

Efficacy and Impact of the Melanocortin Receptor Agonists PL8177 and PL9654 in an STZ-Rat Model of Diabetic Retinopathy

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Purpose

- Melanocortins are a family of hormone agonists that include several melanocyte-stimulating hormones (α , β , and γ -MSH) and adrenocorticotropin hormone^{1,3}
 - These hormones bind to melanocortin receptors (MCRs) to exert their effect
- Melanocortins have a wide range of anti-inflammatory properties^{1,4,5}
 - Inhibition of leukocyte activation by suppressing proinflammatory cytokine production^{4,6}
 - Protection of tissues from the inflammatory response^{5,7,8}
- Melanocortin agonists may represent a new therapeutic avenue to treat inflammatory ocular diseases⁹⁻¹¹

Objective

- The selective MC1R agonist, PL8177 and the MC1R/MC3R/MC4R/MC5R pan-agonist PL9654, were investigated in a streptozotocin (STZ) rat model of diabetic retinopathy (DR) and their effects on ocular inflammation, retinal cell population composition, and gene and protein expression were determined
- The objective was to determine the effectiveness of PL8177 and PL9654 in this model and to characterize its mechanism of action

Methods

- Brown Norway rats were administered STZ via intraperitoneal injection to induce diabetes, and were then randomly assigned to 5 separate study arms and dosed subcutaneously on days 4–113 (**Table 1**)

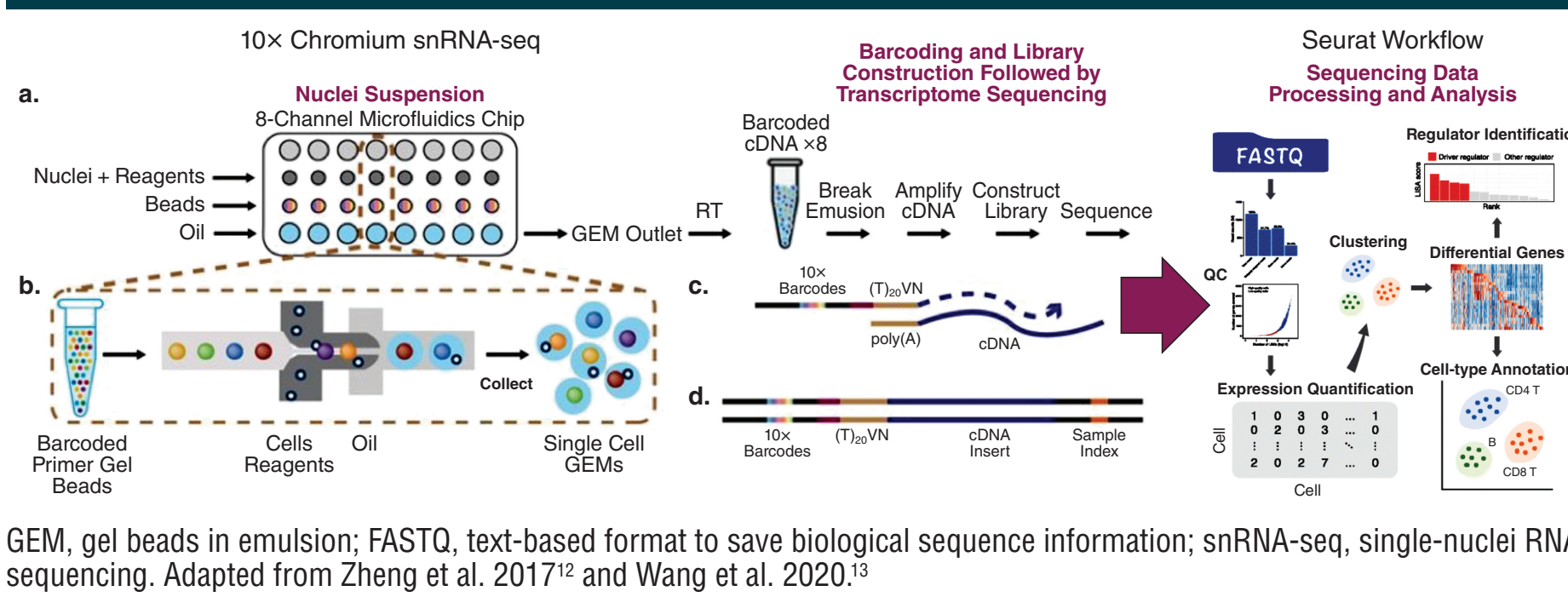
Table 1. Study Arms for PL8177/PL9654 STZ-Treated Rat Model of Diabetic Retinopathy

Treatment	Arm	Dose (SC BID)
Vehicle control (placebo)	1	(0.9% NaCl)
	2	0.05 mg/kg
PL9654	3	0.1 mg/kg
	4	0.5 mg/kg
PL8177	5	1 mg/kg

BID, twice daily; SC, subcutaneous; STZ, streptozotocin.

- Visual function was measured by optokinetic tracking every 2 weeks from days 43–113
 - Cataract images were acquired after each optokinetic session
- Rats were euthanized on day 114
 - Right-eye retinas were dissected and snap-frozen
 - Left eyes were enucleated and fixed for histology. Retinal thickness and photoreceptor degeneration/loss were measured
- Retinas were analyzed by 10× Genomics Chromium Technology single nuclei RNA sequencing (snRNA-seq) (10× Genomics, San Francisco, CA) (**Figure 1**) and by data-independent acquisition tandem mass spectrometry (LC/MS DIA) (**Figure 2**)

Figure 1. snRNA-Seq Workflow using 10× Chromium Technology

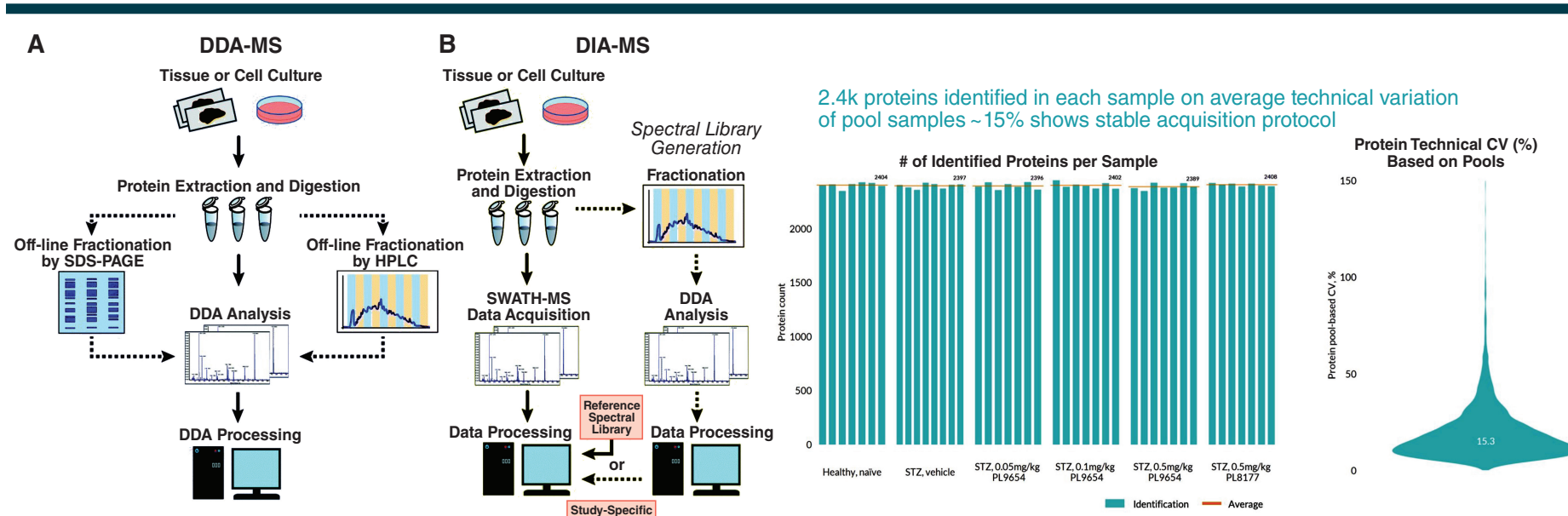


GEM, gel beads in emulsion; FASTQ, text-based format to save biological sequence information; snRNA-seq, single-nuclei RNA sequencing. Adapted from Zheng et al. 2017¹² and Wang et al. 2020.¹³

- snRNA-seq is a powerful technique to interrogate cellular heterogeneity. It uses isolated nuclei instead of whole cells to profile gene expression in cells that are difficult to isolate, as well as archived tissue
- 10× Genomics snRNA-seq technology uses microfluidic partitioning to profile up to tens of thousands of nuclei (typically 1000–6000), by capturing nuclei into nanoliter-scale gel beads in emulsions, where all generated cDNA shares a common 10× barcode used to associate individual reads back to the individual cells

- snRNA-seq data was generated and analyzed for 40 rat retina samples (6 vehicle, 6 healthy control and 7 samples from each treatment group)

Figure 2. LC/MS DIA Proteomic Analysis of Retinas



CV, coefficient of variation; DDA-MS, data-dependent acquisition mass spectrometry; HPLC, high-performance liquid chromatography; LC/MS DIA, data-independent acquisition tandem mass spectrometry; STZ, streptozotocin.

- Proteome profiling using LC/MS was performed on 43 rat retina samples. Specific rat retina spectral libraries were created consisting of 2,683 proteins and 31,682 peptides

Results

- PL9654 doses of 0.05–0.5 mg/kg twice daily (BID) and PL8177 at 1 mg/kg BID showed significant efficacy in reducing vision loss in STZ-treated rats compared to vehicle

Change in Visual Contrast Threshold

- All treatment arms also showed a progressive decline in contrast threshold values. However, the rate was much slower in the PL9654 and PL8177 treatment groups compared to the vehicle group, and at day 113 all PL9654 treatments had significantly reduced loss-of-contrast threshold (**Figure 3**)

Change in Visual Acuity

- All treatment arms showed a progressive decline in spatial frequency threshold (visual acuity) and contrast threshold over the course of the study (**Figure 4**)

Figure 3. Changes in Visual Contrast Threshold Over Days 43–113

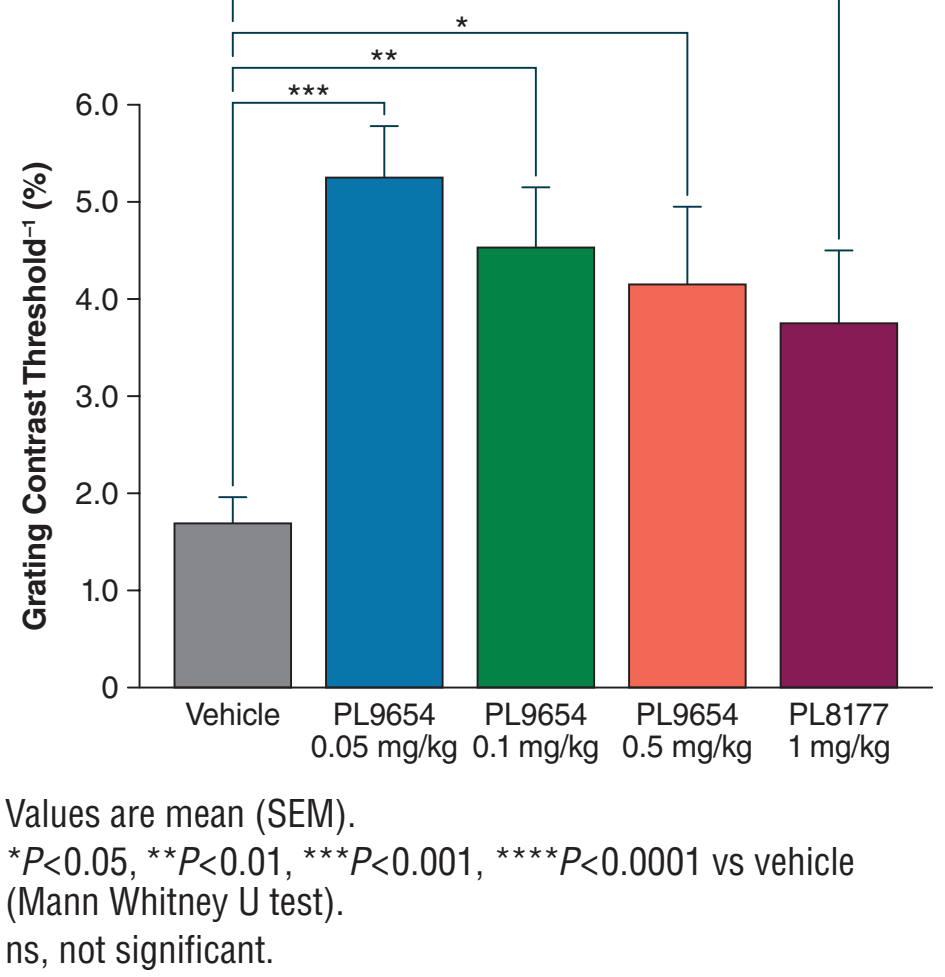
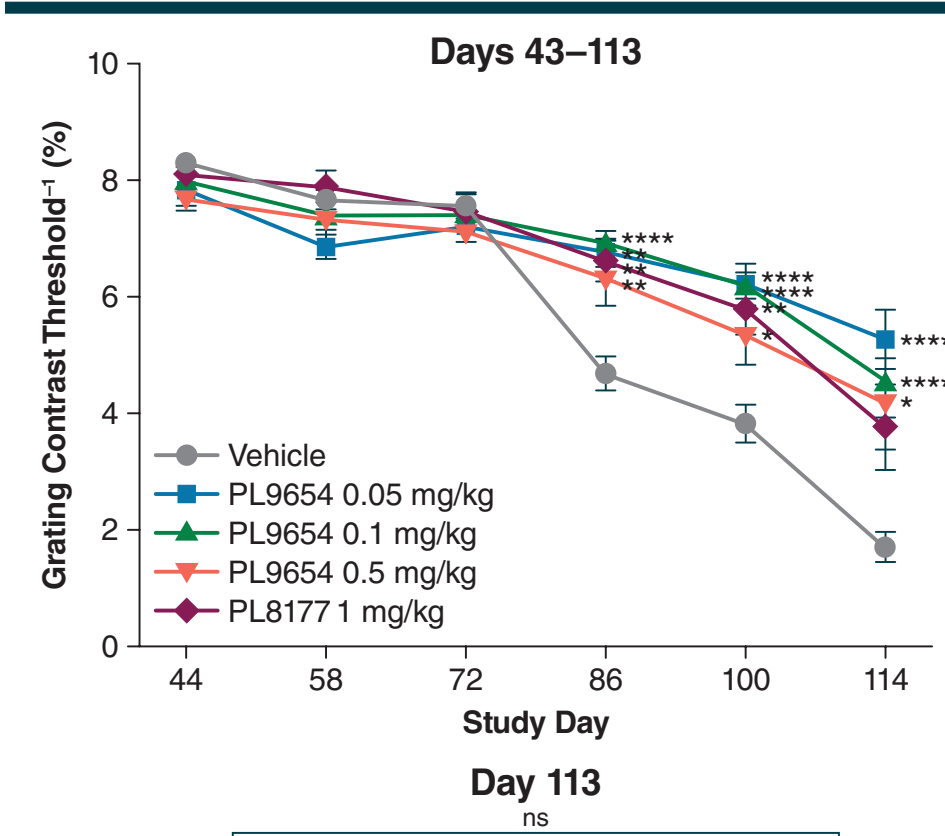
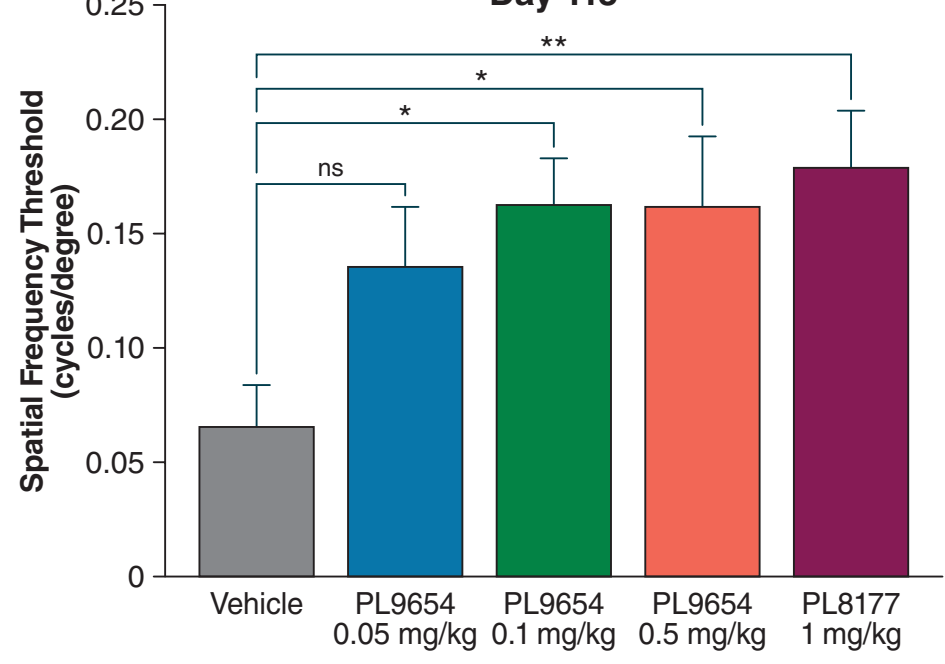
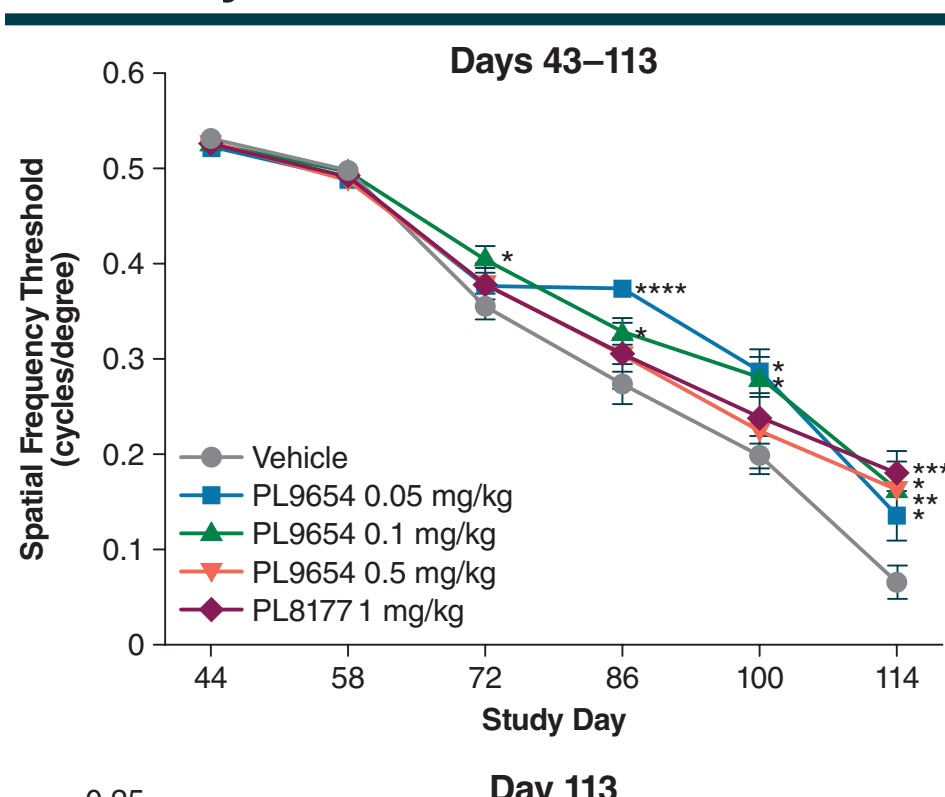


Figure 4. Changes in Visual Acuity Over Days 43–113



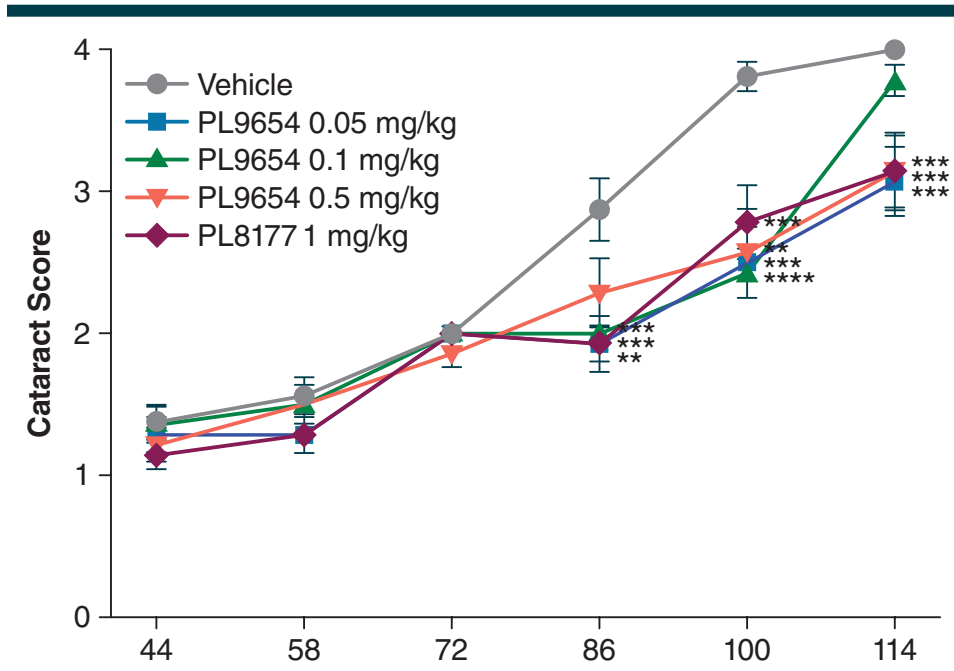
Values are mean (SEM). ** $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$, ***** $P < 0.0001$ vs vehicle (Mann-Whitney U test). ns, not significant.

- PL9654 0.1 and 0.5 mg/kg, and PL8177 1 mg/kg had significantly reduced loss of visual acuity vs vehicle on day 113

Changes in Cataract Severity

- Lens cataracts were scored at 2-week intervals beginning at day 44. The rate of increase in cataract severity (scored from 1 [mild] to 4 [total opacity]) was greatest in the vehicle administered rats with a lower rate observed in the PL9654 and PL8177 treatment groups (**Figure 5**)

Figure 5. Changes in Cataract Severity Scores Over Days 43–113



Values are mean (SEM). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs vehicle (Mann-Whitney U test).

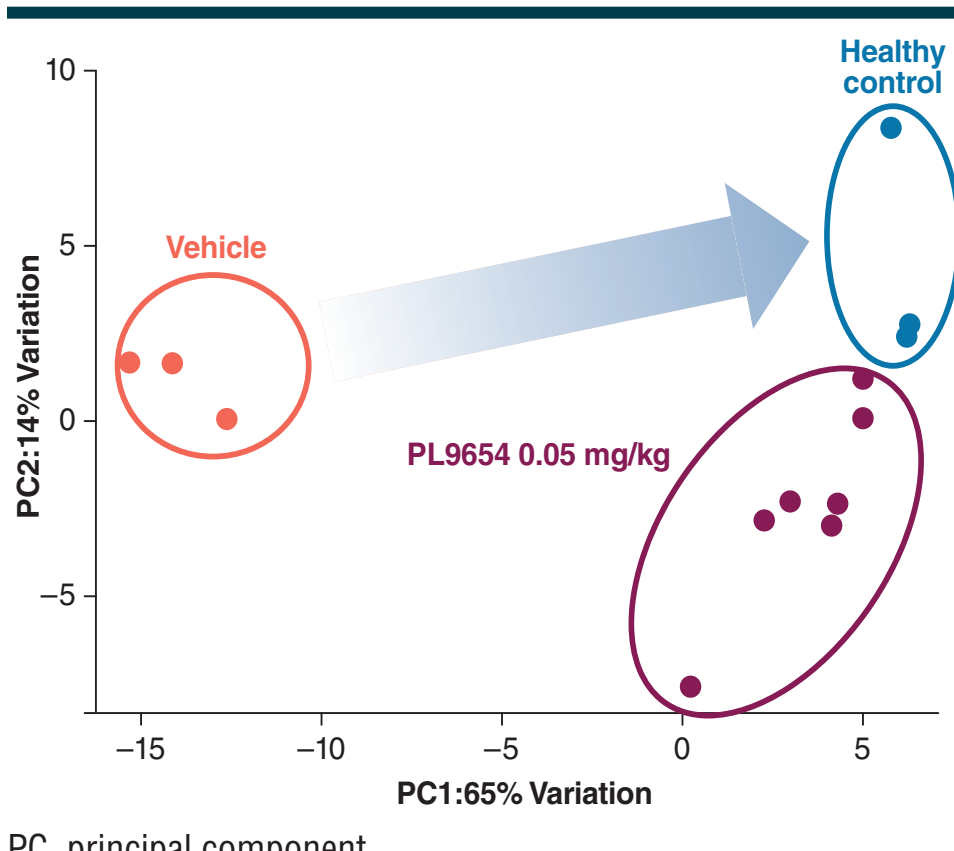
Retinal Histopathology

- Histopathology showed that there was significantly less photoreceptor degeneration with PL9654 0.1 mg/kg vs vehicle ($P < 0.05$)
- Retinal thickness was also significantly improved for the PL9654 ($P < 0.05$) and PL8177 ($P < 0.05$) groups vs vehicle

RNA-seq

- Unsupervised clustering of pseudo bulk RNA-seq data showed separation between treatment groups and close resemblance of PL9654-treated samples to healthy control (**Figure 6**)

Figure 6. Unsupervised Clustering of Bulk Gene Expression Data Shows Separation Between Treatment Groups

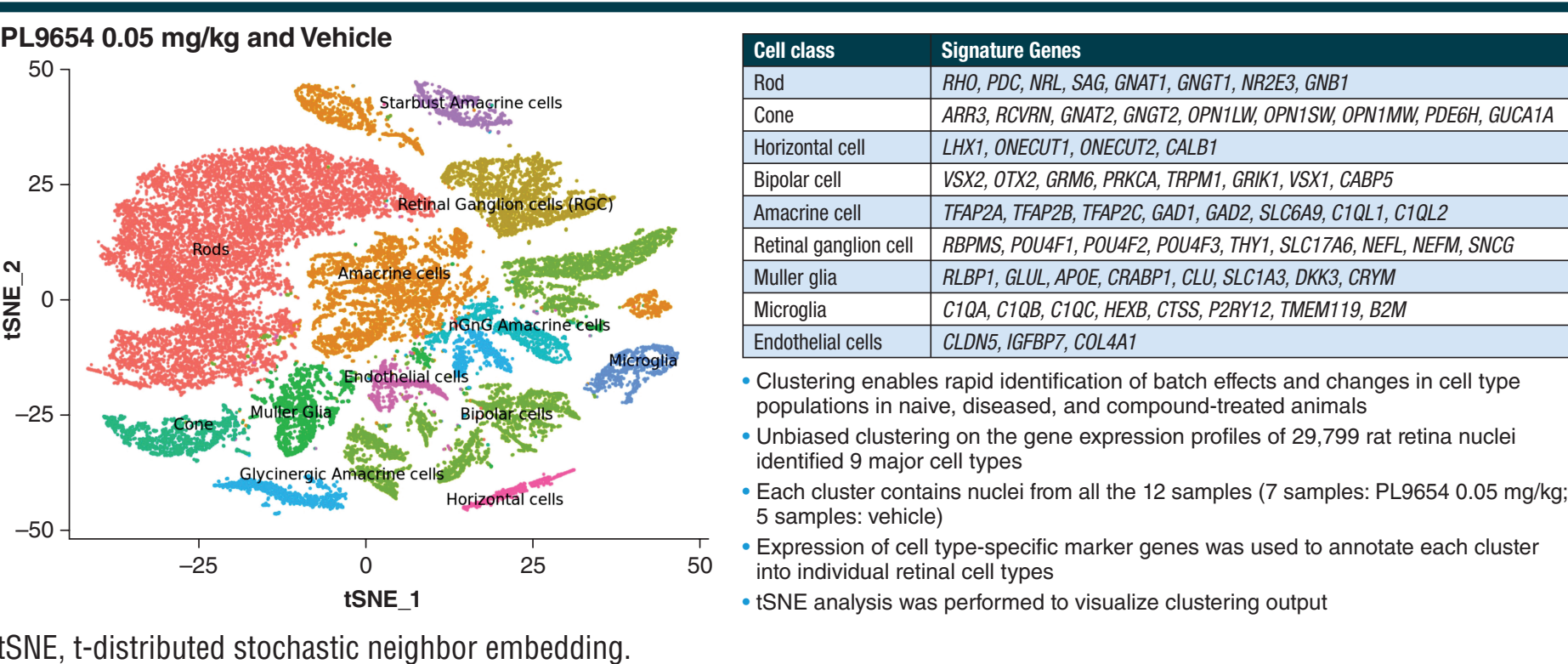


- Principal component analysis results indicate importance of single nuclei study to reveal cellular and pathway changes due to compound treatment

snRNA-seq

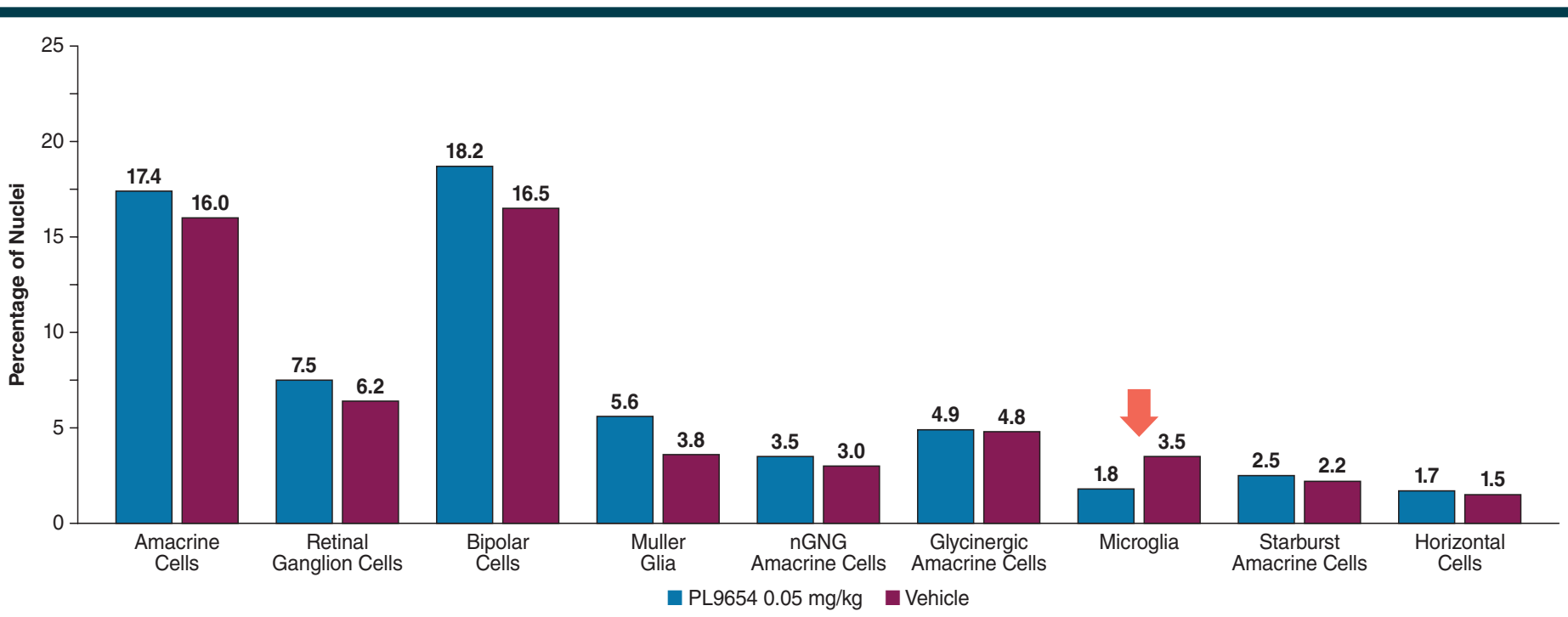
- Unbiased clustering on the gene expression profiles of 29,799 rat retina nuclei identified 9 major retinal cell types
- Expression of cell-type-specific marker genes was used to annotate each cluster into individual retinal cell types
- t-Distributed stochastic neighbor embedding was performed to visualize clustering of single nuclei from different retinal samples (**Figure 7**)

Figure 7. tSNE Analysis of Nuclei in Rat Retinas



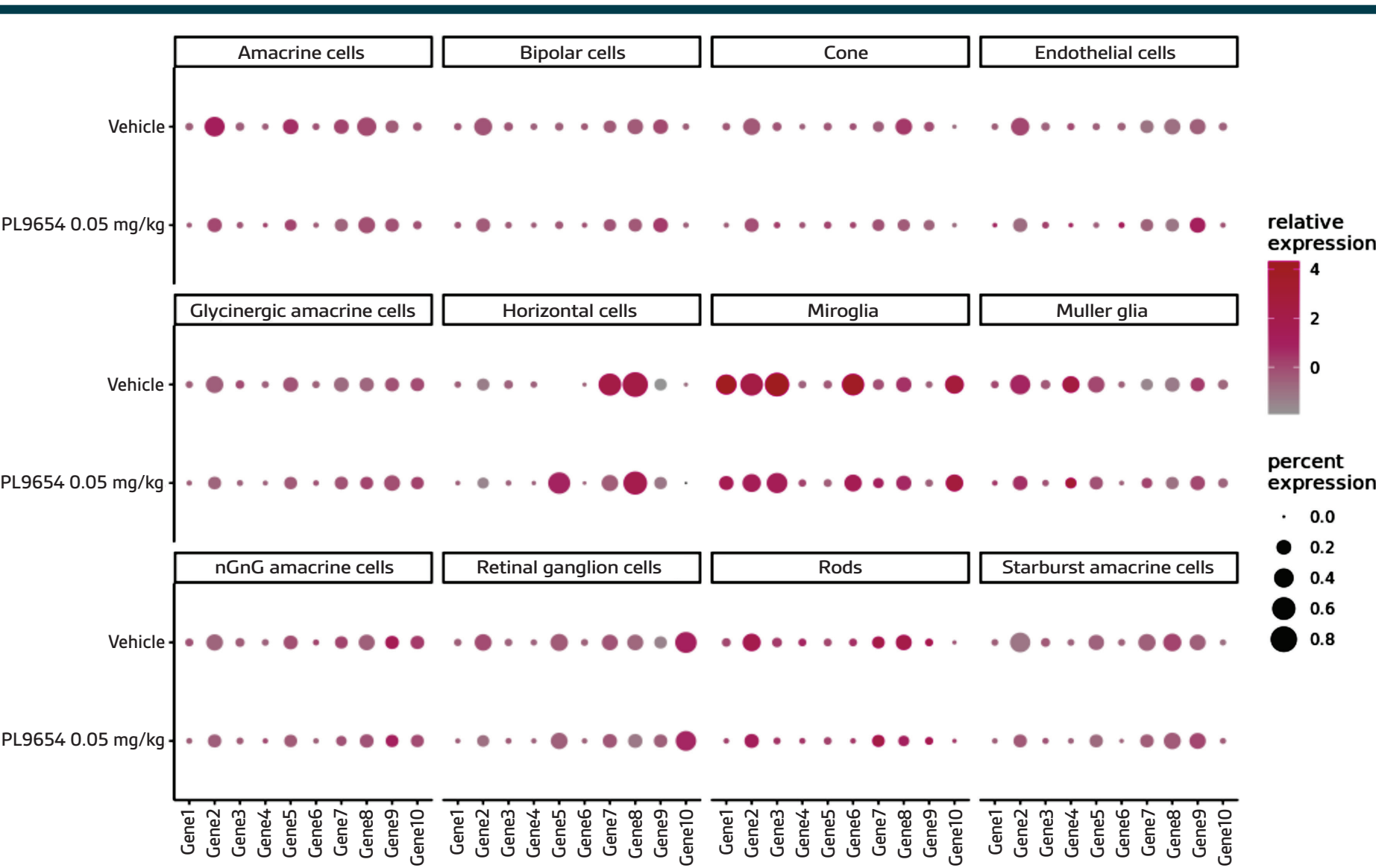
- PL9654 0.05 mg/kg treatment results in depletion of microglia cells and significantly reduced expression of multiple immune marker genes as compared to vehicle treatment (**Figures 8 and 9**)

Figure 8. Retinal Cell Composition and Differential Gene Expression Analysis



- Studies have shown that retinal microglia activation correlates with neuronal damage induced by different retinopathies including DR¹⁴
- Microglial cells show a 2-fold drop in relative representation in the PL9654-treated retinal cell population (**Figure 8**). Similar results were obtained for PL8177 and the other doses of PL9654
- Differential gene expression analysis of individual cell types showed significant down regulation of multiple immune marker genes in samples treated with PL9654 0.05 mg/kg (**Figure 9**)

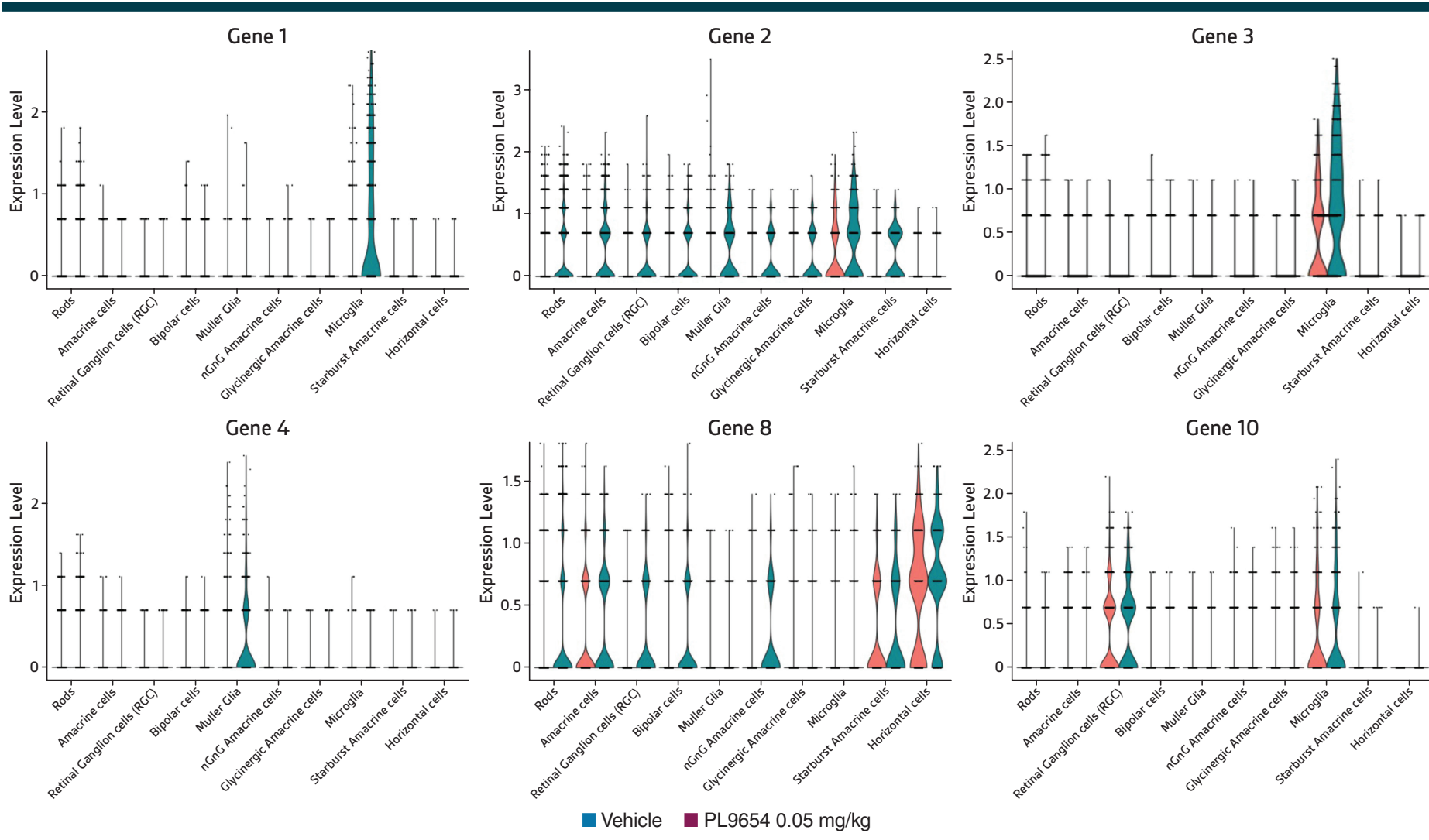
Figure 9. Reduced Expression of Immune Marker Genes in Treated Samples



Dot plot of expression of top 10 significantly differentially expressed immune marker genes in multiple cell types. Y-axis shows treatment groups and x-axis has gene names. Dot size represents percentage of nuclei with non-zero gene expression and dot color represents mean of non-zero expression. Gray dot color indicates low expression and dark pink high expression.

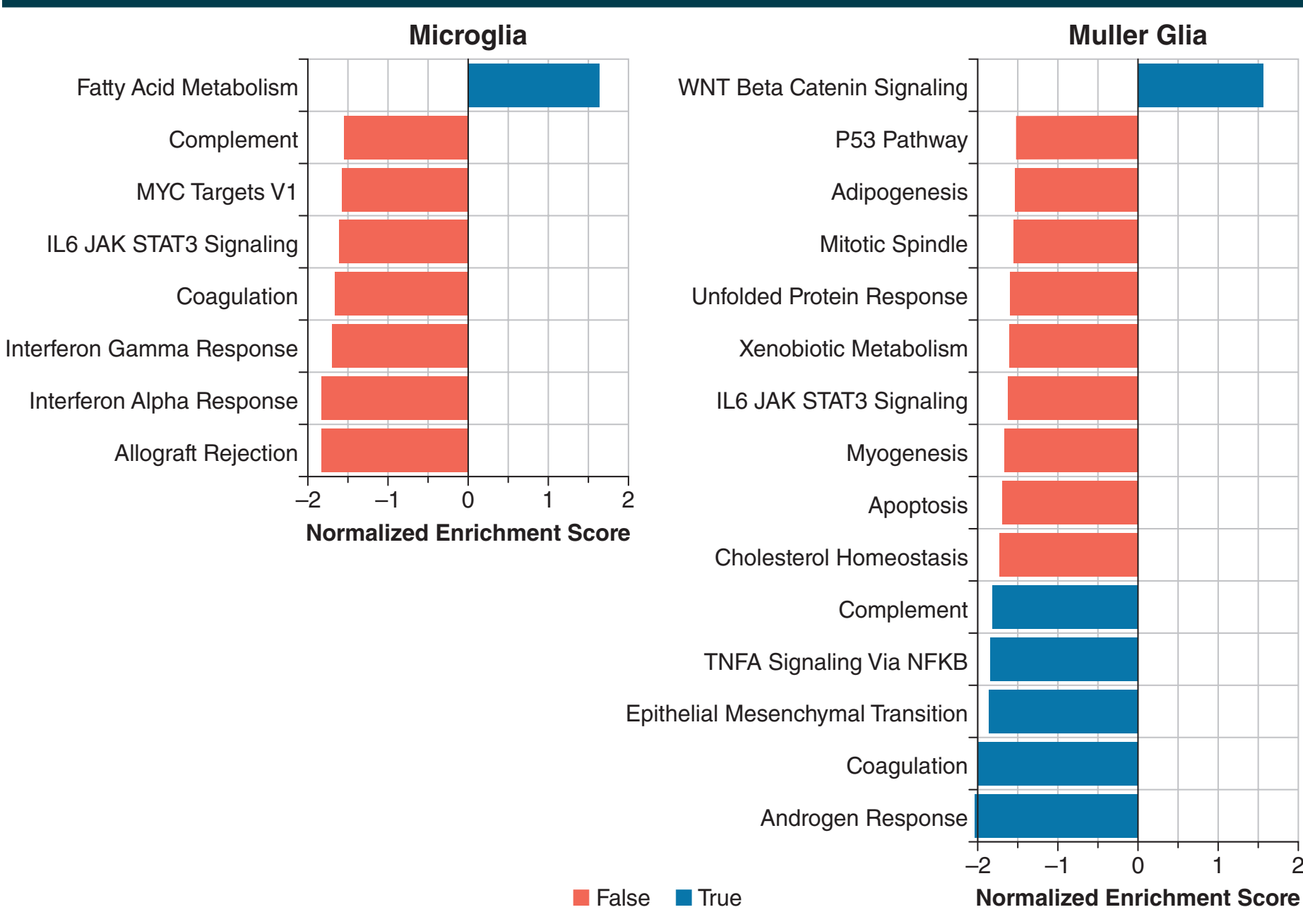
- The top differentially expressed genes in PL9654 0.05 mg/kg treatment vs vehicle by cell type are shown in **Figure 10**

Figure 10. Top Differentially Expressed Genes in Treatment vs Vehicle by Cell Type



- Gene set enrichment analysis identified negative enrichment of diverse immune-related pathways in the PL9654 0.05 mg/kg group vs vehicle (**Figure 11**)

Figure 11. GSEA-Identified Immune Related Pathways in PL9654 0.05-mg/kg Group

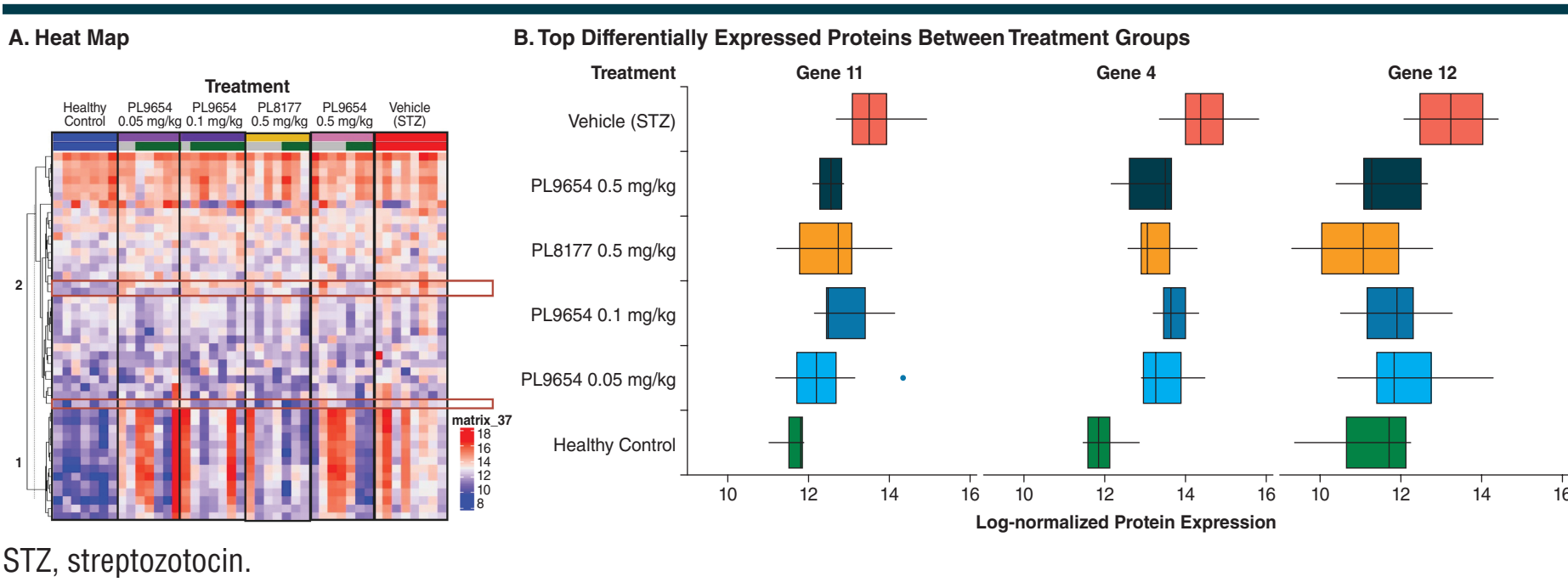


GSEA, Gene set enrichment analysis. All pathways shown have nominal $P < 0.05$ for significance vs vehicle, but pathways shown in green have a significant false discovery rate < 0.05 .

Proteomic Data

- Supervised linear discriminant analysis using top 37 principal components, capturing 95% of variation, showed that the vehicle group separated well from the healthy control and treated samples (**Figure 12**)
- Out of 2600 unique proteins identified across all samples, 46 proteins explain maximum variability between the treatment groups (**Figure 13**)

Figure 13. Retinal Cell Proteome Analysis



- Figure 13A** shows a heat map of 46 proteins that explain maximum variability between different treatment groups. X-axis contains samples clustered based on treatment types and y-axis contains proteins. Cell color ranges from blue to red where blue represent low protein expression and red high expression
- Figure 13B** Shows the top 3 differentially expressed proteins between treatment groups. PL9654 and PL8177 treatment groups showed a significant decrease in protein expression vs vehicle. Gene 4 is a major marker of inflammation in DR and its reduced mRNA expression in glial cells results in low protein levels

Safety

- There were no adverse events resulting from administration of either PL9654 or PL8177, with the exception of skin lesions, common to all subcutaneously delivered peptide melanocortin agonists in rat (no other species show this effect)

Conclusions

- Subcutaneous twice-daily administration of the melanocortin agonists PL9654 and PL8177 reduced vision loss and photoreceptor degeneration in the STZ rat model
- Genomic and proteomic analysis shows that PL9654 0.05 mg/kg causes a reduction in microglia and significant negative enrichment of genes associated with immune-related pathways suggesting a reduction in inflammatory processes.
- Together, these results support the continued development of PL9654 and PL8177 for the treatment of DR

Support Palatin Technologies, Inc.

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Disclosures Paul S. Kayne, Priyanka Dhingra, Alison Obr, John H. Dodd, and Carl Spana are employees of Palatin Technologies, Inc.

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