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Behavioral and neuropathological features of Alzheimer's disease are attenuated in 5xFAD mice treated with intranasal GHK peptide

Matthew Tucker[a, b], Gerald Yu Liao[a], Addison Keely[a], Joo Young Park[a], Manuela Rosenfeld[a], Jackson Wezeman[a], Ruby Mangalindan[a], Dan Ratner[b], Martin Darvas[c], Warren Ladiges[a, *]

[a] *Department of Comparative Medicine, School of Medicine, University of Washington, Seattle WA, USA.*
[b] *Department of Bioengineering, College of Engineering and School of Medicine, University of Washington, Seattle WA, USA.*
[c] *Department of Laboratory Medicine and Pathology, School of Medicine, University of Washington, Seattle WA, USA.*

Abstract

Alzheimer's disease (AD) is a complex neurodegenerative disease and a leading cause of morbidity and mortality. Efforts to find disease modifying treatments have met with limited success. The naturally occurring peptide GHK (glycyl-L-histidyl-L-lysine), in its Cu-bound form, supports angiogenesis, remodeling, and tissue repair, has anti-inflammatory and antioxidant properties, and has been shown to improve cognitive performance in aging mice. These features raised the question of whether GHK-Cu could alleviate neurodegeneration observed in AD. Male and female 5xFAD transgenic mice on the C57BL/6J background at 4 months of age were given 15 mg/kg GHK-Cu intranasally 3 times per week for 3 months until 7 months of age. Results showed that intranasal GHK-Cu treatment delayed cognitive impairment, reduced amyloid plaques, and lowered MCP1-mediated inflammation levels in the frontal cortex and hippocampus. These observations provide the rationale for conducting additional studies to investigate the potential of GHK-Cu peptide as a promising treatment for AD.

Keywords: Alzheimer's disease, GHK-Cu, 5xFAD transgenic mouse, intranasal administration, amyloid plaques, neuroinflammation

Introduction

Alzheimer's disease (AD) is a complex disease that was the seventh leading cause of mortality in the US in 2022 [1]. Contrary to most other leading causes of mortality, there is no effective treatment for AD and efforts to find effective treatments have met with limited success in part because the focus has been on testing drugs that target a specific pathogenic mechanism. The probability of effectively targeting several mechanistic pathways would be greatly increased by using a drug that individually targets more than one pathway [2, 3]. In addition, the Connectivity Map gene profiling software developed by the Broad Institute, showed that out of several thousand biological molecules, the naturally occurring peptide glycyl-L-histidyl-L-lysine (GHK), in its Cu-bound form prevented impaired TGFβ1 signaling [4], a pathway associated with Aβ deposition and neurofibrillary tangle formation [5]. This correlation raised the question of whether GHK-Cu could alleviate neurodegeneration observed in Alzheimer's disease (AD).

GHK is a naturally occurring peptide released from secreted protein acidic and rich in cysteine (SPARC) during proteolytic breakdown [6]. In the event of an injury, GHK supports angiogenesis, remodeling, and tissue repair as a copper complex (GHK-Cu) [7-9]. The peptide is clinically approved as a topical application for age-related skin conditions and promoted mainly as a skin rejuvenation drug [10]. GHK has been shown to be an endogenous antioxidant by decreasing hydroxyl and peroxyl radicals [11], presumptively involved in the neuropathogenesis of AD [12] and improve cognitive performance in aging mice [13].

Targeted neurotherapeutics face a challenge in penetrating the blood brain barrier (BBB) [14-16]. This inherent

* Corresponding author: Warren Ladiges
Mailing address: Department of Comparative Medicine, School of Medicine, University of Washington, Seattle WA, USA.
Email: wladiges@uw.edu
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restriction has significantly limited the availability of effective AD treatments and has triggered investigation into methods or strategies to bypass the BBB. In this regard, the intranasal route is a promising delivery method to obtain greater efficacy in maintaining optimal drug concentrations in the brain compared to parenteral injections [17-20]. The intranasal system uses olfactory epithelium and its associated neural pathways to deliver drugs in therapeutically relevant concentrations.

The 5xFAD transgenic mouse line has been genetically engineered to model features of AD through the expression of human amyloid precursor protein (APP) and presenilin-1 transgenes, which are associated with early-onset AD [21]. Mutations in these genes are linked to the regulation of amyloid-beta processing, a crucial component of AD pathogenesis. Transgenic 5xFAD mice have amyloid neuropathology similar to characteristic amyloid-plaque pathology in human patients [21, 22], and have been shown to develop impairment in cognitive behaviors that correspond to part of the dementia syndrome in AD.

Neuroinflammation plays a crucial role in the onset and progression of AD, with chemokines such as monocyte chemoattractant protein-1 (MCP-1) being key mediators in these inflammatory processes. Elevated levels of MCP-1 have been linked to chronic inflammation, which is a hallmark of neurodegenerative diseases. MCP-1 contributes to the recruitment of immune cells to the central nervous system (CNS), potentially exacerbating neuroinflammation and promoting disease progression [23-25]. Additionally, increased MCP-1 levels are associated with heightened blood-brain barrier (BBB) permeability, further complicating disease pathology by allowing more inflammatory cells to infiltrate the CNS [26]. Therefore, evaluating the extent of inflammatory reactivity to amyloid plaques and microglial activity through MCP-1 staining would provide insights into the role of MCP-1 in neuroinflammatory responses in AD.

The aim of this study was to determine if intranasal delivery of GHK-Cu as a neurotherapeutic would effectively delay the onset of cognitive impairment and neuropathology seen in 5xFAD transgenic mice. The advantages of intranasal administration, including reduced dosage loss, enhanced precision in drug delivery, and expedited brain access, were used to show that GHK-Cu was able to mitigate cognitive impairment and features of AD neuropathology.

Materials and Methods

Animals and experimental design

C57BL/6J mice with the transgenic 5xFAD genotype (JAX, Bar Harbor, Maine) of both sexes were used. The 5xFAD mouse line has five mutations: the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations in APP, as well as the M146L and L286V mutations in PSEN1. Collectively, these mutations induce the formation of amyloid plaques. Mice were bred and genotyped following standard procedures from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were group-housed, up to five per cage, in a specific pathogen free facility verified through viral and bacterial tests (IDEXX Bioanalytics). Nestlets (Ancare Corp, Bellmore, NY) were provided for physical and mental stimulation. Mice were monitored for health daily and cages were changed biweekly. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).

The experiment started when mice were 4 months of age and ended when they were 7 months of age for a treatment period of three months (12 weeks). Transgenic 5xFAD and wild type mice of both sexes were stratified across a peptide treatment cohort and a saline control cohort.

GHK-Cu dose and delivery

GHK was used as a GHK-Cu complex (Active Peptide, Cambridge, MA) at a dose of 15 mg/kg body weight. Unpublished observations in our lab suggested that GHK-Cu complex was more effective than unbound GHK while unbound Cu had no effect in several *in vitro* and *in vivo* experiments. However, Cu ions can induce toxicity in mice if dosage exceeds levels of 35 mg/kg [27]. Within the GHK-Cu complex, Cu made up 14% of the total molecular content, so a 15 mg/kg GHK-Cu dose had 2.1 mg/kg copper.

Drug administration occurred under 3% isoflurane anesthesia. Mice were then positioned at a 10-15° decline, and a hand grip was applied to the back, tail, and neck. This lowered the head in relation to the body, which maximized the surface area of the olfactory epithelium for efficient dosage uptake [8]. The drug was then drawn up using a micropipette with a clean tip and applied carefully to the rim of the mouse nostril, one drop at a time. The timing of droplet placement was synchronized with natural inhalation to allow for drops to settle on the olfactory and respiratory epithelium within the nasal cavity [9]. Droplets were alternated between nostrils with each breath until a 20 µL volume was administered, typically requiring three to four droplets per mouse. Mice were then maintained in the declined position for an additional minute, during which stimulation was applied to the sternum region to enhance volume uptake. Intranasal GHK-Cu was administered three times per week using this procedure for the duration of the study. Saline was administered to the control group using similar methods as stated above.

Behavioral Test

Cognitive function was assessed by an attentive working memory task (Y-maze) designed to assess working spatial memory. The Y-Maze was performed as previously described [28]. Briefly, it consisted of three equally spaced arms at 120° angles from each other, each enclosed by raised walls. There was no escape option within the maze, and mice were allowed to freely explore it for five minutes while the path they took through the three arms was tracked by recording their entries into each arm. After completing the Y-maze, the mice were temporarily removed and placed in a separate resting cage while the maze was sterilized. All littermates underwent the assess-

ment in a similar manner and were collectively returned to their home cage at the end of the trial. The Y-Maze test assessed spontaneous alternation, an indicator of working memory, as it measures the tendency of mice to explore novel arms of the maze rather than revisitations. Data from the trials administered at weeks four, eight, and twelve of the study were recorded. Spontaneous alternation data were expressed as a percentage calculated as the number of times a mouse completed a triad or loop through all three arms during the trial divided by the number of entries minus two [29]. This percentage represents the number of triads over the number of possible triads for a particular mouse otherwise known as spontaneous alternation percentage.

Neuropathology

At the conclusion of the twelve-week (3 months) treatment period, mice were euthanized using CO_2 and tissues were collected and processed. Sections of the brain and other major organs were rapidly frozen in liquid nitrogen and stored at -80°C. The remaining brain sections and systemic organs were fixed in 10% buffered formalin for 48 hours. Brain tissues were placed in PBS for 24 hours before being embedded in paraffin wax and sectioned onto histology slides. Brain section slides were stained with Congo red to evaluate amyloid plaques by averaging the number of plaques in ten different fields under 10x magnification in multiple areas of the brain blindly by two separate observers. Additional staining was performed using an Abcam immunohistochemistry kit for MCP-1 as a measure of general neuroinflammation. Stained slides were analyzed using Qu-Path digital imagining analysis to measure staining intensity as optical density on positively stained tissue as previously described [30].

Data analysis

Behavioral groups were compared using either a one-way analysis of variance (ANOVA) or two-way ANOVA test whenever appropriate and presented in the form of mean ± standard deviation. Main trial-number effects were analyzed using the Bonferroni post-hoc multiple comparisons test to quantify the extent of latency differences. A two-tailed t-test was employed to compare results between cohorts for neuropathological data. All data were analyzed using GraphPad Prism software (version 10.0.3, GraphPad Software Inc., San Diego, CA, USA) as standard error of the mean with statistical significance at $P < 0.05$.

Results and Discussion

Intranasal GHK-Cu peptide improved cognitive performance in transgenic 5xFAD mice

This study showed that transgenic 5xFAD mice of both sexes exhibited improved cognitive performance after 8 weeks of intranasal GHK-Cu treatment, compared to intranasal saline treated transgenic mice. This pattern continued through the 12-week treatment duration, corresponding to when the mice were 7 months old.

Transgenic female mice treated with intranasal GHK-Cu had higher alternation percentages in the Y maze, indicating improved cognitive performance, beginning as early as the second month of treatment (Week 8), and continuing through the third month (Week 12) when the study ended, compared to transgenic mice treated with intranasal saline (Figure 1A). For male mice, there were significant increases in alternation percentages in transgenic male mice treated with intranasal GHK-Cu compared to transgenic male mice treated with intranasal saline starting the first month and continuing for the next 2 months of the study (Figure 1B). Similar to females, intranasal treatment with GHK-Cu in transgenic mice resulted in a cognitive performance level comparable to non-transgenic wildtype mice.

GHK-Cu-treated transgenic mice of both sexes exhibited more spontaneous alternations in the Y-maze test, indicative of rescued working memory and prefrontal cortical functions [31]. Similar levels of spontaneous alternations were observed in both sexes of GHK-Cu-treated mice compared to other studies involving AD-related drug intervention [32-35]. However, evidence supporting the long-term effects of intranasal GHK-Cu on cognitive performance of 5xFAD mice is limited [34, 36], emphasizing the need for additional studies.

Intranasal GHK-Cu peptide attenuated features of neuropathology in 5xFAD mice

The 5xFAD mouse genotype is characterized by the onset of amyloid plaques at 3 to 4 months of age, which prog-

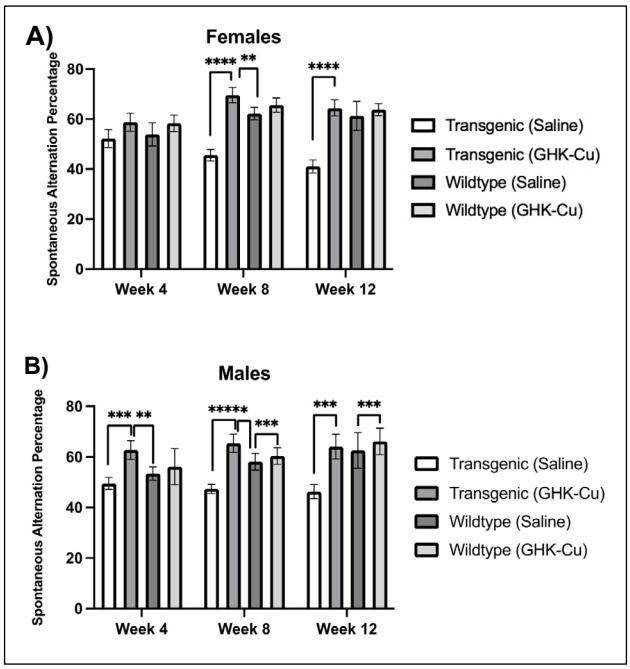

Figure 1. Y-maze percent alternation as a measure of cognitive function over 12 weeks of intranasal GHK-Cu treatment. Cognitive performance was evaluated relative to the 50% threshold. (**A**) A higher alternation percentage was observed for female transgenic mice treated with intranasal GHK-Cu compared to transgenic females treated with intranasal saline at weeks 8 and 12. (**B**) A similar pattern was observed for male transgenic mice treated with intranasal GHK-Cu compared to mice receiving intranasal saline at weeks 4 and 8. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, N = 4-7/cohort, Transgenic = 5xFAD.

ress to substantial and densely concentrated lesions in the brain [37]. Using a Congo Red stain, transgenic mice treated with intranasal GHK-Cu exhibited a reduction in amyloid plaques compared to transgenic mice treated with intranasal saline, irrespective of sex (Figure 2). Both male and female transgenic 5xFAD mice displayed the development of amyloid plaques in the midbrain and thalamus region, frontal cortex, and the hippocampus. Among the transgenic cohorts, those treated with intranasal GHK-Cu had significantly fewer detectable plaques or protein aggregates in comparison to those receiving intranasal saline. Visual observation suggested a pattern where the plaques in saline-treated mice were generally larger and more densely stained compared to the plaques in GHK-Cu-treated cohorts (Figure 3). Wild-type (control) littermates did not display any amyloid plaques, consistent with their genotype.

The accumulation of amyloid plaques is a pivotal factor associated with subsequent neuronal toxicity in AD pathogenesis, eventually associated with synaptic dysfunction and more severe cognitive deficits [38]. Our study revealed that GHK-Cu treated 5xFAD mice exhibited improved cognitive performance compared to saline-treated cohorts while also demonstrating a reduction in amyloid plaques in the midbrain, frontal cortex and hippocampus. While the rescued cognitive abilities in GHK-Cu treated 5xFAD mice may be linked to diminished amyloid plaque formation, the extent of associated neurodegeneration in AD progression was not evaluated. Further investigations targeting this disparity are warranted based on a study protocol designed to assess neurodegeneration and neuronal loss specifically in 5xFAD mice approaching one year of age [39].

MCP-1 staining using immunohistochemistry and Qu-Path digital imaging showed that both male and female transgenic 5xFAD mice that received intranasal GHK-Cu had decreased staining intensity for MCP-1 in the frontal cortex and hippocampus (Figure 4) within tissues registering a positive stain for MCP-1. This correlation indicates reduced neuroinflammation associated with MCP-1 compared to transgenic mice treated with intranasal saline. Heat maps from the Qu-Path generated data show a visual representation of the staining intensity in the frontal cortex from mice treated with intranasal GHK-Cu or saline (Figure 5). The same visual staining intensities were seen in the hippocampus (not shown).

Given that chemokine upregulation can result in chronic inflammation associated with onset and progression of age-related neurodegenerative diseases such as AD, our study employed MCP-1 staining in the frontal cortex and the hippocampus to assess the extent of possible inflammatory reactivity to amyloid plaques and microglial

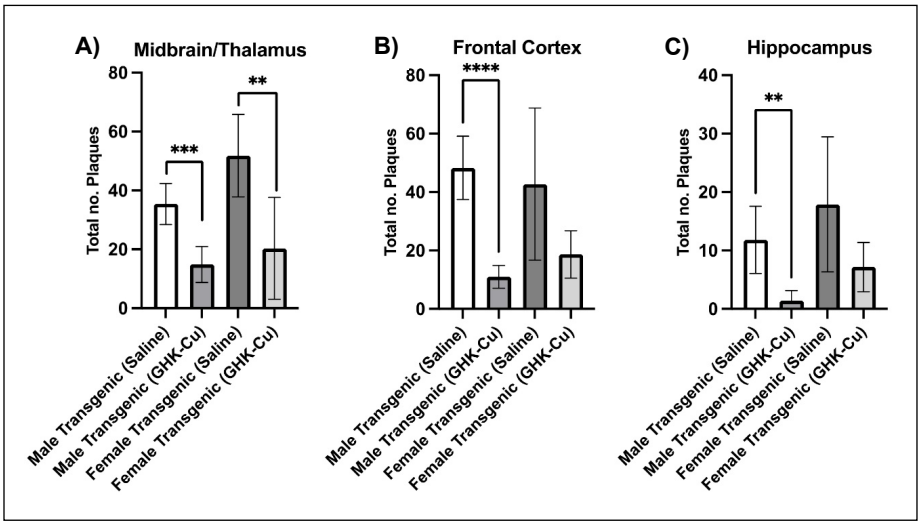

Figure 2. Amyloid plaques were reduced in multiple brain regions of 5xFAD mice following intranasal treatment with GHK-Cu. Quantitative visual count analysis of Congo Red-stained midbrain/thalamus (**A**), frontal cortex (**B**), and hippocampal (**C**) sections from male mice showed a reduction in amyloid plaques in intranasal GHK-Cu-treated transgenic mice compared to intranasal saline-treated transgenic mice while female mice showed a reduction in the midbrain/thalamus region. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. N = 6-7/cohort. Transgenic = 5xFAD.

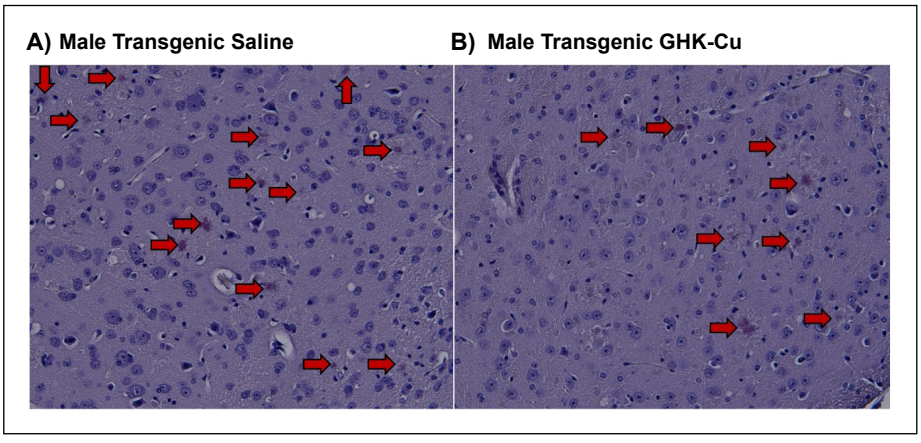

Figure 3. Representative Congo Red staining of frontal cortex sections from (**A**) male transgenic mouse treated with intranasal saline or (**B**) male transgenic mouse treated with intranasal GHK-Cu. Transgenic = 5xFAD. Magnification 200×.

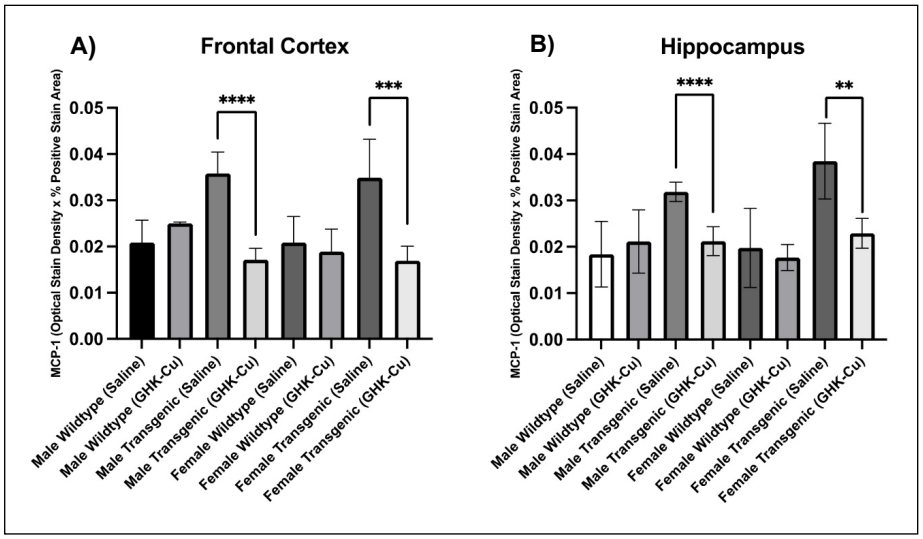

Figure 4. MCP-1 digital stain intensity of positively stained tissues in frontal cortex and hippocampus. (**A**) Transgenic male and female mice treated with GHK-Cu displayed reduced optical stain intensity in positive stained tissues within the frontal lobe when compared to saline-treated cohorts. (**B**) Transgenic male and female mice treated with GHK-Cu also exhibited lower optical stain density in positively stained tissues within the hippocampus in comparison to saline-treated counterparts. Data presented as mean ± standard deviation. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. N = 6-10/cohort. Transgenic = 5xFAD.

activity [23-25]. Furthermore, elevated MCP-1 levels as a result of chemokine upregulation associated with neuroinflammatory diseases have been shown to be associated with increased BBB permeability, which can further exacerbate disease progression [26]. Our results demonstrated a notable decrease in MCP-1 staining intensity in both brain regions in 5xFAD mice that received intranasal GHK-Cu compared to saline-treated cohorts. The observed decrease in MCP-1 staining intensity suggests that intranasal administration of GHK-Cu in transgenic 5xFAD mice had a reducing effect on the AD-induced inflammatory phenotype and could serve as a reliable prototype to study drugs that slow or stop progression of AD [40].

The intranasal administration of GHK-Cu represents a novel approach aimed at overcoming the challenge of bypassing the blood-brain barrier, a common hurdle in traditional targeted neurotherapeutics [14-16]. By leveraging the olfactory epithelium and its associated neural pathways, this method capitalizes on the large surface area, efficient blood flow, and neural connections of the nasal mucosa to provide a relatively non-invasive approach towards efficient peptide delivery into the brain [41-46]. The selection of a three-times-weekly administration schedule accounted for preventing adverse effects of daily anesthesia, and potential localized irritation and toxicity within the nasal mucosa [41, 47]. While systemic toxicity of copper ions was carefully addressed, a comprehensive assessment of inflammation and inflammatory cell infiltration of the nasal epithelium in future studies would further validate the safety and non-toxicity of intranasal GHK-Cu administration.

Conclusions

In conclusion, this study demonstrates the positive therapeutic effect of intranasal GHK-Cu in transgenic 5xFAD mice, a widely used model for AD. Cognitive improvement was observed after 8 weeks of treatment in both females and males, sustained through a 12-week study period, and accompanied by a concurrent reduction in amyloid plaques and neuroinflammation compared to intranasal saline-treated transgenic mice. Behavioral tests, including the Y-maze working memory task and a spatial navigation learning task, helped provide evidence that intranasal GHK-Cu treatment can rescue cognitive performance in a mouse model of AD. The intranasal delivery method allowed a non-invasive approach to efficient drug delivery. These findings suggest that intranasal GHK-Cu has the potential to attenuate features of AD, including cognitive decline, amyloid plaque accumulation, and

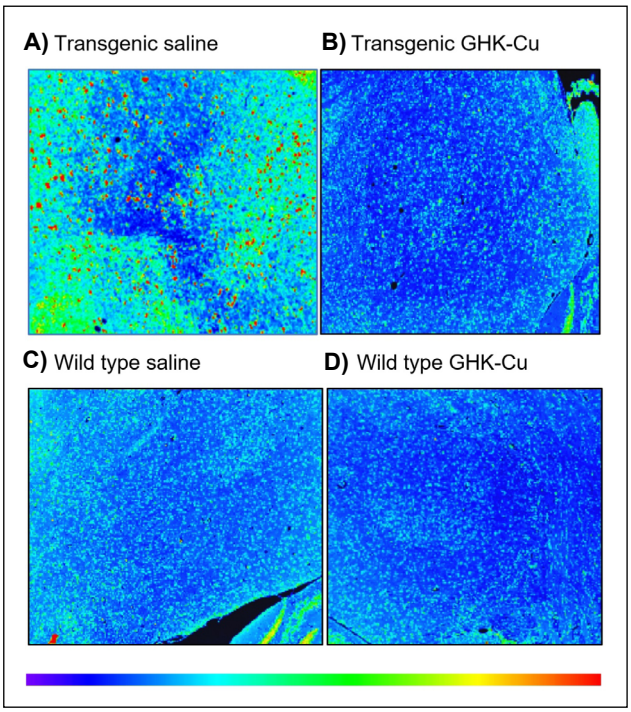

Figure 5. Q-Path generated heat map of an MCP-1 immunohistochemistry stain of frontal cortex from female mice after 12 weeks of treatment. (**A**) Transgenic mouse treated with intranasal saline. (**B**) Transgenic mouse treated with intranasal GHK-Cu. (**C**) Wild type mouse treated with intranasal saline. (**D**) Wild type mouse treated with intranasal GHK-Cu. The heat map legend indicates blue as low intensity staining all the way up to red as high intensity staining. Photos taken at ×40 magnification. Transgenic = 5xFAD; wild type = nontransgenic (age, strain, and sex matched controls).

neuroinflammation, and provide the rationale for further studies in aging mice, for example using an AAV vector containing sequences of Aβ42 and mutant tau [48].

Declarations

Availability of data and materials: Not applicable.

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Conflicts of interest: Warren Ladiges and Martin Darvas are members of the editorial board of *Aging Pathobiology and Therapeutics*. The authors declare that they have no conflicts and were not involved in the journal's review or decision regarding this manuscript.

Ethical approval and informed consent: Not applicable.

Consent for publication: Not applicable.

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