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Lauren Grychowski April 4, 2024

Transient Metabolic Stress Induces Energy and Purine Impairment in Neural Progenitor Cells for Lesch-Nyhan Disease

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Abstract

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Background: Lesch-Nyhan Disease (LND) is a rare neurological disorder characterized by severe behavioral and motor impairments. LND is caused by pathogenic variants in the *HPRT1* gene, which produces an enzyme with a crucial role in purine salvage synthesis. Existing research has yet to examine the impact of acute metabolic stress during neurodevelopment as a potential factor contributing to the pathogenesis of LND. The present study aims to leverage a cell culture model involving neural progenitor cells (NPCs) under conditions that mimic transient glycolytic and purine metabolic stress to determine whether LND cells are more vulnerable to metabolic challenges.

Methods: NPCs were generated from patient-derived induced pluripotent stem cells (iPSCs) and confirmed through immunostaining for neural stem cell markers. Initial experiments were conducted to determine optimal dosage and timing of glycolytic and purine metabolic inhibitors on NPCs to induce metabolic stress while preventing cell death. At these determined conditions, NPCs were again challenged, and purines were quantified utilizing high performance liquid chromatography (HPLC).

Results: Increasing concentrations of glycolytic and purine metabolic inhibitors led to a trend of decreasing ATP levels in both control and LND cell lines. Acute exposure to high concentrations of glycolytic inhibitor resulted in significant differential reduction in total purine content and GTP levels in LND NPCs compared to controls, revealing defects in purine metabolism. Transient exposure to both glycolytic and purine metabolic inhibitors compromised energy metabolism, as demonstrated through reductions in energy transfer purines such as ATP; however, the differences between control and LND NPCs were minimal.

Conclusion: This study suggests that challenging LND NPCs with acute metabolic stressors can reveal impairments in purine and energy metabolism that aren't evident at baseline, which indicates that this model may be a beneficial tool for investigating LND pathogenesis. Further research is required to identify the optimal conditions and developmental stages of cells that can elucidate the role of energy impairments during LND neurodevelopment.

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Introduction

LND Background

Lesch-Nyhan Disease (LND) is a rare, X-linked genetic disorder resulting from pathogenic variants in the *HPRT1* gene, which encodes an enzyme that is important for the salvage synthesis of purines known as hypoxanthine-guanine phosphoribosyltransferase (HGprt). More specifically, HGprt catalyzes the addition of a phosphoribosyl group to metabolize guanine and hypoxanthine into guanosine monophosphate (GMP) or inosine monophosphate (IMP), respectively (Figure 1). Although only one gene is implicated in the disease, researchers have discovered over 600 mutations that can influence the HGprt activity and the severity of LND's symptoms (Fu et al., 2014). Classic LND refers to the most severe disease phenotype resulting from the complete deficiency of HGprt activity (Jinnah et al., 2010, 2013). A characteristic of the disease includes excessive uric acid production, referred to as hyperuricemia, which leads to gouty arthritis and kidney stones. Neurological manifestations include cognitive disability, severe motor impairment due to involuntary movements, and behavioral anomalies such as impulsivity, aggression, and self-injurious behaviors (Jinnah et al., 2006; Schretlen et al., 2005).

The diagnosis of LND is confirmed through clinical assessment of symptoms and genetic testing for pathogenic variants in the *HPRT1* gene. While there is no cure for LND, treatments exist to help manage symptoms. These treatments may include medication to control uric acid levels, physical therapy to improve motor function, and behavioral interventions for self-injurious behaviors. Current medications effectively mitigate hyperuricemia, which is a consistent symptom across all disease variants of *HPRT1* deficiency. Thus, the occurrence of hyperuricemia in milder forms of the disorder that lack neurobehavioral symptoms suggests that it is not the cause of neurological effects seen in LND (Nanagiri & Shabbir, 2024). The hallmark

feature of LND and the most profound neurological defect is self-injurious behaviors, which emerge in the first few years of life and can lead to significant bodily disfigurement (Mohapatra & Sahoo, 2016; Nanagiri & Shabbir, 2024). These behaviors typically manifest as biting of lips, fingers, and cheeks, along with limb-banging and eye-poking. Notably, self-injurious behavior continues in these individuals despite no apparent deficits in pain perception (Torres & Puig, 2007). While prior research implicates the reduction of dopaminergic levels in the basal ganglia in LND, medications targeting dopamine do not provide the anticipated relief for these symptoms. The current standard of treatment often involves physical restraints or dental extraction to prevent self-injury and mutilation (Goodman et al., 2014). Alternative therapies such as deep brain stimulation, botulinum toxin, and S-adenosylmethionine have been explored (Dolcetta et al., 2013; Garcia-Romero et al., 2022; Visser et al., 2021); however, they demonstrate inconsistent outcomes and adverse side effects that discredit their effectiveness. To summarize, the therapeutic strategies for addressing neurological symptoms, particularly self-injurious behaviors, are suboptimal. Further research is necessary to understand the underlying pathogenesis of LND and develop more effective therapeutic approaches.

The pathogenesis of LND remains largely unclear; however, existing research points to a deficit in dopamine. Studies involving HGprt-deficient mice uncovered significant reductions in dopamine levels in the basal ganglia, accompanied by a decrease in tyrosine hydroxylase (TH), which is the rate-limiting enzyme in dopamine synthesis (Jinnah et al., 1994). This decrease in dopamine occurred without effects in other neurotransmitter systems such as norepinephrine and serotonin (Jinnah et al., 1994). Other studies similarly observed a reduction of dopamine in the basal ganglia, particularly in the caudoputamen, without overt degeneration of these dopaminergic neurons (Dunnett et al., 1989; Jinnah et al., 1992). Furthermore, a recent study

revealed neurodevelopmental abnormalities in HGprt-deficient mice that affect the proliferation and migration of developing dopamine neurons during embryogenesis (Witteveen et al., 2022). The same study observed disruptions in dopaminergic projections from the basal ganglia to cortical areas, suggesting that developmental issues may underlie LND as opposed to neurodegenerative mechanisms (Witteveen et al., 2022). Altogether, these insights imply that developmental abnormalities in dopamine production and neural pathways originating from the basal ganglia are likely involved in the pathogenesis of LND. While these mouse models replicate the metabolic disturbances seen in LND, they do not exhibit the distinct neurobehavioral phenotype that is characteristic of LND (Finger et al., 1988).

Patients with LND display basal ganglia dysfunction, similar to the findings observed in mice (Visser et al., 2000). Although the brain structure in LND patients appears to be generally normal, there is a slight reduction in the size of the caudate and putamen, which constitute the basal ganglia, when compared to individuals of the same age without LND (Harris et al., 1998). Moreover, the dopamine levels within the basal ganglia are often abnormal in LND. Postmortem neurochemical analysis of LND brains indicated a significant loss of dopamine, as much as 80%, across the basal ganglia, alongside diminished TH immunoreactivity (Göttle et al., 2014; Lloyd et al., 1981; Saito et al., 1999). Neuroimaging studies support these observations, revealing reduced dopaminergic markers in LND patients (Ernst et al., 1996). Therefore, human research involving autopsy brains from individuals with LND further indicate that midbrain dopaminergic cells may be involved in LND pathogenesis.

Purine Metabolism

The HGprt enzyme plays a key role in purine metabolism. Purines are among the most abundant and critical metabolic substrates for living organisms, serving as key components of many biomolecules. Notably, purines are necessary to produce nucleic acids, coenzymes, signaling molecules, and energy transfer molecules. The most abundant purine is ATP, which is a key energy source for all cells (Traut, 1994). Cells synthesize purines through two main pathways: the *de novo* pathway and the salvage pathway. The *de novo* pathway utilizes pentose phosphate pathway derivatives and other simple biomolecules such as amino acids in an energetically demanding process to produce inosine monophosphate (IMP), which readily converts to other purines such as adenine or guanine, and their derivatives (Moffatt & Ashihara, 2002). In contrast, the purine salvage pathway presents a more energy-efficient alternative to *de novo* synthesis as it recycles preformed purine products. This recycling process depends on the functionality of the enzymes HGprt and adenine phosphoribosyltransferase (APRT) (Figure 1).

LND is among many clinical conditions associated with defects in purine metabolism enzymes. The genetic basis of LND involves pathogenic variants in the *HPRT1* gene, leading to reduced activity of the enzyme HGprt, which is crucial for recycling guanine and hypoxanthine. While earlier studies hypothesized that impaired recycling would result in purine deficiency, investigations in HGprt-deficient cells and mouse models have not revealed significant disparities, suggesting that overactivation of the *de novo* synthesis pathway compensates for this deficiency. Prior animal studies in mice indicated that HGprt-deficient animals appear to have normal brain purine content compared to controls despite their impairment in purine recycling (Jinnah et al., 1993). Using radiolabeled isotopes, these researchers also found that the *de novo* purine synthesis was increased up to fivefold in mutant animals to compensate for the lack of

recycling, causing them to exhibit normal levels of purines (Jinnah et al., 1993). Similarly, studies using cell cultures of HGprt-deficient dopaminergic neuroblastoma cells indicated no significant purine disparities, reinforcing the idea that the activation of *de novo* synthesis can offset the recycling deficiency (Shirley et al., 2007). Therefore, biological challenges likely stem from how completely cellular attempts adjust for purine recycling defects in different types of cells. Given that impairment of dopaminergic neurons from the basal ganglia have been implicated in LND, energetic and functional processes in these cells may be particularly impacted by this compensation of the purine metabolic pathway.

Cell Culture Models for LND using iPSCs

The development of induced pluripotent stem cell (iPSC) technology has significantly advanced the field of *in vitro* disease modeling. These stem cells exhibit pluripotency similar to that of embryonic stem cells and can be derived by reprogramming somatic cells through the introduction of four key transcription factors – OCT4, SOX2, KLF4, and MYC – under conditions similar to those used for embryonic stem cells. (Okita et al., 2007; Takahashi et al., 2007; Takahashi & Yamanaka, 2006). This pluripotent quality allows the iPSCs to differentiate into nearly any cell type in the human body under the appropriate cell culture conditions. This is particularly advantageous for studying disorders of the nervous system, as iPSCs can be differentiated into a diverse array of neural cells, including neurons, neural progenitor cells (NPCs), microglia, oligodendrocytes, and astrocytes by following the principles of developmental biology. These models enable scientists to simulate neurodevelopment *in vitro*, offering a modality to investigate impaired neurodevelopmental processes at specific stages (Ardhanareeswaran et al., 2017; Sabitha et al., 2021). Therefore, iPSC models provide an

opportunity to conduct more biologically relevant studies to investigate human neurological disease, facilitating more valid insights into their mechanisms and developing targeted therapeutics (Engle et al., 2018; L. Li et al., 2018).

The application of iPSCs has recently expanded to modeling LND. Several research groups have successfully established iPSC lines from biopsies of LND patients, verifying their quality through assessments for normal karyotype, pluripotency marker expression, and ability to differentiate into the three germ layers (Y. Li et al., 2024; Sutcliffe et al., 2021). These patient-derived iPSCs demonstrated the expected lack of HGprt activity, alongside other consistent anomalies in gene and protein expression patterns (Sutcliffe et al., 2021). Additionally, the differentiation of these iPSCs into midbrain dopamine neurons was reported and confirmed by a high detection of TH, dopamine, and related metabolites across all cell lines (Seifar et al., 2023). These studies indicate that iPSCs derived from LND patients can be successfully generated and differentiated into dopaminergic neural cells. Importantly, these cell lines preserve the distinctive lack of HGprt activity, affirming the model's potential as a tool to study LND neurodevelopment *in vitro*.

The use of iPSC differentiation into neural cells offers a novel approach for modeling LND and exploring the impact of HGprt deficiency on metabolism. Previous research aimed to elucidate the significance of the two primary purine sources – *de novo* synthesis versus salvage pathways – throughout neurodevelopment *in vitro*, particularly in the context of LND. The preliminary findings revealed a general decline in total purine levels during development, with an increasing reliance on the salvage pathway as neurons mature, highlighting a shift towards salvage pathway dependency in more developed neurons (Seifar et al., 2022). In LND-specific lines, the absence of HGprt activity was linked to elevated levels in *de novo* synthesis enzymes,

supporting a compensatory mechanism for the lack of purine recycling (Seifar et al., 2022). Further studies have focused on NPCs derived from LND patient iPSCs to examine the differential impact of HGprt deficiency across various cell types. This research identified significant changes in gene and protein expression between LND iPSCs and NPCs, with little overlap, underscoring that the effects of HGprt deficiency can vary depending on the cell type (Dinasarapu et al., 2022). Additionally, this iPSC model was employed to screen for molecules that might correct the neuronal phenotype of HGprt-deficient cells by promoting purine metabolism in a way that does not depend on HGprt. Ultimately this led to the identification of several potential pharmacological compounds, including S-adenyslmethionine (Ruillier et al., 2020). Overall, these studies highlight the value of the iPSC model of LND in exploring metabolic complexities and potential therapeutic strategies. Notably, most research to date in this LND model has concentrated on baseline measurements, indicating a need for more in-depth functional studies to better understand the molecular dynamics of the diseased cells.

Energy Impairment Hypothesis

Energy shortage during development has not been extensively studied as a potential cause of neurological disease, however, researchers have recently suggested that mitochondrial or bioenergetic dysfunction may be related to the etiology of LND and other neurological motor disorders (Johnson et al., 2019). Considering what is known regarding the biochemical underpinnings of LND, it seems reasonable to infer that energy-related challenges may contribute to LND pathogenesis. HGprt-deficient cells seem to rely on an exaggerated use of the *de novo* pathway to maintain necessary purine levels, incurring higher energy costs. Moreover, diminished ATP production in HGprt-deficient cells may be a result of reduced available stores

of IMP for ATP conversion due to the lack of hypoxanthine recycling. Therefore, acute energy challenges during midbrain development could serve as a cause for impaired functioning of terminally differentiated dopamine neurons.

Dopaminergic neurons are especially susceptible to impairments due to their high energetic demands, specifically those originating from the basal ganglia. This energetic vulnerability may underlie the pathophysiological basis of neurological disorders such as LND and Parkinson's Disease (Mergenthaler et al., 2013). In response to acute conditions of energy deprivation, such as a lack of oxygen or glucose, dopamine neurons demonstrated reduced ATP production and undergo membrane hyperpolarization, effectively silencing their spontaneous activity, compared to neurons in normal culture conditions (Guatteo et al., 2005). This reduction in synaptic activity due to acute energetic stress underscores the sensitivity of dopaminergic neurons to these conditions. Moreover, dopamine neurons in the substantia nigra of the basal ganglia are distinguished by their extensive axonal fields, requiring the maintenance of action potential propagation over a significantly larger number of synapses compared to other neurons (Bolam & Pissadaki, 2012; Pissadaki & Bolam, 2013). This necessitates higher energetic expenditures to sustain synaptic function, further highlighting the unique vulnerability of these neurons to energy deficiencies. Therefore, this heightened susceptibility of these dopaminergic neurons to transient energy stressors may be especially relevant for individuals with LND. The overactivation of the *de novo* pathway in LND neurons to compensate for purine levels may complicate the maintenance of optimal energy levels within these cells, exacerbating their vulnerability.

A method of addressing this energetic impairment hypothesis could involve transiently inhibiting metabolic processes in LND dopaminergic cells to identify differential changes in energy metabolism compared to normal cells. The drug 2-deoxy-D-glucose (2DG) is a

nonmetabolizable glucose analog that competitively inhibits glycolysis, thereby blocking both aerobic and anaerobic metabolism (Singh et al., 2023). Additionally, azaserine is an analog of glutamine that competitively inhibits two of the limiting reactions in the *de novo* purine synthesis pathway (Figure 1) (Bennett et al., 1956; Ruillier et al., 2020). Therefore, these drugs may be used on LND cells in culture to acutely stress their energetic and purine metabolic processes to examine differential effects due to HGprt deficiency.

Recent investigations have begun examining differential energy impairment in LND, reporting compromised energy metabolism in dopaminergic LND NPCs, with the HGprt deficient cell line exhibiting markedly reduced ATP and GTP levels compared to the control at baseline (Bell et al., 2021). These researchers also conducted continuous measurements of extracellular acidification to examine glycolysis function and found that ATP production and glycolytic capacity was reduced in LND NPCs compared to controls (Bell et al., 2021). Notably, these differences were attenuated in cells of other stages including iPSC and mature neurons. These findings substantiate the idea that LND cells, particularly at the NPC stage, may be more susceptible to energetic challenges due to HGprt deficiency. Therefore, this project was driven by the following primary research question: Are LND NPCs more vulnerable to energy challenges? I hypothesized that if LND dopaminergic NPCs are more vulnerable to energy demands, then they would exhibit significantly diminished ATP levels following exposure to glycolytic or purine pathway inhibitors compared to control NPCs. I further hypothesized that these same trends would be reflected in levels of GTP and its derivatives.

Materials and Methods

Establishment of iPSC Lines

This study utilized in-house iPSC lines generated from human skin biopsies and characterized as previously described (Sutcliffe et al., 2021). Demographic and genetic characteristics pertaining to the human subjects who participated in sample collection are listed in Table 1. LND almost exclusively affects males due to the X-linked genetic nature of the disorder, therefore only males were included in the study. The procedures involved deidentified material, therefore, IRB approval was not required. Human skin fibroblasts were generated from biopsies as previously described (Fu et al., 2015). Fibroblasts were reprogrammed to iPSCs using a non-integrating mRNA reprogramming kit (Reprocell USA #00-0076). The pluripotency status of the iPSCs were evaluated by immunostaining for pluripotency markers and gene expression profiling for pluripotency gene expression. Differentiation of each line to the 3 major germ cell layers of ectoderm, mesoderm, and endoderm was performed and verified by immunostaining with respective markers for each germ cell layer type. Presence of normal or mutant *HPRT1* variant was confirmed using RT-PCR for normal and LND iPSC lines, respectively. Finally, all lines were assessed to ensure normal karyotype (Wicell Research Institute Cytogenetics Lab).

NPC Differentiation and Maintenance

Neural progenitor cells (NPCs) were generated from iPSCs and stored as previously described (GIBCO #MAN0008031). On day 1, high quality iPSCs were passaged using Accutase (Stemcell Technologies #07920) and plated in mTeSR Plus Medium (Stemcell Technologies #100-0274) on a Geltrex-coated (GIBCO #A1413202) 6-well plate. On the following day, the media was changed to complete neuron induction medium (NIM), which consists of neurobasal

medium (GIBCO #A-1647801) with 2% neural induction supplement (GIBCO #A16477-01) and 0.5% PenStrep (GIBCO #15140-122). NIM is changed every other day until day 7 when NPCs have completed development. NPCs are replated on day 7, which is considered passage 0, onto a Geltrex-coated plate in neural expansion medium (NEM), containing 1:1 constitution of neurobasal medium and advanced DMEM/F12 medium (GIBCO #12634-010) with 2% neural induction supplement and 0.5% PenStrep. ROCK inhibitor, or Y-27632 dihydrochloride (Sigma-Aldrich #Y0503), was added to the media during plating for the first 3 passages, starting at passage 0 with a concentration of 1 μL/mL NEM. Media containing rock inhibitor was changed the following day. After that, cells are maintained in NEM with media changes every other day.

After passage 2 or higher, NPCs were cryopreserved for long term storage. NPCs were passaged and resuspended in NEM containing 20% DMSO. The NPC suspension was frozen at -80 °C overnight, and then transferred to a liquid nitrogen tank. To recover NPCs, vials were swirled in a 37 °C water bath and centrifuged at 300 x g for 4 minutes. Then, cells were resuspended in NEM and plated on a Geltrex-coated 6-well dish. After that, NEM was changed at least every other day.

Immunostaining

At passage 4, NPCs are characterized by immunostaining. NPCs are expected to be positive for neural stem cell markers Nestin (Stemcell Technologies #6009.1), Sox1 (R&D Systems #AF3369), and Sox 2 (R&D Systems #MAB2018), and negative for pluripotent marker Oct4 (BD Biosciences #56155). These markers were chosen based on prior studies utilizing these markers to validate NPC stage using the same iPSC differentiation method . NPCs were plated between 100,000 to 300,000 cells/cm^2 on culture chamber slides coated with Geltrex. Once

NPCs reached confluency and were ready for staining, the cells were washed once with Dulbecco's phosphate buffered saline (PBS) (GIBCO #14190-114) for 2 minutes and fixed in 4% paraformaldehyde for 10 minutes. Cells were then washed 3 times with PBS for 2 minutes and permeabilized in 0.1% TritonX-100 for 10 minutes. Cells were again washed 3 times with PBS for 2 minutes followed by 30 minutes incubation in 5% BSA-PBS for blocking. After blocking agent was removed, primary antibody prepared in 5% BSA-PBS (at a concentration of 1:500) was added and incubated at 4C overnight. Cells were washed 3 times with PBS for 2 minutes and incubated in secondary fluorescent antibody (Thermo Fisher Scientific #A-11005) prepared in 5% BSA-PBS (at a concentration of 1:1000) at room temperature in the dark for 1 hour. After that, cells were again washed 3 times with PBS for 2 minutes and slides were gently patted dry with a Kimwipe. Once dry, mounting media containing DAPI was added to cells for 5 minutes and removed. Cells were visualized on an Olympus BX51 upright fluorescent microscope.

Preparation of Inhibitors

2-deoxy-D-glucose (2DG) (Sigma-Aldrich #D8375) was dissolved in cell culture grade water for a final concentration of 50 mg/mL, filtered, and stored at 4 °C. Azaserine (Sigma-Aldrich #A4142) was also dissolved in cell culture grade water for final concentration of 100 mM, filtered, and stored at -20 °C.

Bioluminescent ATP Assay

ATP quantification and cell viability was initially assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega #G7570). In preparation for the assay, NPCs were passaged and counted using Countess 3 (Thermo Fisher Scientific). Black opaque-walled 96-well

plates were coated with Geltrex and 5,000 NPCs were plated per well in NEM. About 36 hours after plating, NPCs were challenged for 6 hours with either 2DG or azaserine at varying concentrations in NEM. During the inhibitor exposure, cells remained in the incubator at standard conditions. After 5.5 hours, the cells were removed and allowed to incubate at room temperature. ATP standards in NEM were prepared from ATP disodium salt hydrate (Sigma-Aldrich #A7699) and added to the plate in triplicates. At the end of the 6-hour exposure, an equal amount of CellTiter-Glo reagent was added to each well. Plates were placed on an orbital shaker for 2 minutes to mix contents and then incubated at room temperature for 10 minutes. White opaque stickers were attached to the bottom of the plates. Luminescence was recorded using Spectromax M5 (Molecular Devices). Standard curve establishing relationship between ATP concentration and luminescence was generated and used to determine ATP concentrations of control and experimental wells. Values were expressed as percent of control, normalizing to no drug condition for each respective cell line, to account for differences in cell growth due to 36-hour delay between plating and ATP assay.

Purine Quantification

Clear 6-well plates were coated with Geltrex. After passaging and counting cells, 250,000 NPCs were plated per well in NEM and maintained until cells were confluent. NPCs were challenged for 6 hours with specific concentrations of either 2DG or azaserine prepared in NEM. At the end of the 6-hour exposure, culture media was collected and stored in 0.2 M PCA for extracellular measurements. Remaining media was removed, NPCs were washed with PBS, and dissociated using Accutase. Cell pellets were collected and stored in 0.2 M PCA for intracellular measurements. Both media and cell pellet samples were stored at -80 °C.

Cell pellet samples were thawed and resuspended on ice, then sonicated for 1 minute at 4 °C. Both media and cell pellet samples were centrifuged at 17,000 x g for 15 minutes at 4 °C. Supernatants of media and cell lysates was removed to an Eppendorf tube. The remaining cell pellets were resuspended in 2% sodium dodecyl sulfate and placed in 37 °C oven overnight and used for Pierce BCA protein determination assay (Thermo Fischer Scientific #23225). Supernatant was neutralized with 4% 2.5M potassium carbonate, incubated for 1 hour on ice, and centrifuged at 17,000 x g for 15 minutes at 4 °C. Supernatant was transferred to 0.45 μM PVDF microcentrifuge filter tubes (Alltech), centrifuged at 8,000 x g for 3 minutes at 4 °C. Filtrate was transferred to high performance liquid chromatography (HPLC) tubes. Purines were quantified using HPLC equipped with photodiode array ultraviolet detection as previously described (Fu et al., 2015; Shirley et al., 2007). This method quantifies the predominant purines of biological significance such as adenine, guanine, and their derivatives.

All graphs representing HPLC purine data either show raw data normalized to protein content or energy charge. Both adenylate energy charge (AEC) and guanylate energy charge (GEC) were calculated using the formula ([NTP + 0.5*[NDP])/([NTP] + [NDP] + [NMP]) where N = adenosine or guanosine.

Statistical Analysis

All statistical analyses were completed using GraphPad PRISM software v10. Quantitative measurements on graphs are displayed as mean ± SEM. The statistical test used was ordinary two-way ANOVA, with factors being cell line group and drug treatment condition. Tukey's multiple comparison test was used for *post-hoc* analysis.

Results

NPC Validation

The first experimental procedures involved validating the protocol's efficacy in generating NPCs from iPSCs. Following differentiation, the cells underwent immunostaining for neural stem cell and pluripotency markers. All cell lines were negative for pluripotency markers (OCT4) and positive for neural stem cell markers (Nestin, SOX1, and SOX2) as expected (Figure 2). Therefore, NPCs were successfully produced.

Reduction of ATP Levels After Inhibitor Exposure in LND NPCs

The first objective was to establish an optimal dosage and timing of glycolysis and purine metabolism inhibitors that selectively influence LND NPC energetic metabolism without triggering cell death. Following the 6-hour drug exposure and prior to cell lysis for the bioluminescent ATP assay, cells were checked under the microscope and no overt changes in morphology or cell death were observed. This means that the any reduction of purines cannot be explained by the drug killing the cells.

As 2DG drug dosage increased, cellular ATP content showed a decreasing trend for both the control and LND cell lines (p-value < 0.0001) (Figure 3). The control cell line exhibited a significant reduction in ATP content at 100 mM 2DG (p-value < 0.01) compared to its respective no drug exposure group. Similarly, the LND line demonstrated significant reductions in ATP content at 50 mM (p-value < 0.05) and 100 mM (p-value < 0.01) compared to its respective no drug exposure group. Analysis of mean values indicated that the control line maintained at least 80% of its cellular ATP until exposure to 75 mM 2DG, whereas the LND line fell below this threshold starting at the 25 mM dose. At the 100 mM 2DG dose, both cell lines exhibited

markedly low ATP levels, around 20% compared to their respective control groups. Generally, the control line showed greater variability in ATP content at any given dose compared to the LND line. No significant difference in ATP concentration was observed between control and LND cell lines, therefore these data cannot firmly conclude a differential reduction in ATP for LND NPCs.

As azaserine drug dosage increased, cellular ATP content exhibited a continuous decrease for both the control and LND cell lines (p-value < 0.0001), which was to a smaller extent than observed with 2DG (Figure 4). The control cell line displayed significant decreases in ATP content at 2.5 μM azaserine (p-value < 0.01) and 5 μM azaserine (p-value < 0.01) relative to its respective no drug exposure group. Similarly, the LND line displayed significant decreases at 1 μM (p-value < 0.05), 2.5 μM (p-value < 0.05), and 5 μM azaserine (p-value < 0.0001) relative to its respective no drug exposure group. Analysis of mean values indicates that the LND line exhibited a more rapid decline in ATP concentration with increasing azaserine dose compared to its control group, with the LND line falling below 80% of control levels starting at the lowest dose of 0.1 uM. In contrast, the control cell line didn't drop below this threshold until the 1 uM dose. No significant difference in ATP concentration was observed between control and LND cell lines, thus this data does not conclusively support a differential reduction in ATP for LND NPCs.

Changes in Purines After 2DG Exposure in LND NPCs

The subsequent objective was to investigate the impact of inhibitor exposure on purines in NPCs at two drug dosages: one that may induce differential effects and another showing a more severe impact on both lines. Based on the data from the preceding ATP assay, 50 mM and 100 mM concentration of 2DG were selected. Overall, exposure to 2DG minimally affected

levels of adenine derivatives in both control and LND cell lines (Figure 5). Specifically, for the control line, ATP levels significantly decreased for both the 50 mM and 100 mM 2DG doses (p-value < 0.05). Similarly, the average ATP levels of LND cell lines showed a significant reduction for both concentrations of 2DG (p-value < 0.01). Since LND cell lines demonstrated a trend for decrease in ATP and an increase in ADP and AMP, a reduced AEC compared to the control cell line for both drug conditions resulted. However, these differences were not statistically significant.

Exposure to 2DG impacted the levels of guanine derivatives in both control and LND cell lines (Figure 6). Cellular GTP levels markedly decreased at 50 mM for the control line (p-value < 0.05) and LND lines (p-value < 0.01). Additionally, GTP levels were significantly reduced for LND lines at 100 mM (p-value < 0.001). Notably, the LND cell lines exhibited a differential decrease in GTP for both 50 mM (p-value < 0.05) and 100 mM (p-value < 0.01) 2DG concentrations. Although the guanylate energy charge (GEC) showed a significant decrease in LND cells at 100 mM 2DG (p-value < 0.05), these differential differences in GTP were not consistently observed in GEC.

Changes in Purines After Azaserine Exposure in LND NPCs

Based on the previous ATP assay, 0.5 μM and 5 μM azaserine concentrations were chosen given that one showed a potential differential impact with LND line being more negatively affected and the other in which both cell lines demonstrated a significant impact. Azaserine exposure did not have a significant impact on adenine derivatives for either control or LND cell lines (Figure 7). However, azaserine did have a slight impact on guanine derivatives. GTP levels significantly decreased in the LND lines at 0.5 μM (p-value < 0.01) and 5 μM (p-

value < 0.05) azaserine concentrations (Figure 8). Also, GDP slightly decreased for the LND lines at both 0.5 μM and 5 μM doses (p-value < 0.05). However, these significant changes were not reflected in the GEC.

Changes in Total Purine Content After Inhibitor Exposure in LND NPCs

Following various exposures to either 2DG or azaserine, total purine content was calculated by adding intracellular ATP, ADP, AMP, GTP, GDP, and GMP. Both control and LND cell lines demonstrated decreasing total purine content as 2DG dose increased (Figure 9). The control cell line had a significant reduction in total purine content at 50 mM and 100 mM (p-value < 0.01) 2DG doses. Similarly, the LND cells lines exhibited markedly reduced total purine content at 50 mM (p-value < 0.01) and 100 mM (p-value < 0.001) doses. Notably, the LND cell lines displayed a differential decrease in total purine content at the 100 mM (p-value < 0.05) 2DG concentration. Furthermore, all cell lines showed a decrease in total purine content as azaserine concentration increased (Figure 9). For the LND cell lines, there was a significant reduction in total purine content at 5 uM azaserine (p-value < 0.05).

Discussion

The primary objective of this study was to determine whether LND NPCs are more vulnerable to energy challenges compared to control NPCs. Initially, I hypothesized that LND dopaminergic NPCs would be more vulnerable to energy demands, thus exhibiting significantly diminished ATP levels following exposure to glycolytic or purine pathway inhibitors compared to control NPCs. I further hypothesized that these same trends would be reflected in levels of GTP. Overall, the results indicated that LND NPCs are impacted by acute energetic stress; however, it remains unclear whether the HGprt-deficient cells are sufficiently affected during this cell stage to contribute to the pathogenesis of LND. Further research is required to achieve a more conclusive understanding.

All cell lines successfully differentiated into NPCs, as confirmed by negative immunostaining for pluripotency markers and positive immunostaining for neural stem cell markers. This method of NPC validation is consistent with other studies utilizing the same method of differentiation (Dinasarapu et al., 2022; Yan et al., 2013).

Initial experiments using a bioluminescent ATP assay were conducted to examine the dose-response relationship between inhibitors and ATP concentrations of the NPCs. A significant inverse relationship was observed between the glycolytic inhibitor 2DG and ATP concentrations, with ATP concentrations decreasing by as much as 80% for the highest dosage of 100 mM 2DG for both cell lines. Similarly, a significant inverse relationship was observed between the purine metabolic inhibitor azaserine and ATP concentrations. NPCs displayed about a 40% and 60% reduction in ATP at the highest dosage of 5 μM azaserine for the control and LND cell lines, respectively. Therefore, the cells exhibited a greater susceptibility to the increased dosage of the glycolytic inhibitor compared to the purine metabolic inhibitor given the concentrations that

were used. These findings confirm that the drugs had the intended effect. However, no significant difference was observed between the control and LND cell lines at any dose of either inhibitor, therefore, a differential impact on ATP concentration due to HGprt deficiency was not seen.

HPLC measurements revealed that intracellular purine levels were affected by increasing concentrations of inhibitors for both control and LND NPCs. Transient exposure to glycolytic inhibitor 2DG slightly impacted adenine derivatives, with significant differences only apparent in ATP. For both control and LND cell lines, ATP markedly decreased as 2DG concentration increased; however, no differential reduction was detected. AEC values were consistently lower in LND cell lines at both 2DG doses compared to the control lines, however not to a significant extent. In contrast, significant variations were detected in guanine derivatives at baseline and in response to 2DG doses. GTP significantly decreased compared to no drug conditions in the LND NPCs. Notably, a differential reduction in GTP was detected at both 2DG conditions, with the most substantial difference occurring at 100 mM 2DG. Therefore, these results indicate that GTP levels may be differentially affected in LND cells, particularly under acute energetic stress. However, this differential affect was not paralleled in the GEC. Furthermore, transient exposure to purine metabolic inhibitor azaserine displayed no significant impact on levels of adenine derivatives. Increasing azaserine dosage exhibited significant reductions in GTP and GDP in LND cells, but not to the extent of differential importance.

In summary, the results indicate a potential impairment of purine derivatives in diseased NPCs, particularly ATP and GTP, in response to glycolytic and purine metabolic inhibitors. These results are supported by existing research reporting that HGprt deficiency impacts energy impairments later in neural development, such as during the NPC stage (Bell et al., 2021). However, in contrast to the referenced publication, the present study did not reveal significant

differences between any purines except GTP at baseline. Another study involving protein expression measurements in NPCs *in vitro* found that NPCs exhibited abnormalities in energy related proteins such as mitochondrial F1F0 ATPase (Dinasarapu et al., 2022). Therefore, the results of the present study are consistent with existing literature that LND NPCs may be more vulnerable to energy impairment, thus demonstrating a potential cause for the early stages of the disease's pathogenesis.

LND cells contain nonfunctional HGprt and, therefore, are unable to recycle the purines guanine and hypoxanthine. A previous hypothesis suggested that this dysfunctional enzyme may reduce total purine levels in the cells, thereby contributing to the pathogenesis of LND. However, prior studies discredited this idea as they consistently demonstrated that LND cells compensate for HGprt deficiency by upregulating the *de novo* pathway to maintain normal purine levels at baseline (Jinnah et al., 1993; Shirley et al., 2007). In contrast, the present study demonstrated that total purine content markedly reduced in LND NPCs compared to controls at the highest 2DG dose. Therefore, the findings are novel in the sense that they identified a discrepancy in purine content due to HGprt deficiency in response to acute glycolytic stress. This result indicates that the NPC stage, the transient energetic stress, or its combination exacerbated the difference in total purine content, thereby reducing the availability of these essential molecules for vital biological processes such as energy metabolism and DNA replication. Overall, this novel finding supports the hypothesis that transient glycolytic challenges may alter cellular purine levels in maturing neural cells, contributing to the pathogenesis of LND. This suggests that a cell culture model of LND involving a neurodevelopmental stage in conjunction with metabolic inhibitors might provide a more meaningful model for exploring the nuances of LND that could influence disease outcomes in humans.

The findings of this study may have wider implications on dopamine-related disorders. Dopaminergic neurons, due to their high energy demands, are particularly vulnerable to metabolic impairments (Bolam & Pissadaki, 2012; Guatteo et al., 2005; Pissadaki & Bolam, 2013). Thus, by demonstrating purine and energy dysfunction in NPCs, the present study initiates the potential link between energy metabolism impairment and abnormal dopamine phenotype in LND. Recent hypotheses suggest that this energetic vulnerability could be a foundational aspect of the pathophysiology in a range of neurological disorders such as LND and Parkinson's Disease (Mergenthaler et al., 2013). Therefore, transient stressors that disrupt the normal functioning of developing neurons and dopamine function, may contribute to the basis of these conditions. The present study underscores the importance of investigating acute energy impairment in dopamine-related diseases to determine their potential role in disease pathogenesis.

Limitations

A limitation of this study includes the inherent experimental variability present in using patient-derived lines. In this study, one control and three LND cell lines were used, which previously underwent gene expression profiling and demonstrated significant heterogeneity (Sutcliffe et al., 2021). The principal distinction between the control and LND is the normal or mutant form of the *HPRT1* gene, respectively. Consequently, other genetic variations within these individuals' genomes might modify, diminish, or intensify purine alterations. Similarly, the use of one control cell line may not be ideal due to possible variations at other genetic loci that could affect quantitative purine measurements. A potential approach to mitigate this issue could involve genetically engineering established iPSC cells lines to contain the same *HPRT1*

pathogenic variant as the patients. This strategy would produce genetically identical control and LND cell lines, excluding the alteration in the *HPRT1* gene, offering a more biologically controlled comparison.

Other limitations of this study stem from its technical aspects, including cell culture environment, the determination of optimal inhibitor exposure, and the developmental stage of the cells. The *in vitro* maintenance of cells does not replicate the complex *in vivo* environment, which could influence outcomes. Additionally, initial experiments of the present study aimed to identify an optimal dosage and timing to alter cellular metabolism without causing cell death; however, extra experiments and validation may be required to establish the conditions that could elicit the hypothesized effects more effectively. Finally, this study focused on the NPC stage to examine the impact of acute energetic stress on metabolic processes. Previous research has indicated that different stages of neuronal development *in vitro* may have varying metabolic requirements (Bell et al., 2021; Dinasarapu et al., 2022; Engle et al., 2018). As a result, cells at later stages of neuronal development than NPCs, such as neurons, could experience more significant effects.

Future Directions

Future studies should further explore the impact of transient energetic stress on developing LND neural cells. The present study leveraged 2DG and azaserine as glycolysis and purine metabolic pathway inhibitors, respectively. Dosages of inhibitors were optimized for a brief 6-hour exposure due to time constraints for this project, although a longer exposure may be necessary to assess the full effects of these drugs. Further investigation should attempt a broader range of dosages and exposure durations, such as 12 or 24 hours, to identify conditions that

render developing neural cells most susceptible to energetic vulnerabilities. Additionally, experiments could investigate other strategies to transiently disrupt energy metabolism. Potential approaches include using inhibitors of the electron transport chain, such as rotenone and antimycin A, reducing glucose levels in the culture media, or triggering excessive action potentials in more mature neurons. Thus, further optimizing cellular conditions involved in the acute metabolic stressors may help elucidate differential energetic impairment in developing LND neural cells.

Another important avenue for study includes investigating how cells react to acute stressors at various neurodevelopmental stages. Prior research has reported that metabolic demands differ across various phases of neuronal development when observed *in vitro* (Bell et al., 2021; Dinasarapu et al., 2022; Engle et al., 2018). Therefore, these different cell stages, such as iPSC, NPC, or immature neurons, may be differentially vulnerable to the same transient metabolic stressors. Similar studies involving neurons at more advanced developmental stages could reveal whether they experience more pronounced impairment. Furthermore, the use of dopaminergic neurons enables other important measurements such as changes in dopamine and its metabolites due to acute stressors, offering a more direct method to investigate a possible cause of dopamine dysfunction in LND cells.

Additional research directions could involve altering the *in vitro* environment to mimic the biological experience *in vivo* more accurately. Recent findings indicated that cultured patient-derived fibroblasts do not exhibit changes in purines when maintained in regular culture media with artificially high levels of folic acid; however, the use of more physiological accurate media in regards to folate levels identified a depletion of ATP in LND these cells (Escudero-Ferruz et al., 2024; López et al., 2020). Therefore, the use of media containing biologically correct levels

of folic acid in conjunction with the transient energetic stressors on developing neural cells could provide more valid model to identify differential changes in purine metabolism in LND cells *in vitro*.

Conclusions

To my knowledge, this is the first study to examine the differential effects of energy metabolism in LND NPCs due to acute glycolytic and purine metabolic stress *in vitro.* The results indicated that total purine content and GTP levels were differentially reduced in LND NPCs at the highest concentration of 2DG, revealing that transient glycolytic stress can induce impairments in purine metabolism. The results also indicated that energy metabolism in both control and LND NPCs were affected by increasing concentrations of these inhibitors; however, differential outcomes regarding energy impairment in NPCs were minimal. Overall, the findings from this study indicate that developing LND neural cells may be more susceptible to acute stressors. Further research is required to optimize conditions that can elucidate whether energetic impairment is a contributing factor to the pathogenesis of LND.

Tables and Figures

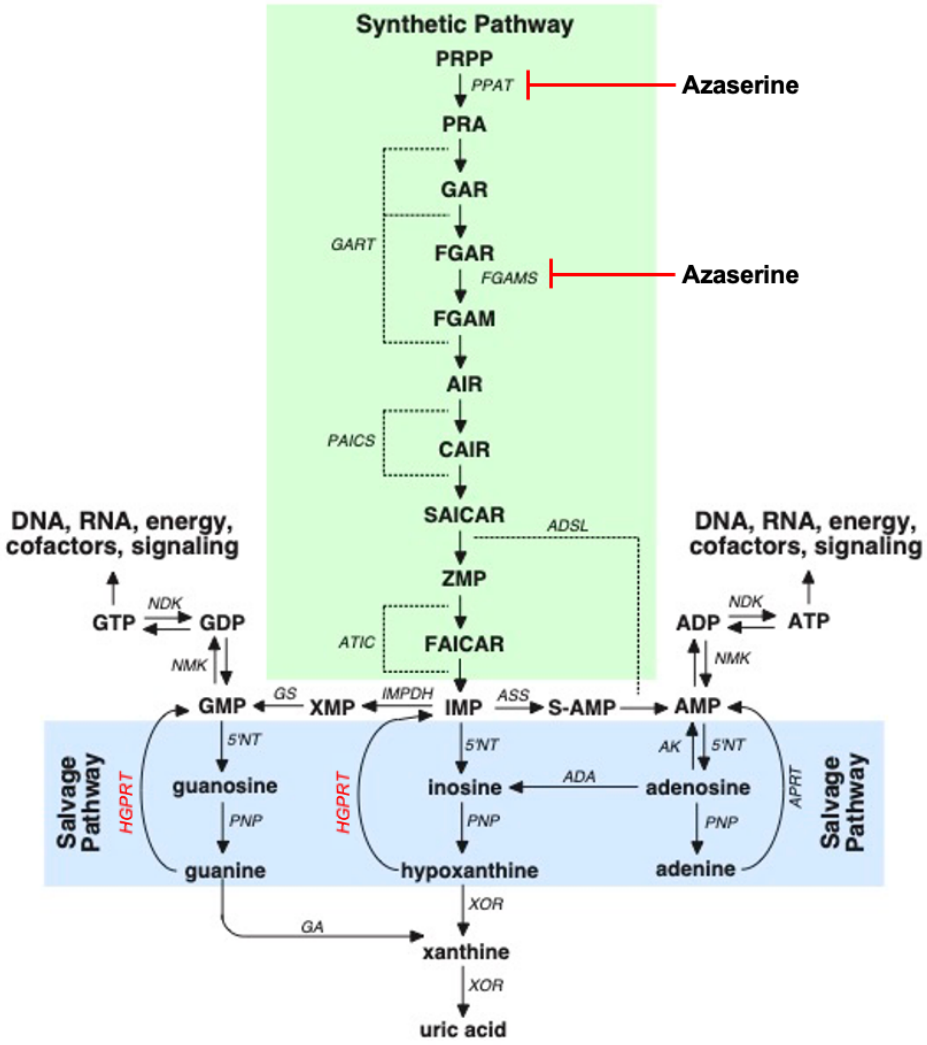

Figure 1: Purine Metabolic Pathway. LND is caused by pathogenic variants in the *HPRT1* gene, which produces nonfunctional HGprt and effectively prevents guanine and hypoxanthine from being recycled into GMP and IMP, respectively. This figure was modified and included with permission from Dr. Jinnah.

	Healthy Control Case	LND Cases		
	CON	LND 1	LND 2	LND 3
Sex	Male	Male	Male	Male
Age	21	10	46	15
HPRT1 Gene Variant	None	c.508C>T	c.371insTT	c.151C>T
HGprt consequence	Normal	Premature Stop	Frame shift, Premature Stop	Premature Stop

Table 1: Subject Characteristics. The iPSCs used were derived from skin biopsies from the described individuals. Age corresponds to the subject's age at the time of the skin biopsy.

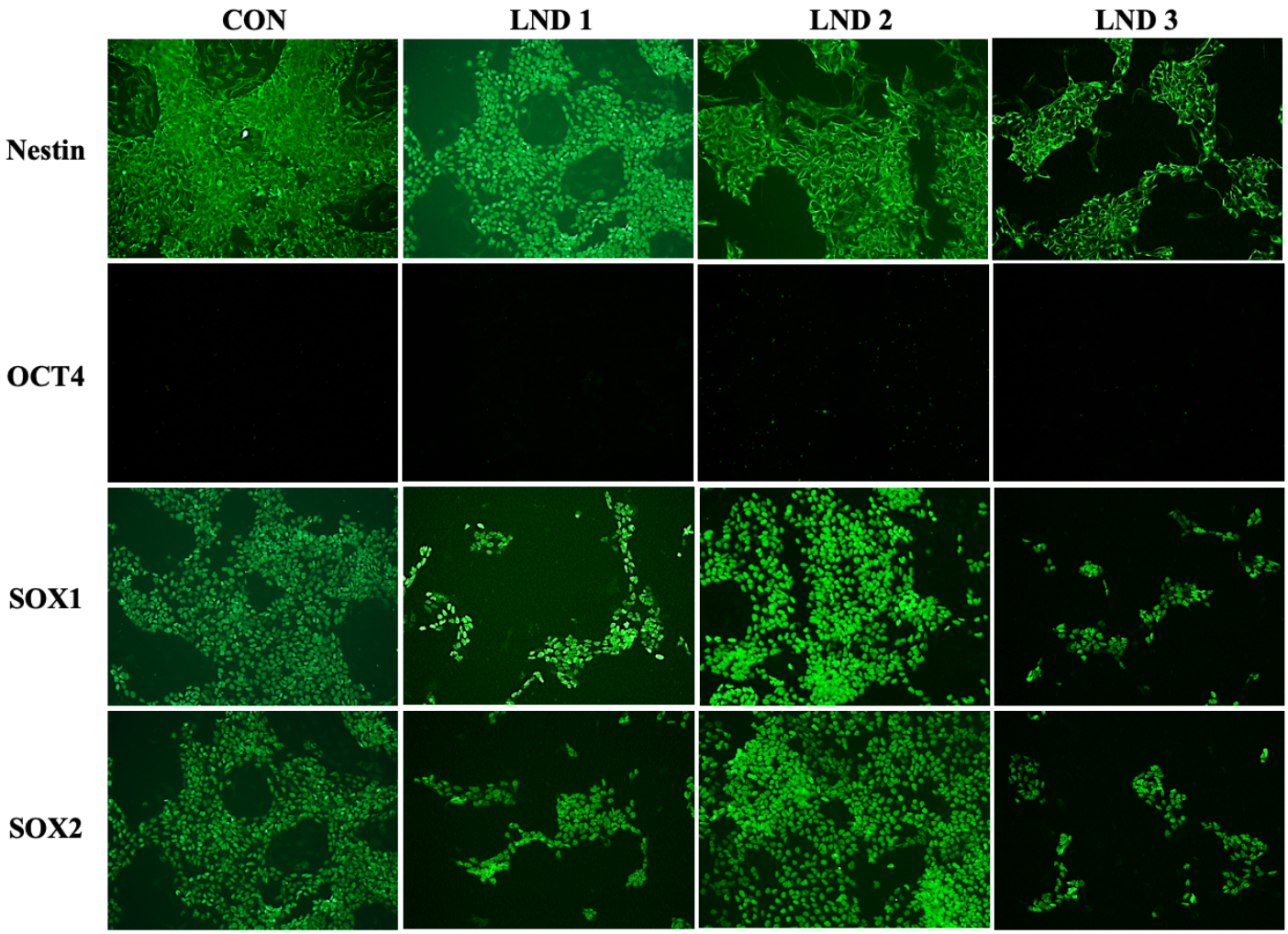

Figure 2: NPC Staining. After differentiation, NPCs were stained with Nestin, OCT4, SOX1, and SOX2 for validation. Images were taken at a magnification of 20X.

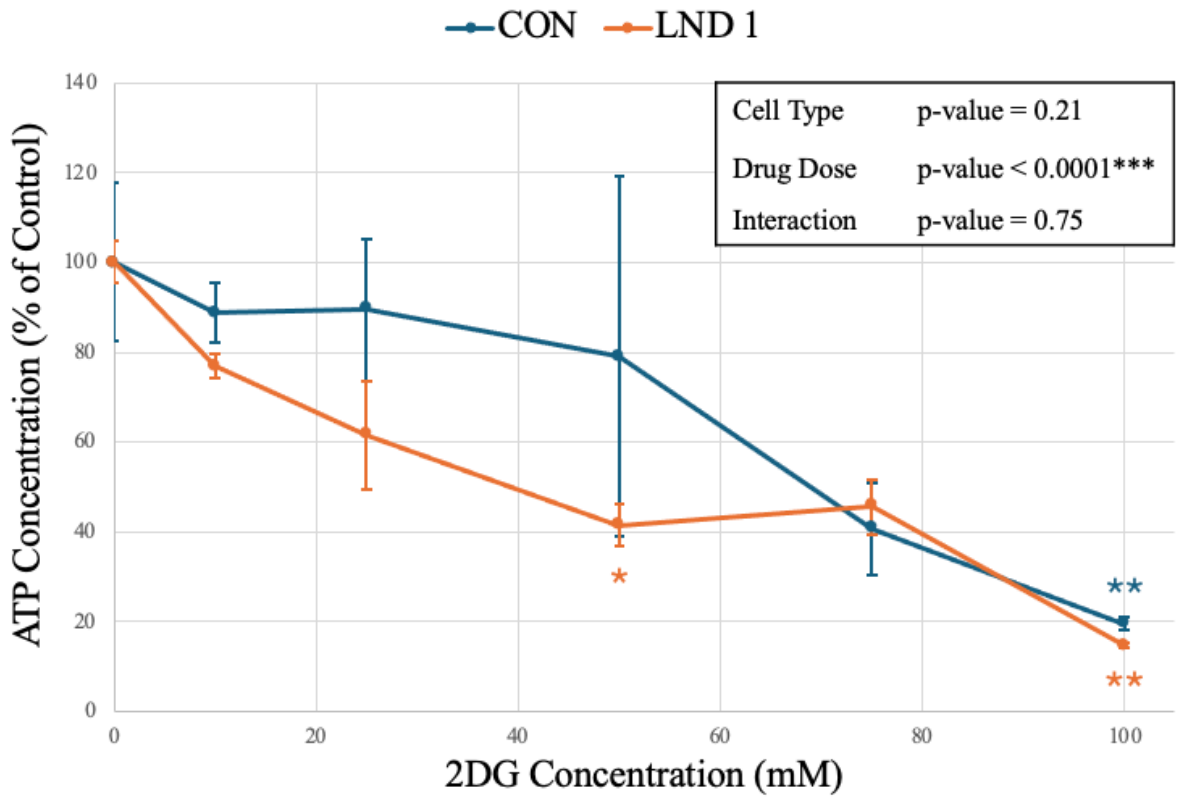

Figure 3: Cellular ATP Content After 2DG Exposure. Data on the line graphs is expressed as percent of control and shows mean values ± SEM. Statistical significance on the line graph refers to significant changes compared to no drug condition based on two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$). n = 1 cell line used for both control and LND groups, with at least three replicates per drug condition.

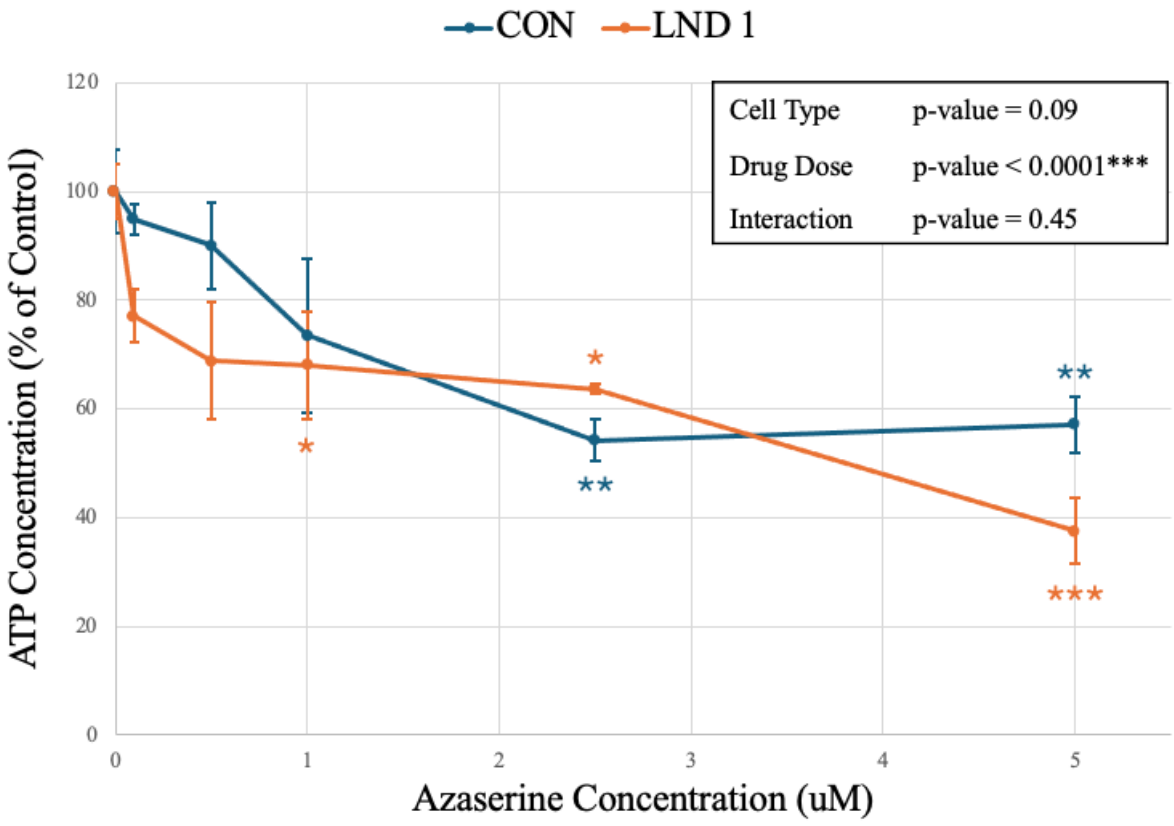

Figure 4: Cellular ATP Content After Azaserine Exposure. Data on the line graphs is expressed as percent of control and shows mean values ± SEM. Statistical significance refers to significant changes compared to no drug condition based on two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.0001). n = 1 cell line used for both control and LND groups, with at least three replicates per drug condition.

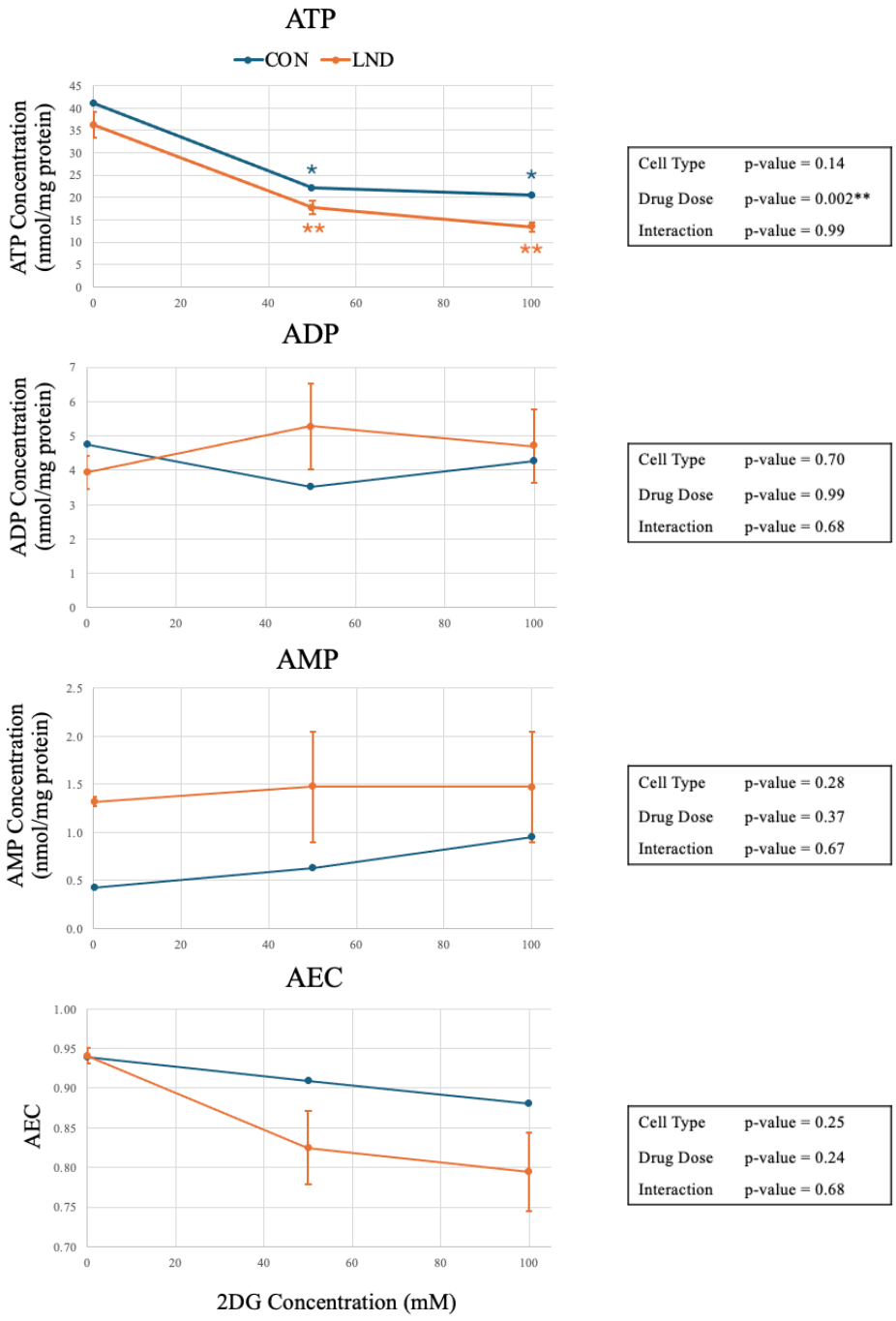

Figure 5: Concentration of Adenine Derivatives After 2DG Exposure. Data on the line graphs shows mean values ± SEM. Statistical significance refers to significant changes compared to no drug condition based on two-way ANOVA (*p < 0.05, **p < 0.01). For control line n = 1 and for LND lines n = 3.

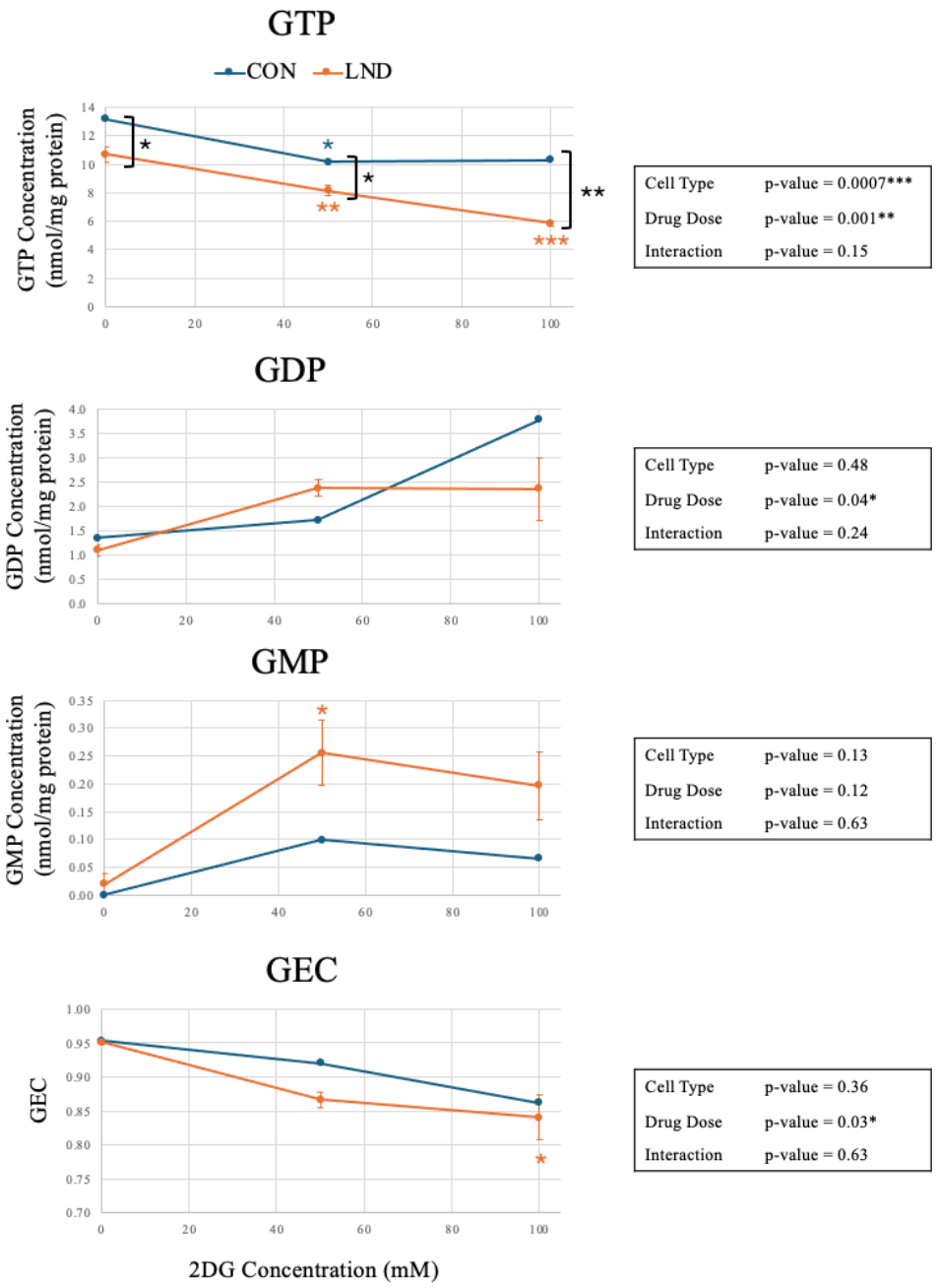

Figure 6: Concentration of Guanine Derivatives After 2DG Exposure. Data on the line graphs shows mean values ± SEM. Statistical significance refers to significant changes compared to no drug condition based on two-way ANOVA (*p < 0.05, **p < 0.01, ***p <0.001). For control line n = 1 and for LND lines n = 3.

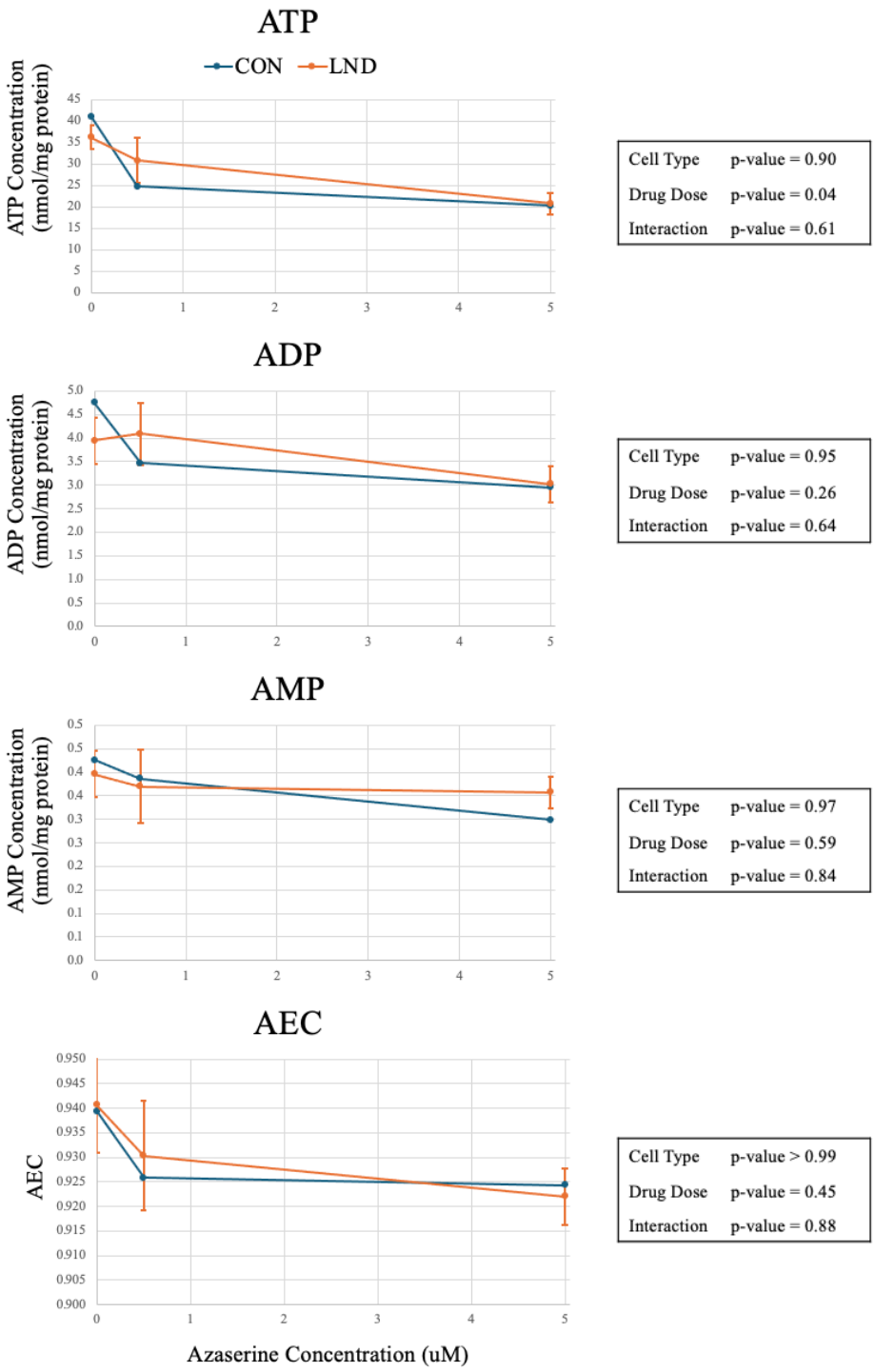

Figure 7: Concentration of Adenine Derivatives After Azaserine Exposure. Data on the line graphs shows mean values ± SEM. Statistical significance refers to significant changes compared to no drug condition based on two-way ANOVA. For control line n = 1 and for LND lines n = 3.

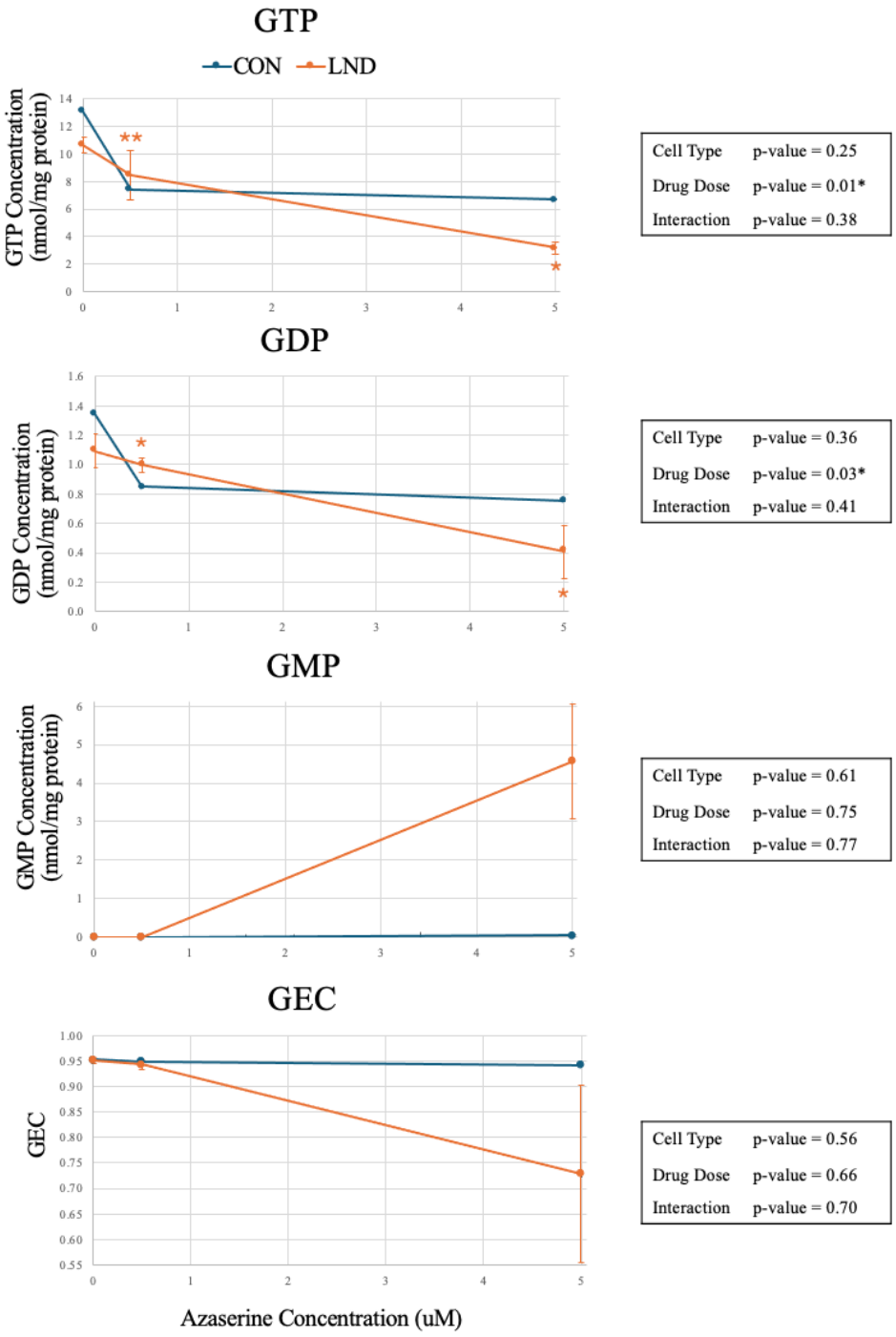

Figure 8: Concentration of Guanine Derivatives After Azaserine Exposure. Data on the line graphs shows mean values ± SEM. Statistical significance refers to significant changes compared to no drug condition based on two-way ANOVA (*p < 0.05, **p < 0.01). For control line n = 1 and for LND lines n = 3.

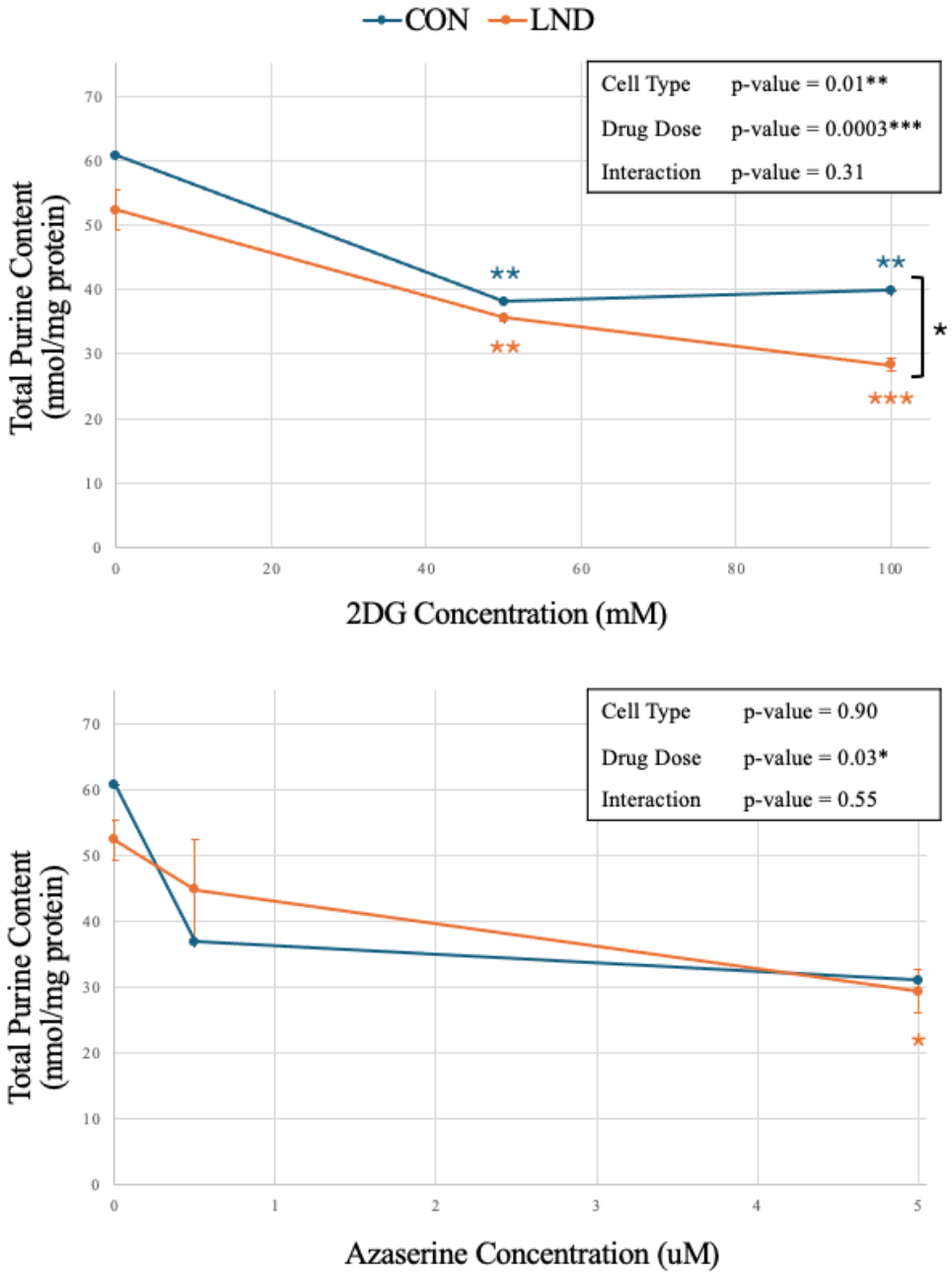

Figure 9: Total Purine Content After Inhibitor Exposure. Total purines content refers to the sum of intracellular ATP, ADP, AMP, GTP, GDP, and GMP. Data on the line graphs is shows mean values ± SEM. Statistical significance refers to significant changes compared to no drug condition based on two-way ANOVA (*p < 0.05, **p < 0.01, ***p<0.001). For control line n = 1 and for LND lines n = 3.

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