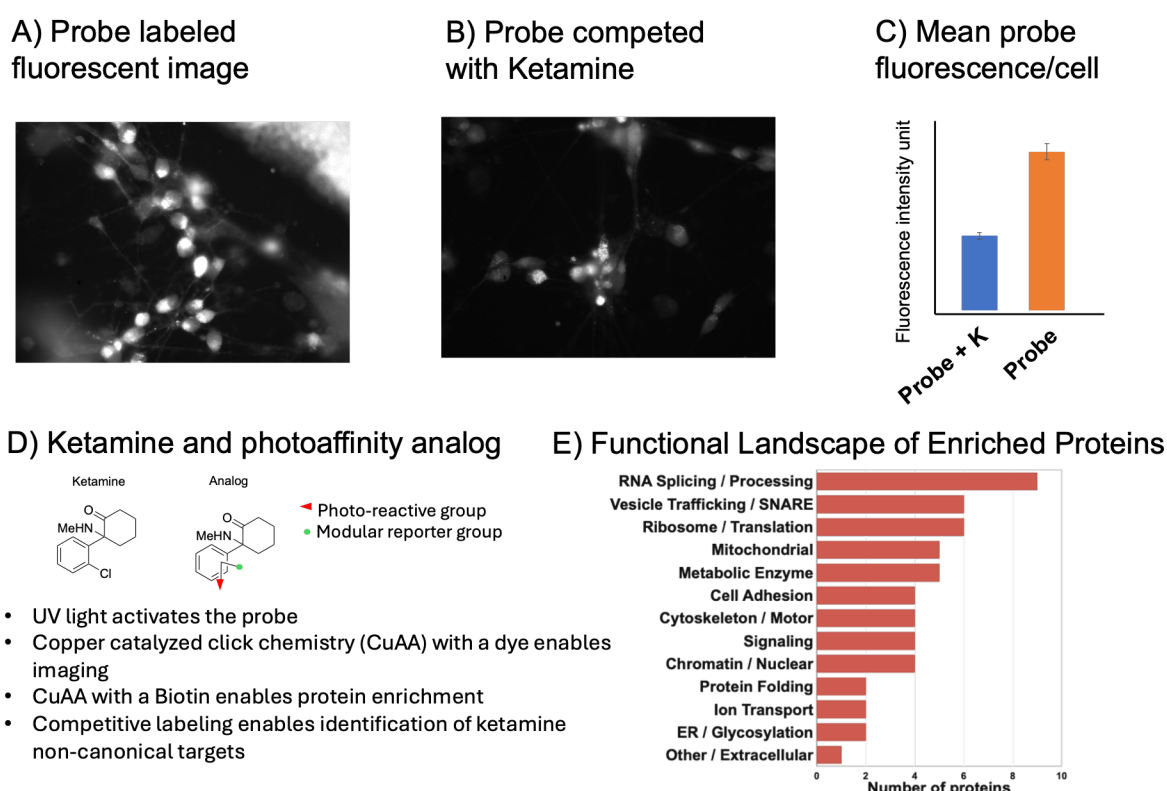


Figure 6. Competitive photoaffinity profiling identifies candidate ketamine-interacting proteins in human cortical neurons. (A) Representative fluorescence image of cortical neurons labeled with the ketamine-mimicking photoaffinity probe. Probe labeling was performed in intact cells, followed by UV crosslinking and installation of a fluorescent reporter by click chemistry. (B) Representative image following competition with ketamine, showing reduced probe-associated fluorescence. (C) Quantification of mean cellular probe fluorescence in probe-only and ketamine-competition conditions. (D) Ketamine photoaffinity analog used in photoaffinity-based protein profiling. (E) Functional landscape of proteins identified by photoaffinity enrichment and competitive chemoproteomic analysis. Proteins whose enrichment was reduced by ketamine competition were prioritized as candidate ketamine-interacting proteins.

2.6 Raman-Active Ketamine Probe and Live-Cell SRS Imaging Reveal Dynamic Chemical Changes

Photoaffinity labeling enabled visualization and capture of ketamine-associated protein interactions but required UV crosslinking, fixation, and subsequent reporter installation. To extend the chemical-probe strategy to living cells, the project sought to develop minimally modified ketamine analogs carrying small vibrational reporters that could be detected directly by stimulated Raman scattering (SRS) microscopy. Alkyne and nitrile groups are attractive Raman tags because of their small size and characteristic vibrational signals within the relatively cell-silent spectral region, allowing molecular imaging without installation of a bulky fluorophore (Zhao et al. 2017).

Candidate Raman-active ketamine analogs were first evaluated computationally by docking to an NMDA receptor structure (PDB 5UN1) to prioritize modifications expected to retain interactions comparable to the parent molecule. Ketamine showed a calculated binding energy of -6.70 kcal/mol, while the candidate analogs ranged from -6.54 to -7.33 kcal/mol. Analog 3, which had the most favorable calculated binding energy among the evaluated structures (-7.33 kcal/mol), was synthesized for experimental characterization (Figure 7A). Raman spectral analysis of the resulting alkyne-tagged probe showed a distinct signal at approximately 2148 cm^{-1} , confirming that the incorporated alkyne provided a spectroscopically detectable vibrational reporter.

In parallel, live-cell SRS imaging was used to examine chemical changes in cortical neurons during ketamine exposure (Figure 7B). SRS measurements included the CH-stretch region associated with endogenous cellular molecules, including signals collected across $2950\text{--}2980\text{ cm}^{-1}$ and $2850\text{--}2875\text{ cm}^{-1}$. Bright-field and SRS images obtained from control neurons and neurons following ketamine exposure showed treatment-dependent changes in the distribution and relative intensity of these spectral features. Spectral profiles extracted from selected cellular regions likewise changed over the course of exposure, indicating dynamic alterations in the chemical composition of the neuronal cells.

The observed changes were most consistent with alterations in lipid-associated cellular signatures. This result complements the independent lipidomics measurements, which also demonstrated time-dependent remodeling of lipid classes following ketamine treatment. Together, the mass-spectrometry and SRS measurements provide orthogonal evidence that lipid composition and/or organization represents a dynamic component of the neuronal response to ketamine.

Importantly, the final SRS measurements should be distinguished from definitive mapping of the intracellular ketamine probe itself. Although the synthesized analog generated the expected Raman signal near 2148 cm^{-1} and established the feasibility of a Raman-active ketamine reporter, the live-cell imaging results presented here primarily demonstrate ketamine-associated changes in endogenous cellular chemical signatures rather than validated subcellular localization of the ketamine analog. Additional optimization of probe sensitivity, exposure conditions, and spectral acquisition will be needed to directly map probe accumulation and localization in living neurons.

Nevertheless, this work established a complementary live-cell imaging capability that extends the molecular measurements developed in this project. Multi-omics profiling provided global

molecular responses, reactivity-based probes reported changes in protein chemical state, photoaffinity probes established pathways for identification of non-canonical ketamine targets, and SRS microscopy added spatially resolved measurements of dynamic cellular chemistry. Together, these approaches provide a framework for investigating neuromodulator responses across molecular, functional, and spatial scales.

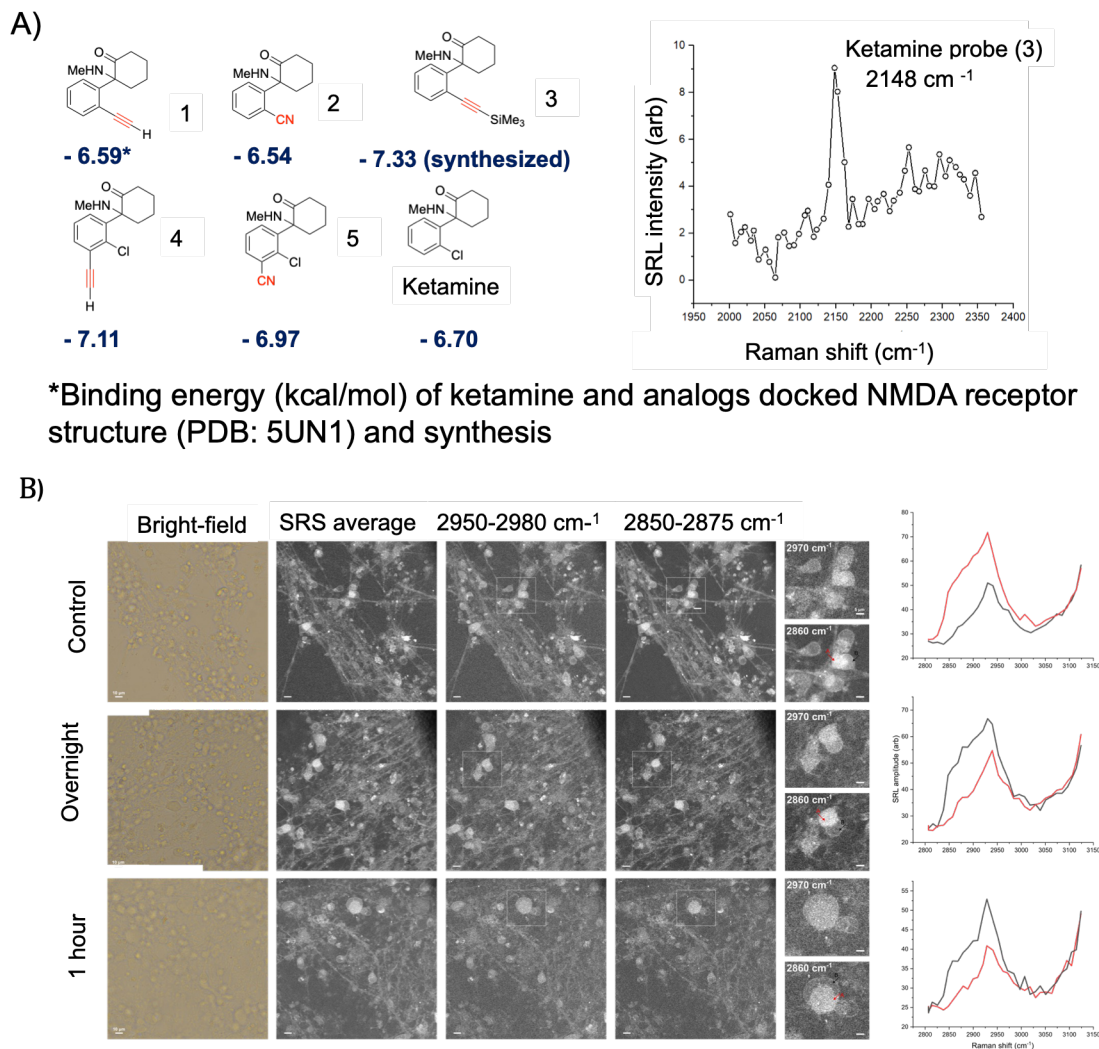


Figure 7. Development of a Raman-active ketamine probe and live-cell SRS imaging of ketamine-associated chemical changes. (A) Computational evaluation of ketamine and candidate Raman-active analogs docked to the NMDA receptor structure (PDB 5UN1). Analog 3, with a calculated binding energy of -7.33 kcal/mol, was synthesized and exhibited a characteristic alkyne Raman signal at approximately 2148 cm^{-1} . (B) Representative bright-field and SRS images of live human iPSC-derived cortical neurons under control conditions and following ketamine exposure. SRS images collected across the $2950\text{--}2980$ and $2850\text{--}2875\text{ cm}^{-1}$ regions and corresponding representative spectra demonstrate exposure-dependent changes in lipid-associated cellular chemical signatures.

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