PREPRINT

# GHK peptide prevents sleep deprived learning impairment in mice

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**Abstract**
Sleep deprivation is known to cause memory impairment and is associated with inflammation and cell damage linked to neurodegenerative diseases. GHK (glycyl-L-histidyl-L-lysine) is a naturally occurring tripeptide found in mammalian plasma. GHK has anti-inflammatory activity and can pass through the blood brain barrier suggesting the potential to prevent neuroinflammation associated with sleep deprivation. In this study, mice were injected with 15mg/kg GHK per day for five days and sleep deprived on the last two days of treatment. Sleep deprived mice treated with GHK did not show the acute learning impairment seen in sleep deprived mice treated with saline. GHK prevented an increase in MCP-1 and nitric oxide levels in the hippocampus of sleep deprived mice suggesting that anti-inflammatory and anti-reactive nitrogen species activity could be a therapeutic target for learning impairment associated with short term sleep deprivation.

**Key words.** Sleep deprivation, learning impairment, GHK, neuroinflammation, nitric oxide

## Introduction

Sleep deprivation is an increasing problem in modern society. Over the past 25 years, there has been a significant decrease in sleep duration among American adults. Sleep deprivation has become a norm of the American working world, and researchers are finding that a decrease in overall sleep can cause serious health issues. Both acute and chronic habitual sleep loss are related to negative impacts on mental health, cardiometabolic risk factors and pain (Conklin et al., 2019). Sleep deprivation is also known to cause cognitive impairment as well as inflammation and oxidative damage within systemic tissues which can contribute to the development of age-related disease, such as Alzheimer's and other neurodegenerative conditions. Insufficient sleep can also cause increased cytokine secretion provoking an inflammatory response (Periasamy et al., 2015). Inflammation is a non-specific immune response that directs components of the immune system to the site of injury and is often accompanied by reactive oxygen/nitrogen species productions.

GHK (glycyl-L-histidyl-L-lysine) is a human plasma copper-binding peptide which is known to possess wound healing, antioxidant, anti-inflammatory, and anti-aging conditions (Pickart et al., 2015). Because of its antioxidant and anti-inflammatory properties, GHK is a promising peptide for use in the treatment of anti-aging and neurodegenerative disease. This report describes preliminary observations on the ability of GHK to prevent adverse neuropathological effects of short-term sleep deprivation in mice.

## Methods

*Animals*

CB6F1 female mice were obtained from the National Institute on Aging aged rodent colony at 12 months of age. Mice were housed 3-4 per cage in an SPF facility at the University of Washington under a 12-hour light-dark cycle starting at 6 am. Room temperature was 25℃±4. Reverse osmosis water and irradiated food (Picolab Rodent Diet 20, 5053) were supplied. All studies were approved by IACUC.

*GHK treatment, sleep deprivation and learning paradigm procedures*

Mice were started on intraperitoneal injection of GHK 7.5 mg GHK/kg/day (n=8), or ip saline twice daily (n=12) for five days. On day 4 and 5 of treatment, the GHK group and 6 of the saline group were sleep deprived for 4 hours as described (Mukherjee et al., 2019). Six of the saline group were not sleep deprived. On the fifth day, following sleep deprivation, each group was put through a spatial navigation learning test called the Box maze as described (Darvas et al., 2020). Each mouse was put into a clear rectangular plastic box with 7 fake holes and 1 escape hole leading to a safe cage. The mouse was given 120 seconds to find the escape hole and tested continuously for 4 trials with escape time for each trial recorded. The first trial served as acclimatization and learning ability was quantified as the slope of the escape times in the last three trials.

*Immunohistochemistry (IHC) Analysis*

Mice were euthanized by $CO_2$ and fixed in formalin immediately after the Box maze test. Samples of the brain were dissected and paraffin sections (4 μm) were mounted on slides for IHC staining. Slides were stained using an Anti-Rabbit HRP-DAB Cell & Tissue Staining Kit by R&D Systems 2013. Each slide

was rehydrated and incubated in a citrate buffer at 95℃ for 30 minutes for antigen retrieval. The slides were then stained with MCP-1 and nitrotyrosine antibody to qualify the inflammation and the nitric oxide (NO) production in the tissues, respectively. Hippocampus sagittal sections were taken at 4x magnification. CA3 and dentate gyrus (DG) sections of hippocampus were taken at 20x magnifications for stain intensity analysis. Images were processed through the program ImageJ with IHC toolbox and IHC profile plugin (Varghese et al., 2014).

**Results and Discussion**

Mice treated with GHK showed little learning impairment after sleep deprivation in line with saline treated mice that were not sleep deprived, and significantly less than sleep deprived mice treated with saline (Figure 1). The learning ability in the Box maze was calculated as the slope of escape times of each mouse. More negative numbers indicated faster escape times and less learning impairment. The negative slope value of sleep deprived mice treated with GHK (SD+GHK) was similar to the negative slope value of non-sleep deprived mice treated with saline (Control). In contrast, sleep deprived mice treated with saline (SD) had a significant decrease in the learning curve slope indicating severe learning impairment in the absence of GHK.

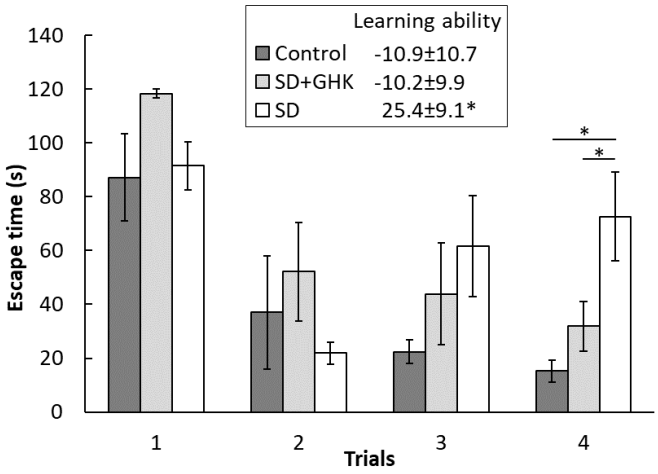


**Figure 1.** The GHK treatment prevented learning impairment caused by sleep deprivation. The SD+GHK mouse group had escape times and a learning curve slope similar to the non-SD+saline (control) group but significantly less than the sleep deprived saline treated mice (SD) *: $p<0.05$.

Inflammation and NO production in sleep deprived mice were measured by MCP-1 and nitrotyrosine staining and quantitated by ImageJ digital analysis. MCP-1 levels in sleep deprived mice treated with GHK were significantly lower in the CA3 region compared to sleep deprived mice treated with saline, indicating GHK's anti-inflammatory activity (Figure 2A). Notably, sleep deprived mice treated with GHK had lower inflammation compared to both sleep deprived and control mice in the DG region. Sleep deprivation significantly increased NO production in the hippocampus and GHK treatment successfully prevented such effect (Figure 2B). These results indicate that GHK can suppress inflammatory and nitric oxide changes induced by sleep deprivation, particularly in the DG region of the hippocampus suggesting that GHK might preferentially target the DG region.

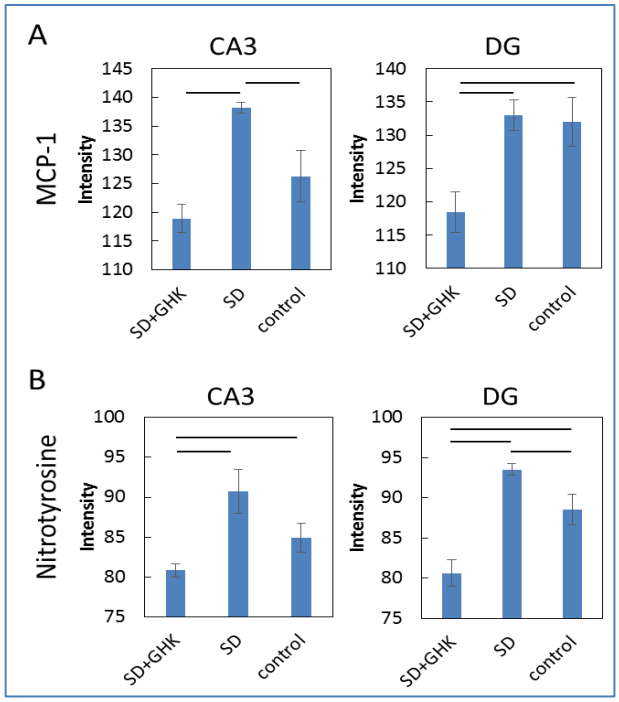


**Figure 2.** Immunohistochemistry images were quantified by staining intensity using ImageJ. Sleep deprived mice treated with GHK had **(A)** decreased MCP-1 and **(B)** and decreased nitrotyrosine staining intensity in the hippocampus. Results connected by horizontal line are significantly different (*p<0.05*).

The MCP-1 positive cells were concentrated in the DG and CA3 regions of the hippocampus (Figure 3A) whereas nitrotyrosine signaling was mainly absent in these areas in sleep deprived mice treated with GHK and non-sleep deprived mice (Figure 3B). GHK is known to work as an anti-inflammatory and antioxidant agent by decreasing inflammatory cytokines and reducing reactive oxygen species (ROS) levels (Dou et al., 2020). The data show that GHK reduced inflammation and NO production in the brain caused by sleep deprivation.

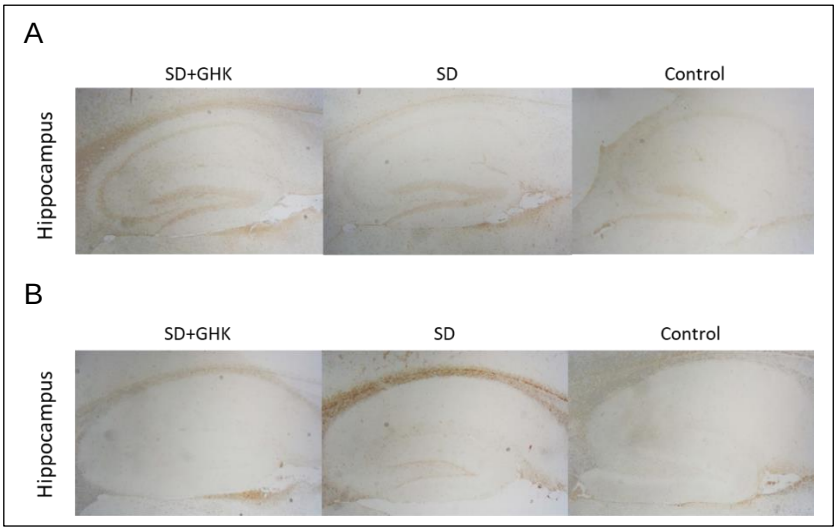


**Figure 3.** A. MCP-1 stain of hippocampus (4x) sections. B. Nitrotyrosine stain of hippocampus (4x) sections.

Sleep deprived mice treated with GHK had lower inflammation and NO levels in the hippocampus associated with prevention of learning impairment compared to control mice. Therefore, GHK could be a potential treatment for preventing the adverse neuropathological effects of sleep deprivation.

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