ARTICLE

BIOL
THE ALL RESULTS JOURNALS

4-phenylbutyric acid, metformin, terpin and phenoxyethanol do not reduce Lafora bodies in murine Lafora disease

Lori Israelian, Peixiang Wang, Berge A. Minassian*

All Res. J. Biol., **2024**, 15, 1-9

The publication cost of this article might be covered by external sponsors. More info for sponsors at: innovacion@sacsis.es

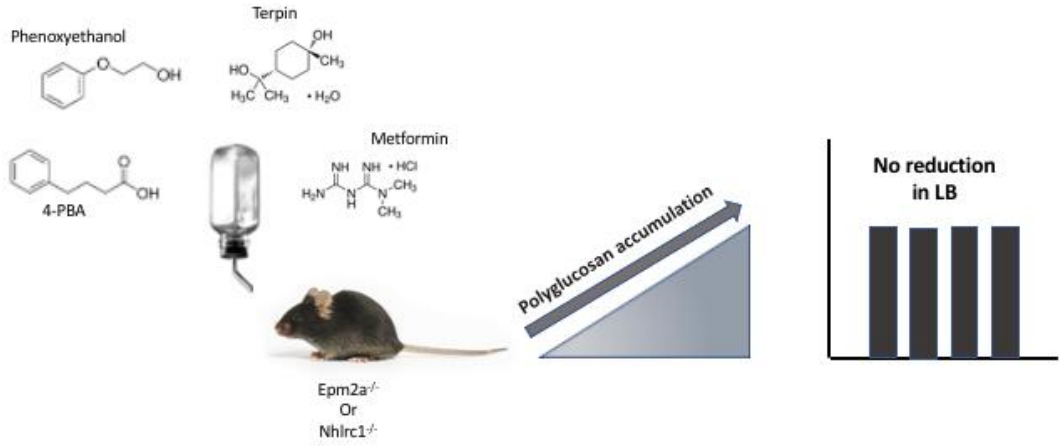
All your results are good results!

Issue 1, vol 15, 2024, 1-9

4-phenylbutyric acid, metformin, terpin and phenoxyethanol do not reduce Lafora bodies in murine Lafora disease

Lori Israelian[1], Peixiang Wang[1], *Berge A. Minassian[1,2]

[1]*Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, Ontario, Canada.*
[2]*Division of Neurology, Department of Pediatrics and Dallas Children's Medical Center, University of Texas Southwestern, Dallas, Texas 75390-9063*



Abstract: Lafora disease is a fatal neurodegenerative epilepsy that results from the accumulation in the brain of insoluble glycogen aggregates (polyglucosans) into Lafora bodies (LB). It is caused by loss of function mutations in either *EPM2A or NHLRC1*, encoding the glycogen phosphatase, laforin, and the E3 ubiquitin ligase, malin, respectively. The actions of laforin and malin in glycogen homeostasis are interdependent, deficiency of either leading to dysregulated glycogen synthesis. Several studies have demonstrated that genetic lowering of glycogen synthesis in mouse models of LD is therapeutic, reducing LB, neurodegeneration and behavioral phenotypes. Two FDA-approved drugs, 4-phenylbutyric acid and metformin were recently tested in LD mice and reported to reduce LB accumulation. Glycogen branching enzyme deficiency is a second disease characterized by polyglucosan aggregates. Two additional compounds, terpin and phenoxyethanol, were recently reported to reduce polyglucosan formation in fibroblasts from mice and humans with this disease. In the present study, we administered the above four compounds to genetic mouse models of LD until 3 months of age. At sacrifice we found no reduction in polyglucosan and LB accumulation compared to controls. Our results supplement available data on the above drugs as potential therapies for Lafora disease.

Keywords: *Lafora disease; polyglucosan; terpin; phenoxyethanol; 4-phenylbutyric acid; metformin*

Introduction: Lafora disease (LD) is a neurodegenerative, progressive myoclonus epilepsy with onset in adolescence. Recessively inherited loss-of-function mutations in the gene encoding the glycogen phosphatase laforin (*EPM2A*) or the E3 ubiquitin ligase malin (*NHLRC1*) impair proper glycogen synthesis. Pathognomonic to LD is the profuse presence throughout the brain of aggregates of malstructured (poorly branched) insoluble glycogen (polyglucosan) termed Lafora bodies (LB). The progressive neurodegeneration, worsening of seizures and cognitive decline in LD correlate with increasing spread and sizes of LB[1]. Several independent groups have confirmed that LB accumulation, and LD progression, in genetic mouse models of LD mice can be halted or attenuated by genetic knockout or knockdown of glycogen synthase, the rate limiting enzyme in glycogen synthesis, or its activator (PTG, protein targeting to glycogen, which targets the pleotropic phosphatase PP1 to dephosphorylate and activate glycogen synthase)[2,3,4]. Collectively, these results suggest that LB are pathogenic in LD, and that compounds that inhibit glycogen synthesis, or otherwise reduce LB formation or accumulation, may be therapeutic to the disease.

LB contain a minor component of various proteins[6]. Whether these are merely non-specific proteins trapped in the overwhelming mass of polyglucosans composing the bodies or are a significant contributor to the formation of LB, is unknown. The compound 4-phenylbutyric acid (4-PBA; $C_{10}H_{12}O_2$) counters protein misfolding and aggregation in several models of neurodegenerative diseases due to misfolded protein aggregation[7,8]. It was recently tested in the malin-deficient mouse model of LD and reported to result in LB reduction in the hippocampus[9]. In this study, we sought to replicate this result.

It has been speculated, but never confirmed, that LB may be cleared through autophagy. Evidence has been presented that a primary defect in autophagy might at least in part underlie LD[10,20], although this was not confirmed in a subsequent study[12]. Metformin, a widely used drug in type II diabetes, has been tested in the context of LD with the intent to promote autophagy through its activation of AMP-activated protein kinase (AMPK)[9]. AMPK is as a cellular energy sensor that promotes autophagy through its inhibition of rapamycin complex 1 (mTORC1)[12,13]. AMPK is also a GS kinase and its activation should inhibit glycogen synthesis[14]. Administering metformin to malin deficient mice reduced LB in the hippocampus[8]. We sought to confirm this finding in laforin deficient mice.

Glycogen was then quantified indirectly following the protocol of Lowry and Passonneau[18]. The amount of glucose was related back to the original weight in grams of the brain sample. Metformin, phenoxyethanol and terpin treatments had brain glycogen quantified in a similar manner, where glycogen quantity was based off the protein-content of the sample.

Statistics

Data are presented as mean with SEM bars. One-way ANOVA analyses detected no significant differences among the brain glycogen quantities of 4-PBA, NaCl, and water treated mice. Polyglucosans very similar to LB form in a second disease, namely glycogen branching enzyme (GBE) deficiency. Terpin ($C_{10}H_{20}O_2$) is a United States Food and Drug Agency (FDA) approved expectorant, and phenoxyethanol ($C_8H_{10}O_2$) a germicidal agent, identified in a cell-based screen for compounds that lower polyglucosan accumulation in fibroblasts derived from mice or patients with GBE deficiency by over 50% (reference[19] and O. Akman and O. Kakhlon, *unpublished observation*). Since the possible modes of action of terpin and phenoxyethanol against polyglucosans in GBE deficiency are not clear, and since GBE and LD have a common disturbance in glycogen structure (insufficient branching), we elected to test a potential effect of these two compounds in murine LD.

Methods

Ethics Statement

All animal procedures were approved by the Toronto Center for Phenogenomics Animal Care Committee.

Histochemistry

The laforin KO[17] and malin KO[16] mice were previously described. Sections were stained with periodic acid-Schiff (which stains polyglucosans) and briefly treated with diastase (amylase) to digest and exclude normal glycogen from analysis; the combined staining and amylolysis is termed PASD. Pathology images were acquired at 20x magnification, and LB were quantified on slides scanned with the 3DHistech Pannoramic 250 Flash II Slide Scanner. The HistoQuant module of the 3DHISTECH digital pathology software detects LB based on their hue and saturation values following PASD treatment. LB accumulation was quantified by dividing the total area of LB on a section of hippocampus by the total area of the hippocampal section and converting the value into a percentage. Size exclusion was applied on HistoQuant to avoid quantifying objects that were stained with PASD but are not LB, such as blood vessels. We utilize hippocampi for LB quantification for its ease of anatomical recognition and framing. Our studies have shown that hippocampal LB counts reflect counts in other brain regions.

Glycogen quantification

Mice were sacrificed by cervical dislocation and brain harvested and placed in -80°C immediately. For 4-PBA brain samples, glycogen was quantified as µmol glucose/g fresh weight (FW) tissue, where brain tissue was first boiled in 30% KOH and precipitated in 67% ethanol three times prior to an amyloglucosidase digestion to release glucose.

Similarly, LB quantitation was also found to not differ significantly by one-way ANOVA. A student's t-test was conducted to analyze brain glycogen content and LB quantities in metformin treated mice, and no differences were found in comparison to control mice. Lastly, one-way ANOVA detected no significant differences between the brain glycogen or LB quantities of mice treated with phenoxyethanol or terpin as compared to control mice. N = 3-9 for experiments, and statistics were conducted using Graphpad Prism version 7.04.

Results

4-PBA does not lower total brain glycogen or hippocampal LB in malin KO mice

Through the time of weaning and until sacrificing at 3 months of age, malin knockout (KO) mice were administered water, or, in lieu of water, either a 50 mM solution of NaCl or a 50 mM solution of 4-PBA (pH 7). This concentration is 2.5-fold the one used the previous study (20 mM)[9]. A NaCl control was included in this study because the 4-PBA solution was titrated with an equimolar amount NaOH for both solubilization and neutralization purposes. Qualitative histopathology of PASD stained hippocampi showed no apparent difference in LB accumulation between animals that consumed 4-PBA, water or NaCl (Figure 1). Quantitation of LB as the percent of hippocampus stained with PASD revealed no differences between the three groups (Figure 1). Albeit malstructured, polyglucosans are glycogen and therefore raise the total amount brain glycogen in LD. Biochemical measurement of total brain glycogen therefore reflects the extent of polyglucosan accumulation[15]. Quantitation of total brain glycogen in the 4-PBA treated animals showed no difference from the control groups (Figure 1).

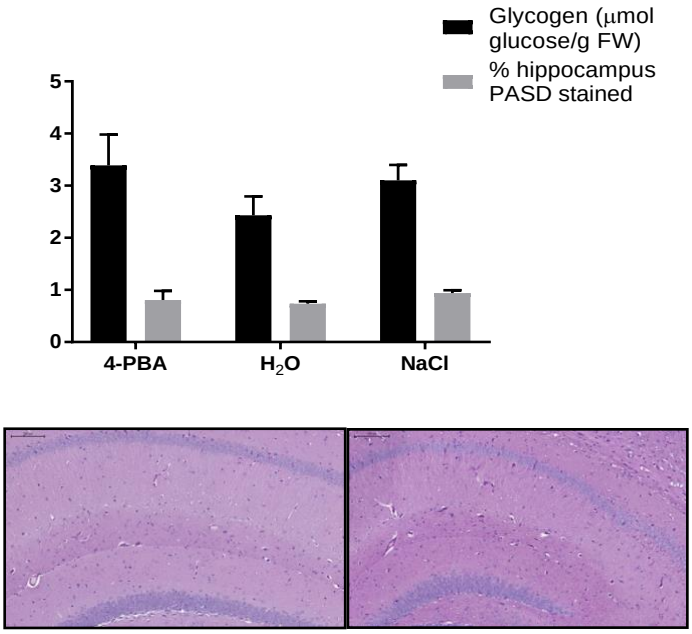


Figure 1. Top row includes data from 4-PBA experiment where (A) is 4-PBA treated MKO mice and (B) and (C) are 4-PBA and water-administered MKO mice, respectively. Scale bars are 100 μm. n = 6-9 for glycogen and 3-5 for LB quantification

Metformin does not lower total brain glycogen or hippocampal LB in laforin KO mice

Laforin KO mice were administered either water or a 2 mg/ml aqueous solution of metformin ($C_4H_{11}N_5$). This concentration is 25% higher than the dose used in the previous study (1.5 mg/ml or 12 mM)[9]. Mice consumed the control or treatment solution for 2 months after weaning and were sacrificed at 3 months-old. Quantitation of LB and brain glycogen revealed no reduction in hippocampal LB or total brain glycogen (Figure 2).

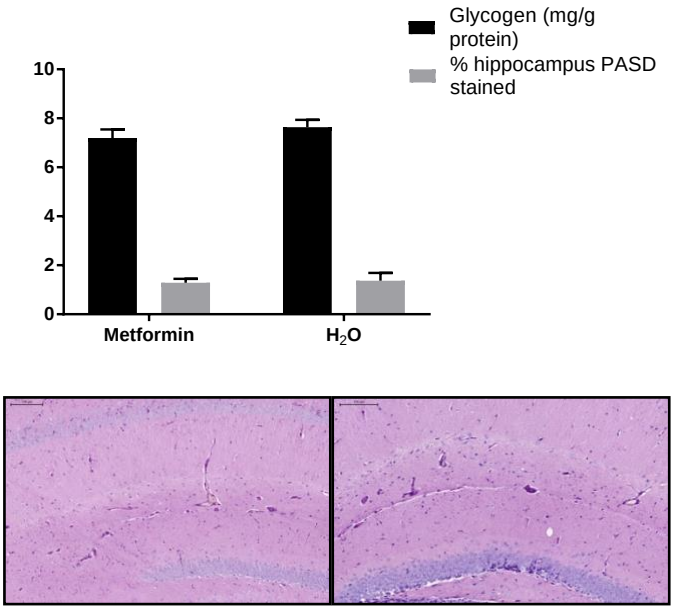


Figure 2. (A) Glycogen and LB quantification of metformin and control treated LKO mice. Images (B) and (C) are PASD stained sections of metformin and water-treated mice, respectively. Small pink inclusions are the Lafora bodies. Scale bars are 100 μm.

Phenoxyethanol and terpin do not lower total brain glycogen or hippocampal LB in laforin KO mice

Terpin (LD50 = 300 mg/kg) was first dissolved in dimethyl sulfoxide and then water to a final concentration of 0.75 mg/ml. Phenoxyethanol (LD50 = 933 mg/kg) was prepared by first dissolving in ethanol and then diluting with water to a final concentration of 3 mg/ml. By administering both compounds via drinking water, the mice are provided a dose beneath the respective oral LD50s of terpin and phenoxyethanol, despite the mouse's constant intake of fluid. Both compounds were administered after weaning and until sacrificing at 3 months of age. There were no differences in hippocampal LB measures or total glycogen between treated and untreated groups (Figures 3 and 4).

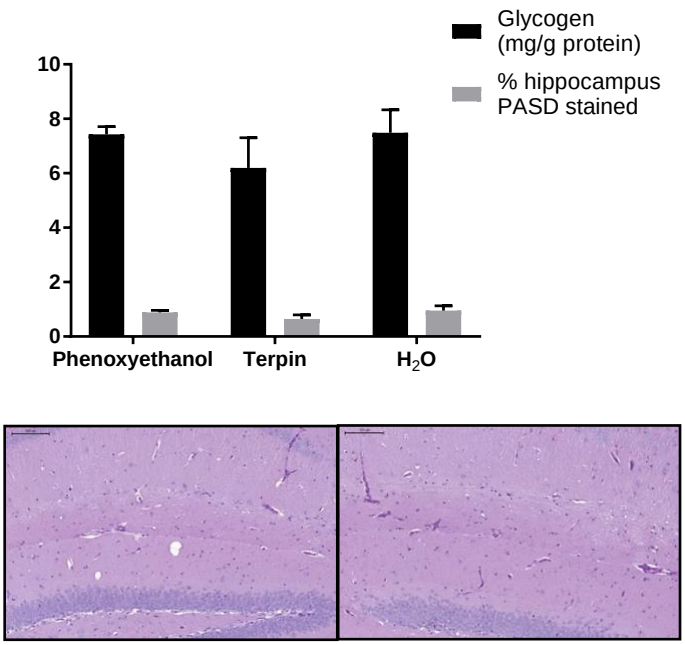


Figure 3. (A) Glycogen and LB quantification of phenoxyethanol, terpin and water-treated mice. Images (B) and (C) represent phenoxyethanol and water-treated mice, respectively. Scale bars are 100 µm. n = 3-7 for glycogen and 3 for LB quantification.

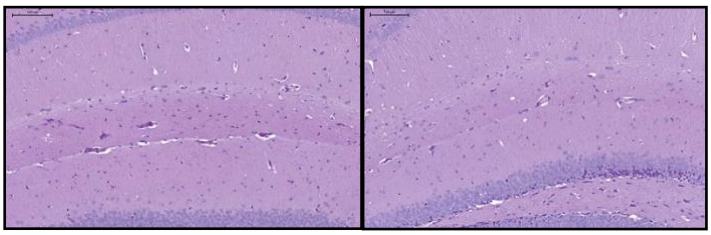


Figure 4. PASD stained hippocampi of (A) terpin and (B) water-treated mice. Scale bars are 100 µm.

Discussion

Several groups have shown that preventing or reducing LB formation (through genetic downregulation of glycogen synthase) rescues neurodegeneration, behavioral deficits and seizure susceptibility to pro-convulsant drugs[2,3,4]. As such, LB are an appropriate screening biomarker of LD. 4-PBA and metformin are FDA approved compounds that, if effective in LD, would represent an immediate potential therapeutic to patients with this devastating disease. We set out to confirm the effect of 4-PBA on malin KO LB, but were unable to do so, despite the use of a dose higher than published. In the case of metformin, we tested the effect in laforin KO, hoping to extend the previously published results in malin KO to the laforin-deficient form of the disease. However, here also we did not find a positive effect on LB counts or brain glycogen quantity. It is possible that metformin impacts LB only in malin KO disease.

In the previous publications, both 4-PBA and metformin showed benefits beyond LB reduction, namely reduced neurodegeneration, behavioral and memory problems and proneness to seizures following convulsant drug administration. We did not test these effects, and it is possible that the drugs have benefits in LD neurodegenerative disease and epilepsy unrelated to effects on LB.

One of the difficulties in screening for small molecules inhibitors of LB formation is that cell lines from LD patients or mouse models do not form LB. Polyglucosans accumulate into LB over a long time within non-dividing cells, and appear to have no time to do so in dividing fibroblasts. However, cell lines from GBE deficient humans and mice have been reported to accumulate polyglucosan bodies. We tested two compounds, terpin and phenoxyethanol, shown to affect polyglucosan accumulation in GBE deficient fibroblasts, but unfortunately neither had a measurable effect on brain LB in the laforin KO LD mouse model.

The potential use of medications approved for other indications by the FDA (and corresponding agencies) in severe diseases like LD is highly desirable. We present our results here to afford a more complete picture of existing data for the benefit of researchers and clinicians assessing these drugs for these patients.

Acknowledgments

This work was funded by the National Institute of Neurological Disorders and Stroke of the NIH under award number P01 NS097197. B.A.M. holds the University of Texas Southwestern Jimmy Elizabeth Westcott Chair in Pediatric Neurology.

References

1. Verhalen, B., Arnold, S., & Minassian, B.A. (2018) Lafora disease: a review of molecular mechanisms and pathology. *Neuropediatrics*, 49(06), 357-362.
2. Turnbull, J., DePaoli-Roach, A. A., Zhao, X., Cortez, M. A., Pencea, N., Tiberia, E., . . . Ackerley, C. A. (2011). PTG depletion removes lafora bodies and rescues the fatal epilepsy of lafora disease. *PLoS Genetics*, *7*(4), e1002037.
3. Pederson, B. A., Turnbull, J., Epp, J. R., Weaver, S. A., Zhao, X., Pencea, N., . . . Minassian, B. A. (2013). Inhibiting glycogen synthesis prevents lafora disease in a mouse model. *Annals of Neurology*, *74*(2), 297-300
4. Duran, J., Gruart, A., García-Rocha, M., Delgado-García, J. M., & Guinovart, J. J. (2014). Glycogen accumulation underlies neurodegeneration and autophagy impairment in lafora disease. *Human Molecular Genetics*, *23*(12), 3147-3156.
5. Lomako, J., Lomako, W. M., & Whelan, W. J. (1988). A self-glucosylating protein is the primer for rabbit muscle glycogen biosynthesis. *The FASEB Journal*, *2*(15), 3097-3103.
6. Sakai, M., Austin, J., Witmer, F., & Trueb, L. (1970). Studies in myoclonus epilepsy (Lafora body form): II. Polyglucosans in the systemic deposits of myoclonus epilepsy and in corpora amylacea. *Neurology*, 20, 160-176.
7. Wiley, J.C., Pettan-Brewer, C., & Ladiges, W.C. (2011) Phenylbutyric acid reduces amyloid plaques

and rescues cognitive behavior in AD transgenic mice. *Aging Cell*, 10, 418-428

8. Zhou, W., Bercury, K., Cummiskey, J., Leong N., Lebin, J., & Freed, C.R. (2011). Phenylbutyrate up-regulates the DJ-1 protein and protects neurons in cell culture and in animal models of Parkinson disease. *Journal of Biological Chemistry*, 286 (17), 14941-14951
9. Berthier, A., Payá, M., García-Cabrero, A. M., Ballester, M. I., Heredia, M., Serratosa, J. M., . . . Sanz, P. (2016). Pharmacological interventions to ameliorate neuropathological symptoms in a mouse model of lafora disease. *Molecular Neurobiology*, *53*(2), 1296-1309.
10. Aguado, C., Sarkar, S., Korolchuk, V. I., Criado, O., Vernia, S., Boya, P., . . . Rubinsztein, D. C. (2010). Laforin, the most common protein mutated in lafora disease, regulates autophagy. *Human Molecular Genetics*, *19*(14), 2867-2876
11. Lahuerta, M., Aguado, C., Sánchez-Martín, P., Sanz, P., & Knecht, E. (2018). Degradation of altered mitochondria by autophagy is impaired in lafora disease. *The FEBS Journal*, *285*(11), 2071-2090.
12. Wang, P., Israelian, L., Xue, Y., Song, S., Attisano, L., & Minassian, B.A. (2016). SGK1 (glucose transport), dishevelled2 (wnt signaling), LC3/p62 (autophagy) and p53(apoptosis) proteins are unaltered in Lafora disease. *All Results Journal Biol*, 7, 28-33.
13. Sánchez-Elexpuru, G., Serratosa, J.M., Sanz, P., & Sánchez, M.P. (2017). 4-PBA and metformin decrease sensitivity to PTZ-induced seizures in a malin knockout model of Lafora disease. *Neuroreports*, 28(5), 268-271
14. Lage, R., Diéguez, C., Vidal-Puig, A., Lopez., M. (2008). AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends in Molecular Medicine*, 14(12), 539-549.
15. Nitschke, F., Sullivan, M.A., Wang, P., Zhao, X., Chown, E.E., Perri., A.M., …Minassian, B.A. (2017) Abnormal glycogen chain length pattern, not hyperphosphorylation, is critical in Lafora disease. *EMBO Molecular Medicine*, 9(7), 906-917.
16. Turnbull, J., Wang, P., Girard, J., Ruggieri, A., Wang, T.J., Draginov, A.G., … Minassian, B.A. (2010). Glycogen hyperphosphorylation underlies Lafora body formation. *Annals of Neurology*, 68, 925-933
17. Ganesh, S., Delgado-Escueta, A.V., Sakamoto, T., Avila, M.R., Machado-Salas, J., Hoshii, Y., …Yamakawa, K. (2002) Targeted disruption of the Epm2a gene causes formation of Lafora inclusion bodies, neurodegeneration, ataxia, myoclonus epilepsy and impaired behavioral response in mice. *Human Molecular Genetics*, 11(11), 1251-1262.
18. Lowry, O. H., & Passonneau, J. V. (1972). A collection of metabolite assays. *A Flexible System of Enzymatic Analysis*, 146-218.
19. Kakhlon, Or., Ferreira, I., Somelsky, L.J., Khazanov, N., Lossos, A., Alvarez, R., . . . Akman, H.O (2018) Guaiacol as a drug candidate for treating adult polyglucosan body disease. *JCI Insight, 3(17)* doi: 10.1172/jci.insight.99694
20. Puri, R., Suzuki, T., Yamakawa, K., & Ganesh, S. (2012) Dysfunctions in endosomal-lysosomal and autophagy pathways underlie neuropathology in a mouse model of Lafora disease. *Human molecular genetics*, 21(1), 175-184

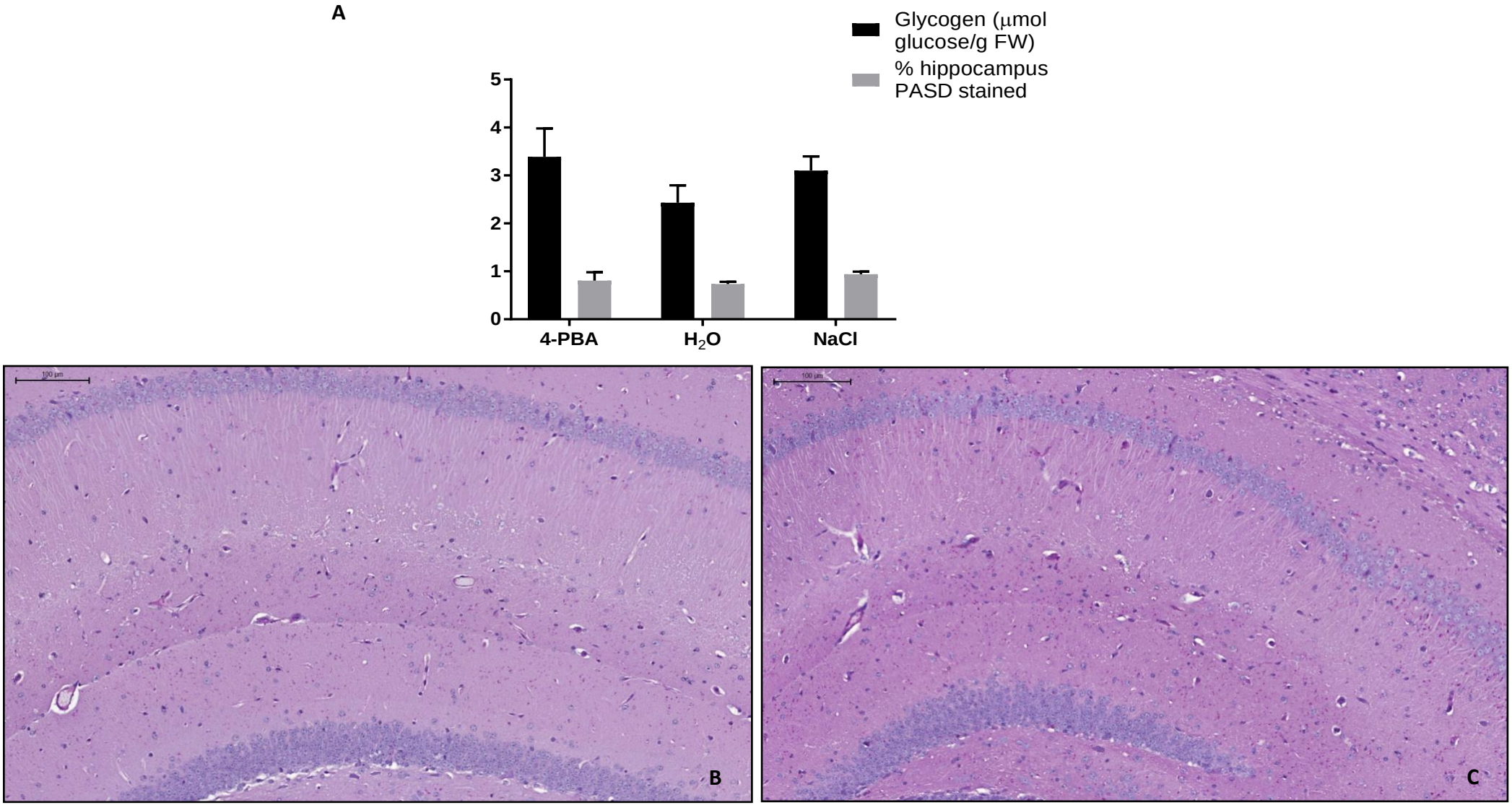


Figure 1. Top row includes data from 4-PBA experiment where (A) is 4-PBA treated MKO mice and (B) and (C) are 4-PBA and water-administered MKO mice, respectively. Scale bars are 100 μm. n = 6-9 for glycogen and 3-5 for LB quantification

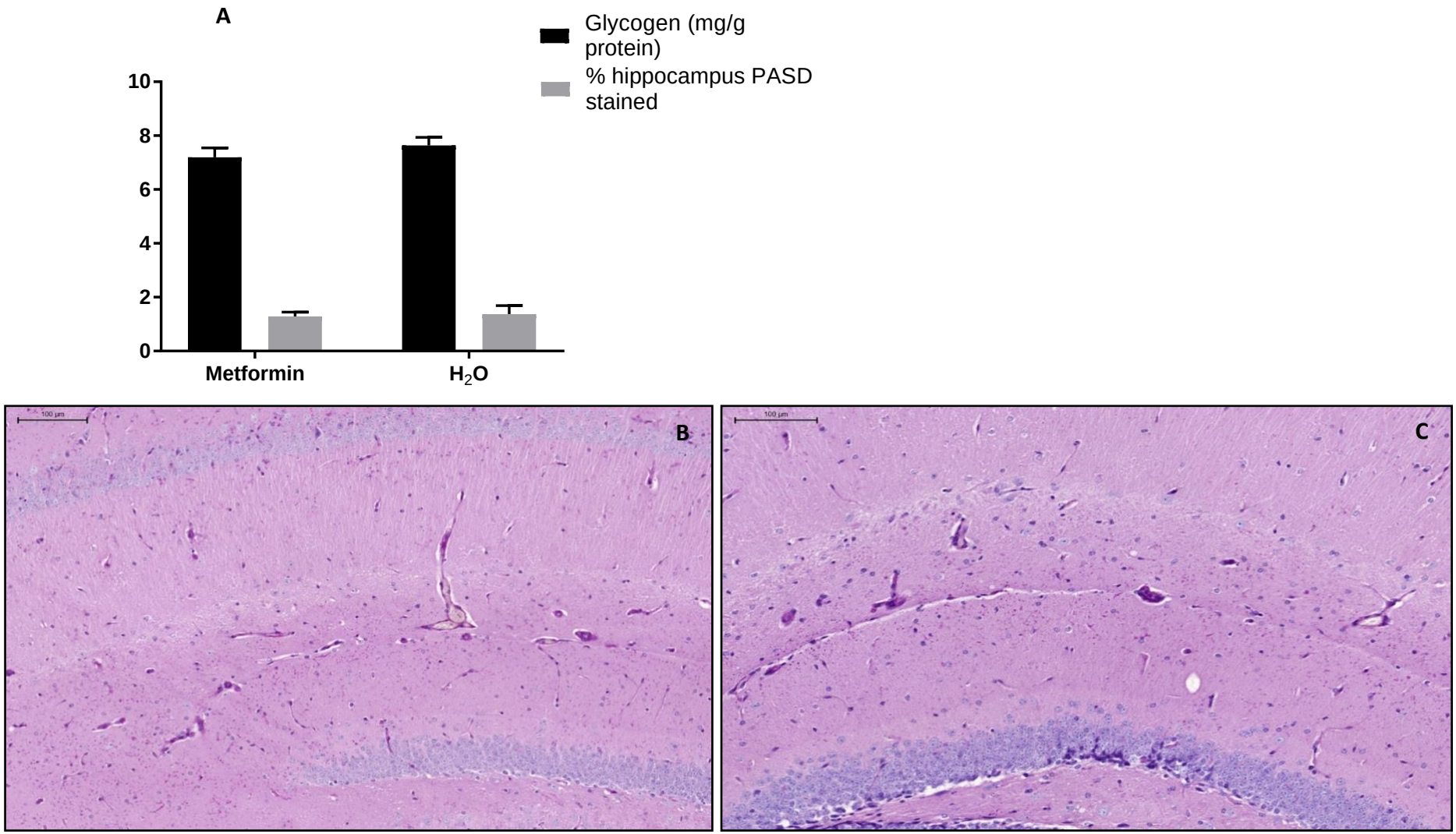


Figure 2. (A) Glycogen and LB quantification of metformin and control treated LKO mice. Images (B) and (C) are PASD stained sections of metformin and water-treated mice, respectively. Small pink inclusions are the Lafora bodies. Scale bars are 100 µm. n = 3-6 for glycogen and LB quantification.

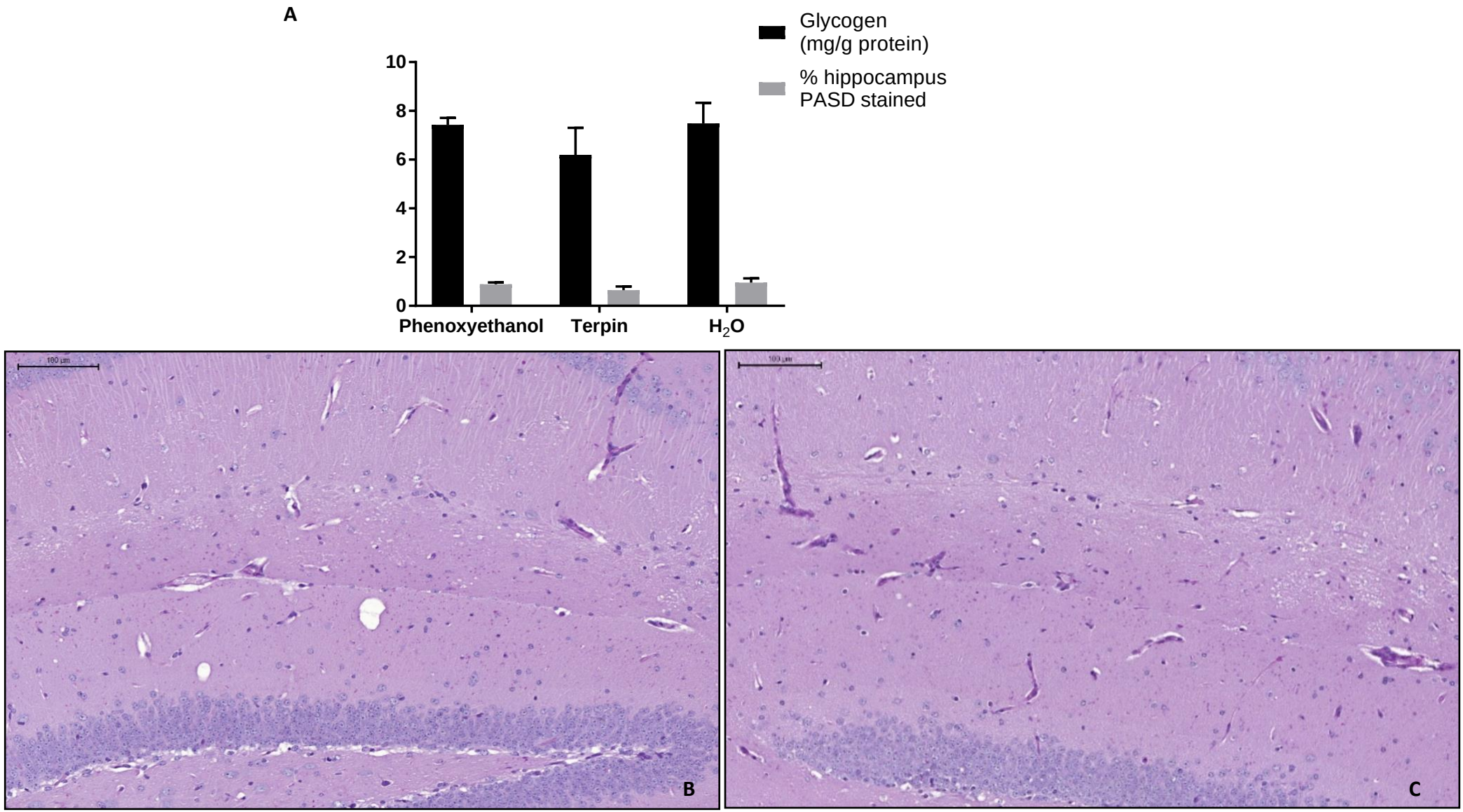


Figure 3. (A) Glycogen and LB quantification of phenoxyethanol, terpin and water-treated mice. Images (B) and (C) represent phenoxyethanol and water-treated mice, respectively. Scale bars are 100 µm. n = 3-7 for glycogen and 3 for LB quantification.

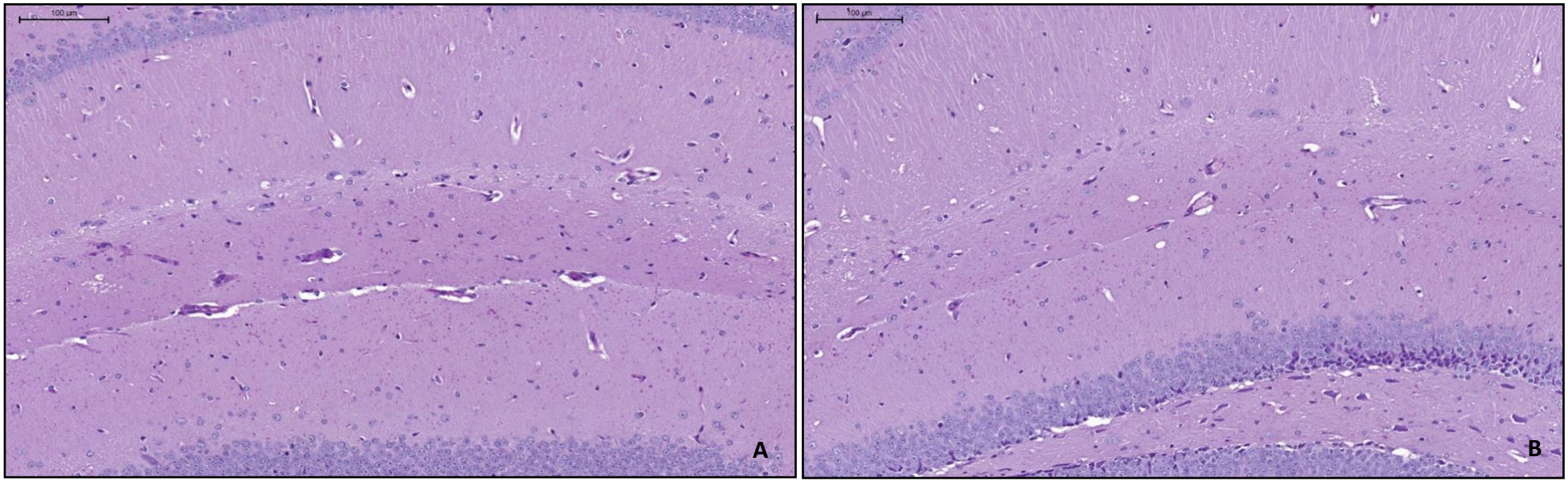


Figure 4. PASD stained hippocampi of (A) terpin and (B) water-treated mice. Scale bars are 100 µm.