Investigating the Presence of Ketohexokinase in Neurons of Mammalian Brain Tissue

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Background

The brain's source of fuels is carbon-based molecules, with increasing evidence that glucose, once believed to be the only contributor, may not be the only metabolic form. However, little is known about other possible metabolic sources and their molecular role in feeding neurons. One of the possible fuels is fructose. Fructose is metabolized in two pathways: the fructose-1-phosphate pathway, which leads to the formation of pyruvate, and the fructose-6-phosphate, which leads to glucose-6-phosphate. It is not known which of these two pathways predominates. Previous studies showed that peripheral fructose injections in mice led to higher levels of glucose in the motor cortex. Other research indicates presence in the brain of a fructose-specific transporter GLUT-5. This project aims to detect the presence of ketohexokinase (KHK), an enzyme that catalyzes fructose-1-phosphate formation from fructose. Further, this research may be extended to uncovering possible effects of current high-fructose diets and diabetes on cognitive brain function.

Research Objective

To localize the presence of Ketohexokinase in neurons and further support the hypothesis that peripheral fructose is present and metabolized as a source of fuel in brain tissue.

Methods

Fixation

CD1 whole mouse brain tissue was perfused with a 4% paraformaldehyde, 0.2% picric acid in a sodium phosphate buffer solution (pH = 6.9). Tissue was further fixed in the same solution for one hour. Alcohol fixation (10% sucrose in sodium phosphate buffer) was done to avoid epitope masking by formaldehyde cross-linking.

Freezing

Brain was frozen in CO_2. Sagittal sections of 14 um were obtained using a cryostat. Tissue was transferred onto charged glass slides and stored in -80 °C.

Retrieval

Antigen retrieval was done on the sections due to previous results suggesting masking of epitopes. Sections were put in a 10mM sodium citrate buffer solution (pH 6.0) and subjected to heating cycles with a maximum temperature of 100 °C.

Primary Antibodies

Sections were immersed in 10mM PBS before immunohistochemistry. Rabbit anti-Ketohexokinase and guinea pig anti–Fox3 were applied.

Secondary Antibodies

Subsequent washing in 10mM PBS, 3 x 5 minutes was done. Alexa 488 rabbit anti-goat for Ketohexokinase and Alexa 594 donkey anti-guinea pig for Fox-3 were applied.

Amplification

Alexa 488 donkey, anti-goat for Ketohexokinase was applied.

Nuclear Staining

Slides were washed in 10mM PBS 3 x 5 minutes. Nucleus was stained with Hoescht 33342. A final 10mM PBS wash was done 3 x 5 minutes before anti-fade was applied to the glass slides. All antibodies were diluted in 0.3% PBS-T and Hoescht 33342 was diluted in 10mM PBS.

Results

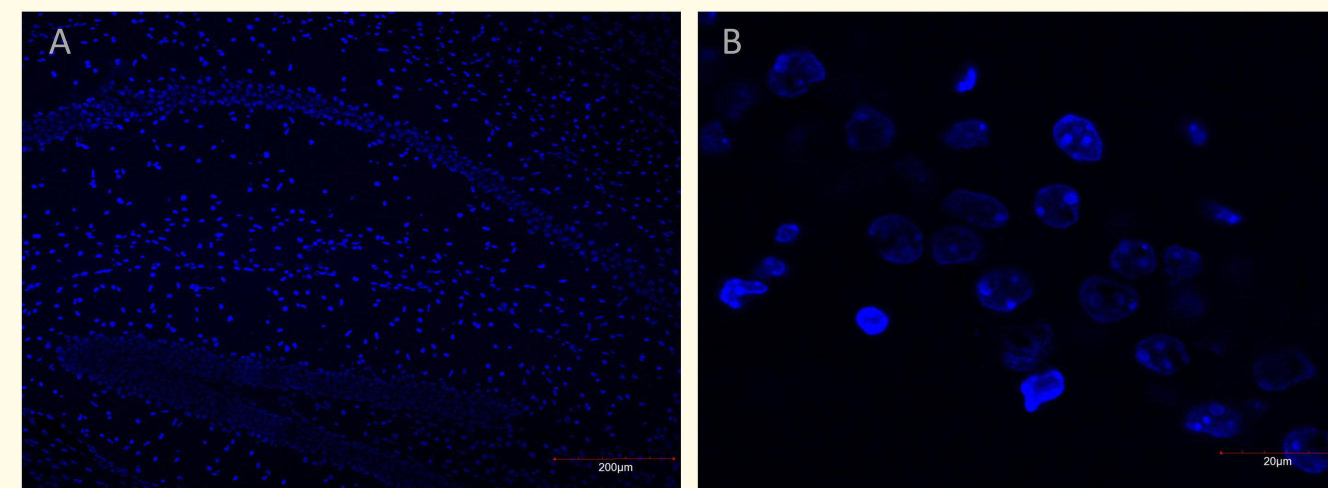


Figure 1. DNA staining with Hoechst fluorescent dye. A) Whole view of nuclei in hippocampus sagittal section (14µm) and **B)** nuclei of cell clusters in CD1 mouse brain tissue. H3570 Hoechst 33342 (Invitrogen) was diluted to 1 µg/ml in 10mM PBS.

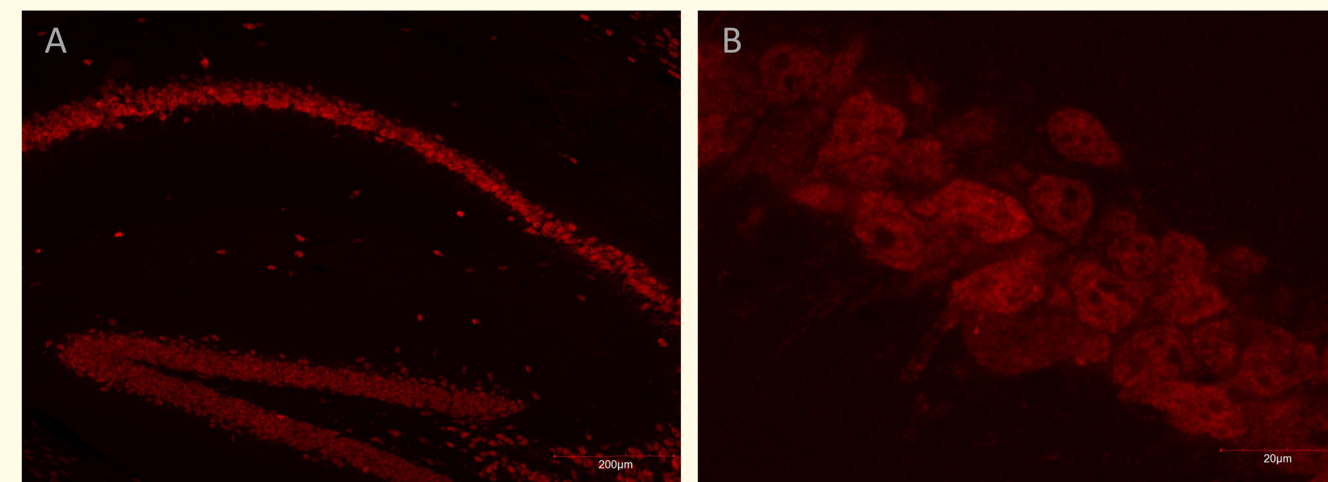


Figure 2. Fox-3 protein neuronal staining. A) Neuron-specific dye in hippocampus sagittal section (14µm) and **B)** neuron clusters in CD1 mouse brain tissue. Guinea pig anti –Fox-3 (1:1000, Millipore) and Alexa 594 donkey, anti-guinea pig (1:000, Invitrogen) were added.

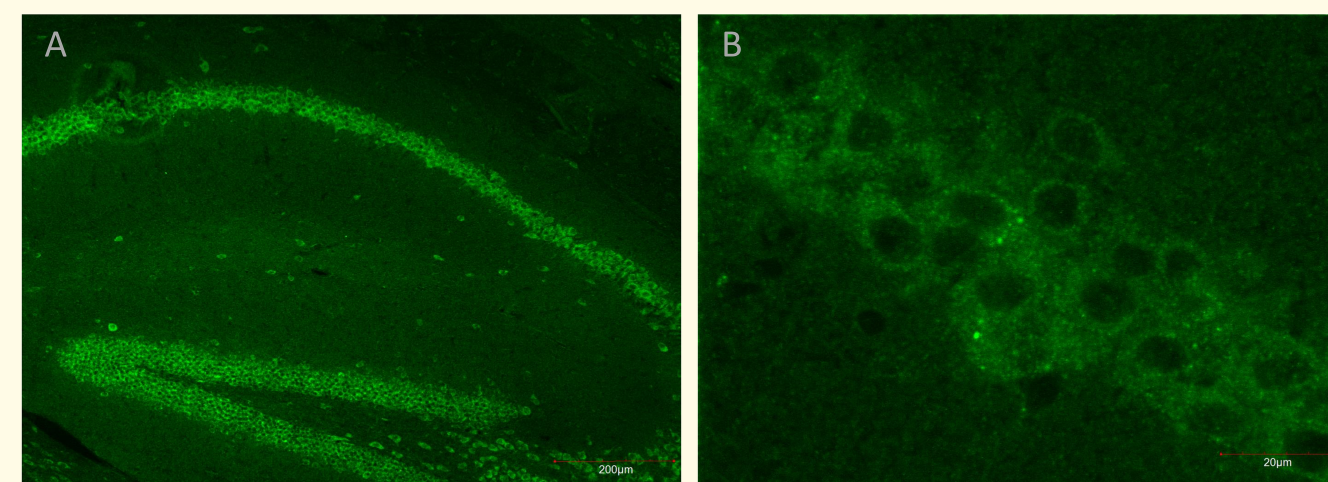


Figure 3. Ketohexokinase staining with amplification. A) Ketohexokinase-specific dye in hippocampus sagittal section (14µm) and **B)** clusters in CD1 mouse brain tissue. Rabbit anti-KHK (1:500, Abcam), Alexa 488 goat anti-rabbit (1:800, Invitrogen) and Alexa 488 donkey anti-goat (1:1000, Invitrogen) were added.

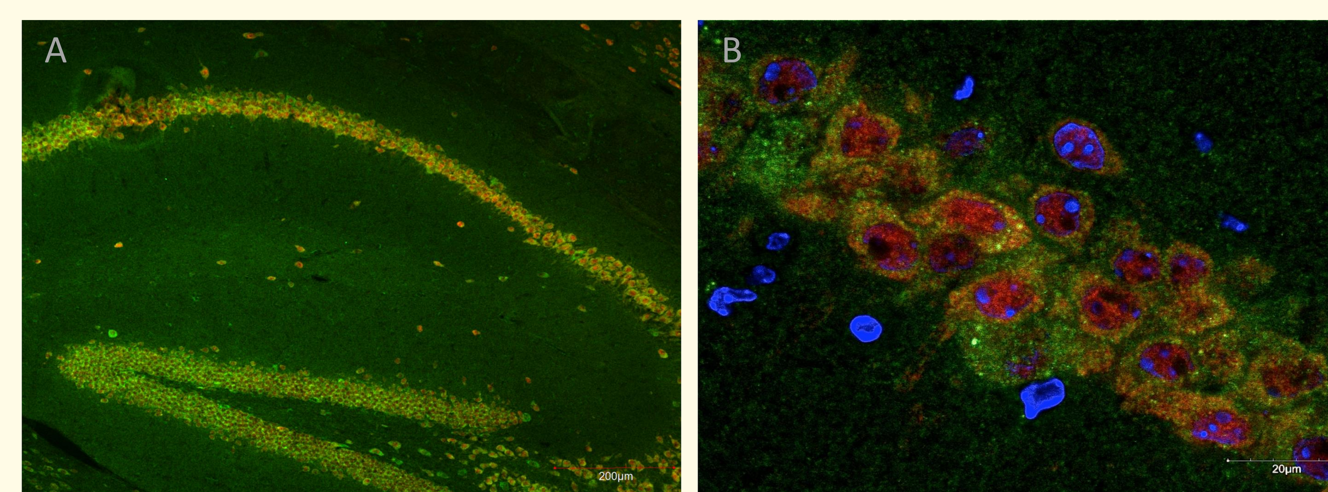


Figure 4. Ketohexokinase, Fox-3 and DNA staining. A) Hippocampus sagittal section (14µm) and **B)** clusters in CD1 mouse brain tissue distribution of Ketohexokinase in neurons. Rabbit anti-KHK (1:500, Abcam), Alexa 488 goat anti-rabbit (1:800, Invitrogen) and Alexa 488 donkey anti-goat (1:1000, Invitrogen) were used for KHK analysis. Guinea pig anti –Fox-3 (1:1000, Millipore) and Alexa 594 donkey, anti-guinea pig (1:000, Invitrogen) were used for neuron staining. H3570 Hoechst 33342 (Invitrogen) was diluted to 1 µg/ml in 10mM PBS.
Blue = DNA, Red = Neurons, Green = KHK.

Presence of KHK in Neurons

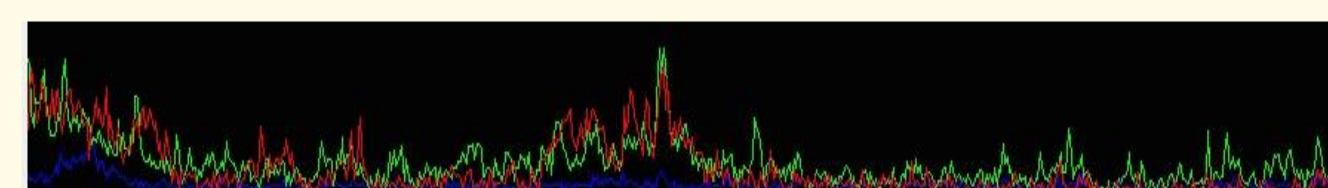


Figure 5. Analysis of the relative distributions of Ketohexokinase and NeuN. KHK = Green, NeuN = Red.

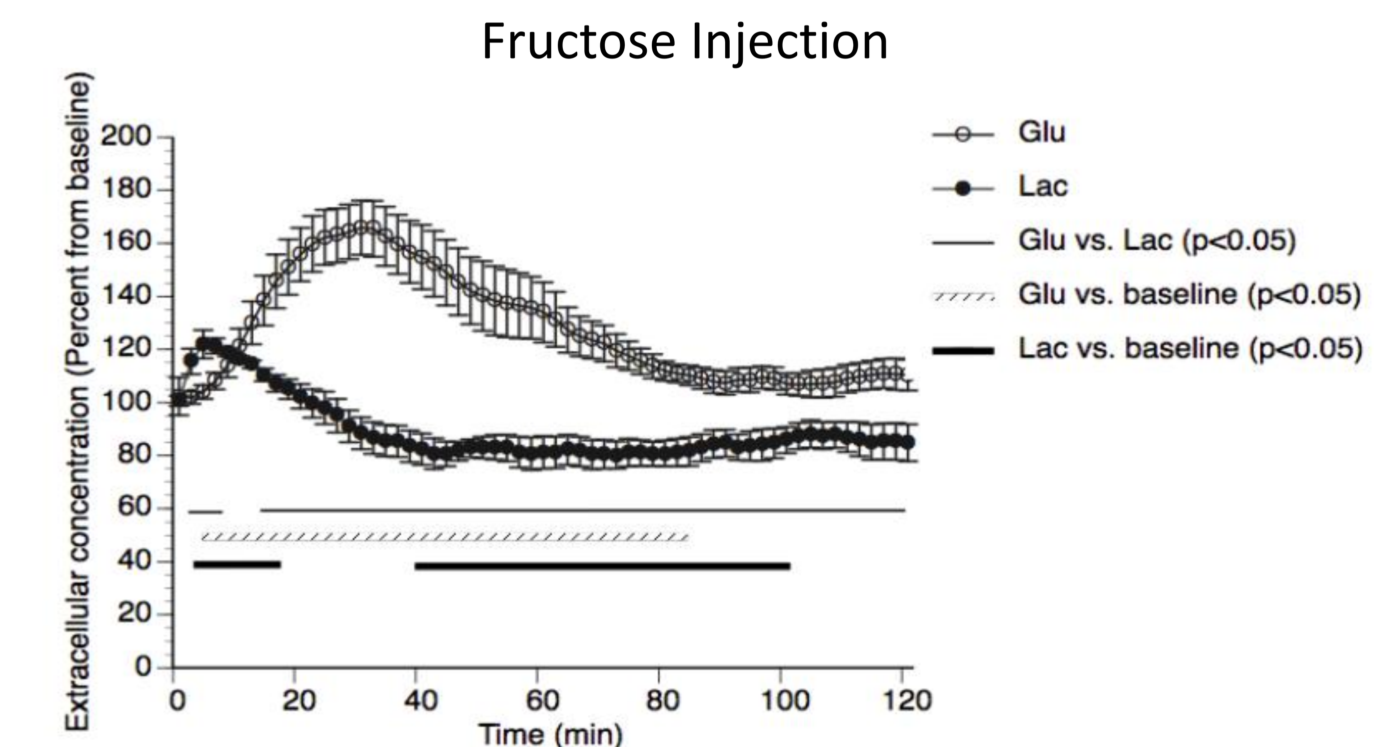


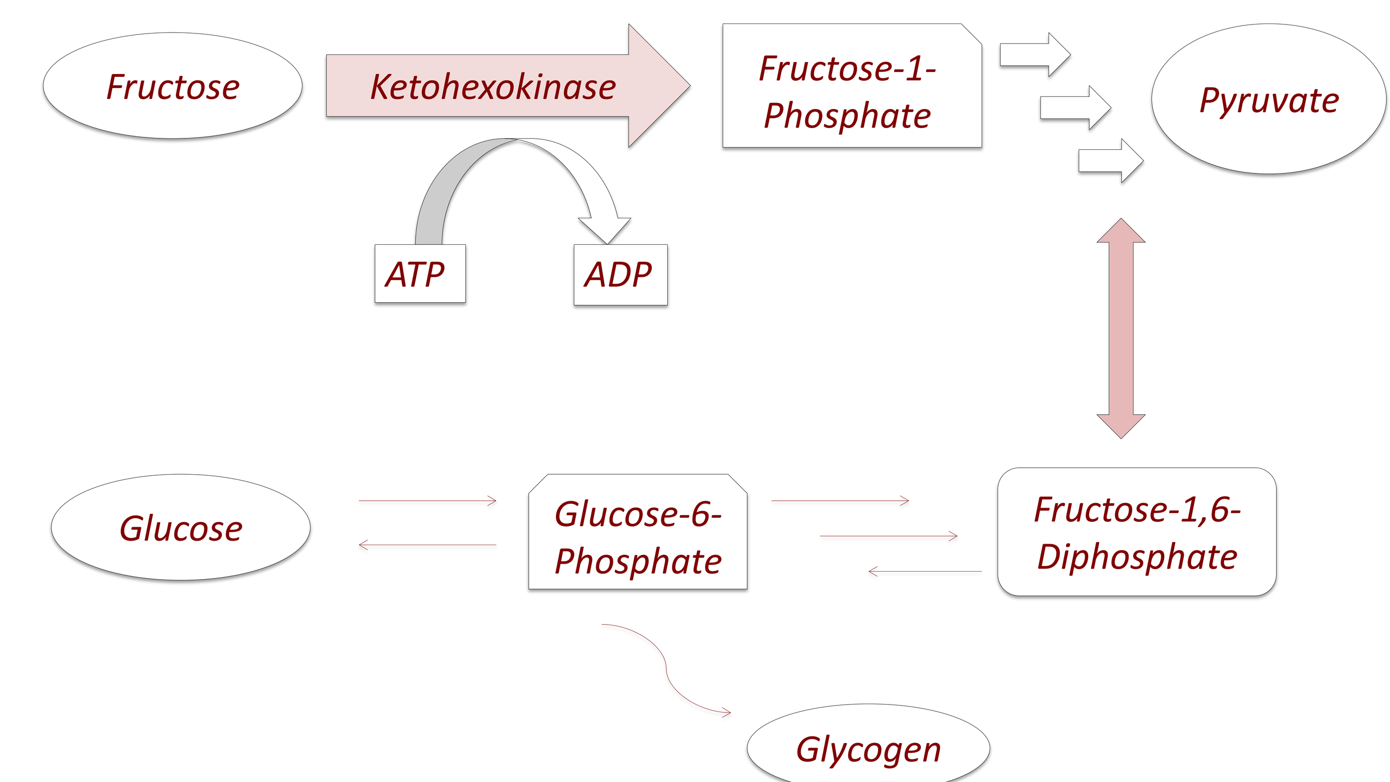
Figure 6. Motor cortex extracellular concentrations of glucose and lactate after a 2g/kg ip injection of fructose in awake mice (n=6). Error bars represent standard error of the mean. Taken from Justine Courtemanche, M.D. Candidate.

Conclusion

The expression of Ketohexokinase in the brain is confirmed through fluorescence in indirect immunohistochemistry. Secondary antibodies of Ketohexokinase are expressed conjointly with secondary antibodies of the Fox-3 protein/NeuN, a neuronal nuclear antigen which acts as a biomarker for neurons. Negative control slides without the primary antibodies show some fluorescence, but at significantly lower levels. Additionally, some background noise (green) is evident in secondary antibody staining of Ketohexokinase. Further analysis may be required. Since Ketohexokinase is an enzyme specific to the metabolism of fructose, its presence supports previous research suggesting that fructose is present in brain cells and plays a role in fueling the brain. The exact pathway of this metabolism is still unknown.

Future Applications

Although the presence of Ketohexokinase in the brain is an indicator of the use of Fructose as a fuel beyond the liver, it does not exclusively distinguish the metabolic path it takes. Radio-isotopic analysis may be applied to follow the exact pathways configured below. Better insight into the metabolism of sugars and their effect on the brain is important in today's increasingly high fructose diets.



Acknowledgements

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