Dysregulation of *SLC1A3* in AUD and alcohol-associated behaviors

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Introduction

Alcohol consumption induces persistent changes in glutamatergic signaling in both humans and rodents, including changes in glutamate reuptake and metabolism. The main glutamate transporter is SLC1A2, which is also one of the most abundant proteins in the forebrain. SLC1A2 has been well studied in addiction and has been shown to regulate addiction-like behaviors in rodents, also for alcohol. Other glutamate transporters have not received the same attention, and in our experiments *SLC1A3* is frequently found dysregulated following alcohol consumption; more so than *SLC1A2*.

Multiple lines of evidence from both humans and rodents suggest that *SLC1A3* dysregulation being specific to astrocytes, and, to a lesser extent, microglia:

- Following an every other day 2 bottle choice (EOD-2BC) drinking model, *Slc1a3* was found significantly downregulated in astrocyte-enriched cells but not in bulk tissue from mouse medial prefrontal cortex (mPFC) (Erickson et al 2019.a).
- Following chronic intermittent exposure (CIE) to alcohol vapor, *Slc1a3* was downregulated in both astrocyte-enriched cells as well as bulk tissue, but not in microglia-enriched cells from mouse mPFC (Erickson et al 2019.b).
- In another CIE experiment, *Slc1a3* downregulation was observed 24h after last exposure to EtOH vapor, but normalized after 72h (Farris et al 2020).
- In a single nucleus transcriptome profiling of dorsolateral PFC from alcohol-dependent humans, *SLC1A3* was significantly upregulated in several cell types, including astrocytes and microglia (Brenner et al 2020).
- We recently identified an astrocyte-specific co-expression module that is upregulated in mouse mPFC following CIE (Salem *et al.,* 2024)

Aims

Here, we aimed to specifically target *Slc1a3* in the mPFC and to functionally probe *Slc1a3* in alcohol-related behaviors in mice.

We further aimed to identify genes that co-express with *Slc1a3*, and that might act in concert with *Slc1a3* to regulate behaviors.

Lastly, we aimed to investigate the consequences of *Slc1a3* KD on gene expression.

Methods

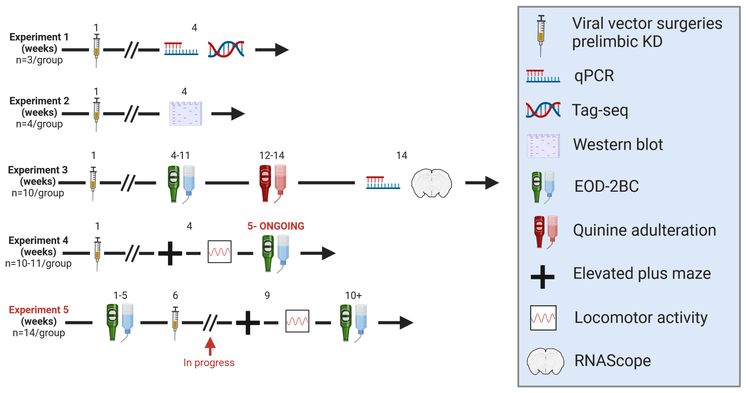

Figure 1. Three experiments have been completed on C57BL/6J mice, and two more are in progress.

VIRUS DESIGN AND VALIDATION

We utilized a single nuclei RNAseq dataset from the mPFC of male C57BL/6J to identify expression patterns of *Slc1a3* along with possible astrocyte specific cell markers to use as promoters (data not shown). For validation, C57BL/6J mice were infused with the knock-down (KD) virus or a scrambled control (SCR) into the dmPFC and were given 3 weeks for the virus to express before the tissue was collected. Gene expression was then assessed with qPCR (experiment 1), and protein expression levels were assessed with Western Blot (experiment 2).

ROLE OF *Slc1a3* IN VOLUNTARY ALCOHOL CONSUMPTION

Male C57BL/6J were injected with anti-*Slc1a3* miRNA or SCR into the dmPFC. Following 3 weeks of recovery and viral expression (experiment 3), they were tested for locomotion and anxiety-like behavior (experiment 4) and subjected to a 15% EtOH EOD-2BC drinking model.

ROLE OF *Slc1a3* IN COMPULSIVE-LIKE BEHAVIOR

Following the EOD-2BC model in experiment 3, the same animals were tested for compulsive-like behavior in a model of quinine adulteration. Mice were given a 2-bottle choice (15% EtOH or water), with increasing concentrations of quinine added to the EtOH. Each concentration was given for 2 days and is presented as an average.

ROLE OF *Slc1a3* IN ANXIETY-LIKE BEHAVIOR

In experiment 4, locomotion and anxiety-like behavior were assessed prior to the EOD-2BC drinking model. Anxiety-like behavior was assessed with an elevated plus maze (EPM) for 5 min. Locomotion was assessed for 15 min in an open field arena, and % time spent in the center was extracted from this as a secondary measurement of anxiety-like behavior.

CO-EXPRESSION ANALYSIS OF *SLC1A3* IN HUMAN AUD CASES AND A MOUSE CIE EXPERIMENT

We constructed gene co-expression modules in each cell type using high dimensional weighted gene co-expression network analysis on single nuclei RNAseq dataset from 73 AUD cases and controls (n=36-37/group), and 14 CIE exposed mice (n=6-8/group).

Tag-seq

Sequencing libraries were constructed from dmPFC (prelimbic cortex) RNA using a 3' Tag-based approach (Tag-seq). Libraries were sequenced at The Genomic Sequencing and Analysis Facility at UT Austin.

Selective KD of *Slc1a3* to probe function in mouse dmPFC

Viral vector design and validation

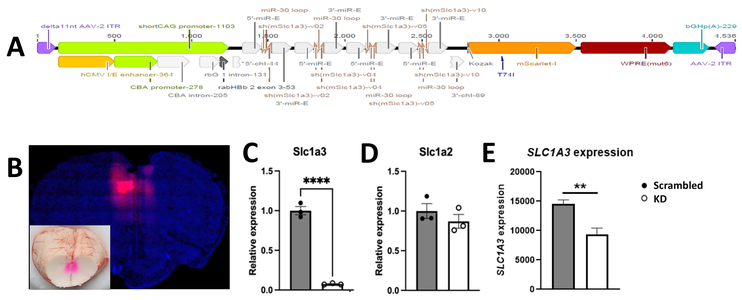

Figure 2. Schematic showing the viral construct designed. The vector contains 4 different miRNAs targeting different regions of the Slc1a3 gene. The vector was constructed and packed into an AAV5 by Zürich viral vector Facility, Switzerland (**A**). Viral vector infusion into the dmPFC (**B**) resulted in a robust KD of *Slc1a3/SLC1A3* (**C**/**E**) without affecting the expression of *Slc1a2* (**D**).

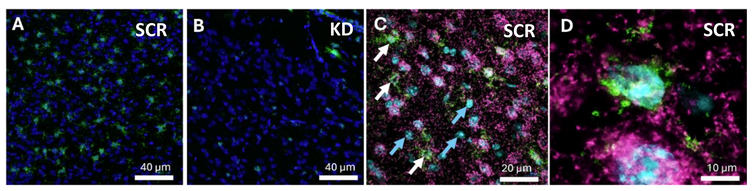

Figure 3. Representative micrographs of in-situ hybridization (RNAScope©) for *Slc1a3* (**green**) and *Slc1a2* (**pink**). *Slc1a3* expression in the dmPFC is reduced following viral vector infusion containing miRNA against the transcript compared to SCR (SCR: **A**; KD: **B**). Co-localization of *Slc1a3* with *Slc1a2* in a SCR shows their distribution in cells of the dmPFC and the lower abundance of *Slc1a3* compared to *Slc1a2* (**C**). **Blue arrows** point to cells not expressing either of the transcripts, and **white arrows** point to examples of cells only expressing *Slc1a3*. **D**) Super resolution image (100x) highlights the distribution of *Slc1a3* and *Slc1a2* in cells of the dmPFC, and in particular their abundance outside of the nucleus. **Blue**: DAPI.

Slc1a3 KD in the dmPFC resulted in a small but stable increase in alcohol consumption in mice

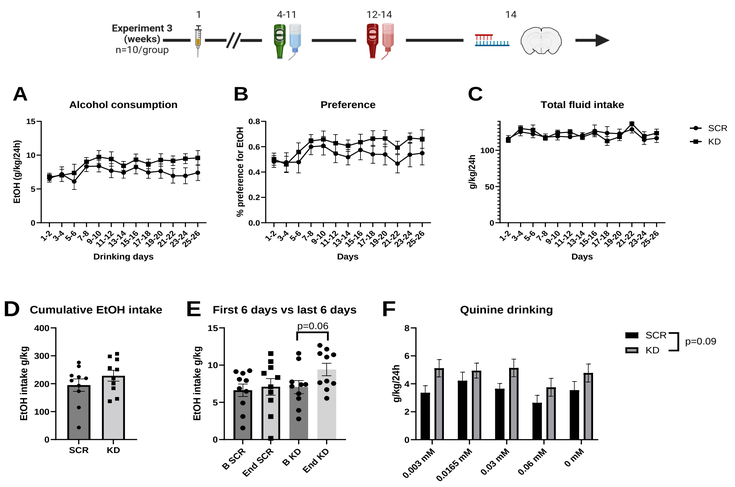

Figure 4. KD of *Slc1a3* in the dmPFC promotes a small, but not statistically significant, increase in voluntary ethanol intake (A) and preference for alcohol over water (B), with no observed effect of total fluid intake (C), compared to SCR. D) cumulative EtOH intake and E) the first and last 6 drinking sessions were compared. *Slc1a3* KD animals did not show any differences in compulsive-like behavior when tested for quinine adulterated EtOH (F). The trend for increased EtOH intake, however, remained in the presence of quinine.

Expression levels of *Slc1a3* inversely correlated with cumulative voluntary EtOH intake

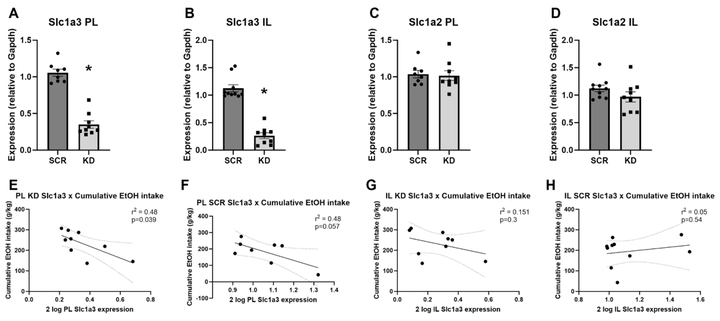

Figure 5. Viral vector-mediated KD of *Slc1a3* showed a robust downregulation of the transcript in the dmPFC, both the prelimbic- (PL)(**A**) and infralimbic cortex (IL)(**B**), without affecting levels of *Slc1a2* (**C**-**D**). We analyzed the PL and IL separately as they are known to sometimes have opposing roles in regulating behaviors. Expression levels of *Slc1a3* inversely correlated with cumulative voluntary EtOH intake in the PL (KD: **E**; SCR: **F**) but not the IL (KD: **G**; SCR: **H**).

In a separate experiment, KD of *Slc1a3* in the dmPFC showed no effect on alcohol consumption or anxiety-like behavior

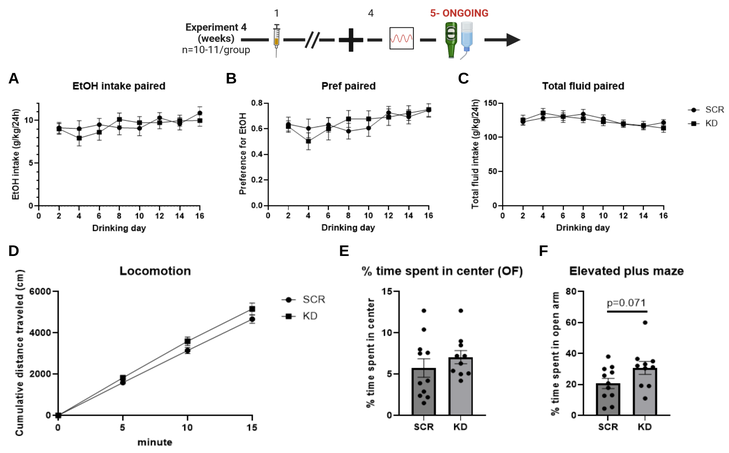

Figure 5. In a separate experiment, no effect was observed on voluntary EtOH consumption (A), preference for alcohol over water (B) or total fluid intake (C) following *Slc1a3* KD, compared to SCR. No effect was observed on locomotion (D) or anxiety-like behavior (E-F), however a trend for decreased anxiety-like behavior was observed in the EPM (F).

SLC1A3 is a high-ranking hub gene in a cross-species, astrocyte specific co-expression module

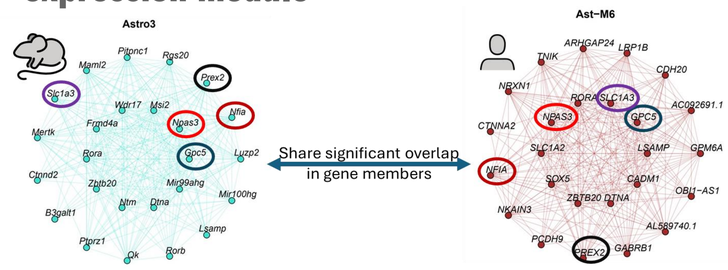

Figure 6. In parallel with the investigation into the role of *Slc1a3* in alcohol-associated behaviors, we identified a cross-species, astrocyte-specific co-expression module in single nucleus RNAseq datasets, in which *Slc1a3* was a high-ranking hub gene. Network plots showing top connected gene members of the astrocyte-specific gene module identified in a mouse CIE experiment (left) and human AUD cases (right).

KD of *Slc1a3* does not affect other genes in the astrocyte co-expression module

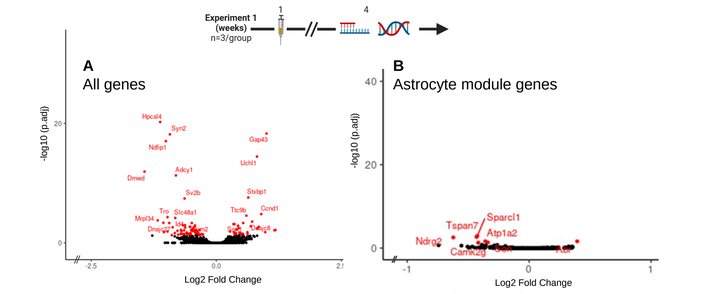

Figure 7. We identified 98 differentially expressed genes after *Slc1a3* KD in the dmPFC (adj. p-val <0.05; **A**). However, few genes from the identified astrocyte-specific co-expression module were changed following *Slc1a3*-KD (adj. p-val <0.05; **B**)

Conclusions

- ***Slc1a3* is dysregulated following alcohol consumption in both humans and mice, more so than *Slc1a2* in our data**
- **We selectively targeted *Slc1a3* in the dmPFC without affecting the expression of *Slc1a2***
- **Single gene KD of *Slc1a3* does not appear to change alcohol-related behaviors or expression of other astrocyte-module members**
- **It is possible that *Slc1a3* has a more direct role in regulating behaviors in other drinking models and brain regions**
- **Gene co-expression analysis and Tag-seq suggest that *Slc1a3* may act in concert with other module genes to regulate behavior**

Ongoing and future experiments

- One outstanding question is whether a prior exposure to alcohol is required for *Slc1a3*-KD in the dmPFC to affect behaviors. We aim to address this question with experiment 5
- Map viral spread and correlate transcript levels with EtOH consumption
- We aim to target multiple transcripts identified in the astrocyte module simultaneously using Perturb-seq to address the possibility of multiple module genes acting in concert to modulate alcohol drinking behaviors

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