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Exploration of the Neuronal Subtype Specificity of an Ethanol Responsive Gene: Glycogen Synthase Kinase 3 Beta (Gsk3b)

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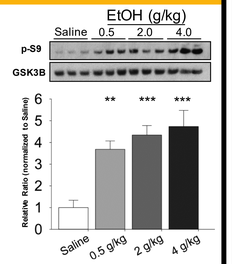
Introduction



Our laboratory has previously revealed that Glycogen Synthase Kinase 3 Beta (Gsk3b) functions as a hub gene in a network of genes regulated by acute ethanol in the medial prefrontal cortex (mPFC) across a mouse genetic panel (Wolen et al, 2012). GSK3B is a serine/threonine kinase that has been shown to regulate dendritic activity and synaptic plasticity (Nelson et al, 2013). It is also an ethanol responsive gene, where adult mice treated with acute ethanol showed increased phosphorylation of GSK3B on the Ser9 residue in prefrontal cortex (**Right** - van der Vaart et al, 2018).

It also modulates ethanol consumption, showing an increase as a result of viral-mediated overexpression of Gsk3b in mouse mPFC, and a decrease with pharmacological inhibition of GSK3B (van der Vaart et al, 2018). However, it is unknown what neuron subtypes are driving this change in behavior. In this study, we provide evidence that deletion of Gsk3b in Camk2a+ glutamatergic neurons of mouse mPFC decreases voluntary ethanol consumption. Furthermore, we constructed a plasmid for Cre-dependent overexpression of Gsk3b, along with a Cre-dependent control plasmid. These are planned for use in conjunction with different Cre drivers for viral-mediated expression in any specified neuron type. Dissection of the neural circuitry of this pathway can lead to a better assessment of Gsk3b as a potential target for the treatment of alcohol use disorders.

Materials and Methods

Tamoxifen-inducible Gsk3b knockout mice (Camk2a-Cre-ERT) Mice on a C57BL/6J background were bred from a cross of Gsk3b fl/fl X Camk2a-Cre-ER (Cre+ or Cre-). Mice received IP injections of Tamoxifen (75mg) across 5 days to induce Cre expression and Gsk3b deletion. Three weeks were given for recovery and ample GSK3B turnover.

Drinking Paradigm Mice were studied on a two-bottle choice model between water and 15% w/v ethanol to measure voluntary ethanol consumption. A combined daily-access (days 1-15) and intermittent access (5 weeks, M-W-F, access days 15-30) drinking model was implemented.

Molecular Cloning The backbone of a pAAV-hSyn-dF-HA-KORD-IRES-mCitrine vector (Addgene, Plasmid #65417) excluding the KORD open reading frame was isolated by Phusion high fidelity PCR (New England Biolabs). The Gsk3b ORF was isolated from C57BL/6J cDNA from prefrontal cortex. These fragments were combined using Gibson Assembly (NEB) with the Gsk3b ORF in reverse orientation to generate a Double-floxed inverted Orientation (DiO) Gsk3b plasmid. The same backbone was amplified with high fidelity PCR, excluding the HA-Gsk3b-IRES region, and recircularized using the blunt-end Quick Ligation Kit (NEB), to generate a DiO mCitrine reporter plasmid.

Sequencing Plasmid constructs were sequenced using Illumina MiSeq v2 Nano at the VCU Genomics Core. Sequencing results were aligned to the reference sequences using Burrows-Wheeler Aligner. Sequencing coverage and identity was displayed through the Integrated Genomics Viewer.

Immunocytochemistry HEK293FT cells were plated and transfected with Lipofectamine 3000 with either the DiO-Gsk3b plasmid, a Cre-GFP plasmid, or both plasmids together. Two days post-transfection, cells were fixed with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes, then blocked with 5% normal goat serum (Cell Signaling Technologies) containing 0.2% Triton X-100 for 30 minutes. Primary antibody incubation for the HA-tag (1:200, Abcam, ab18181) followed by secondary antibody incubation with Alexa Fluor 594 anti-mouse (1:500, A11005) were each performed in 1 hour incubations at room temperature with 3% NGS and 0.2% Triton X-100 .

Gsk3b Deletion in Camk2a+ Neurons

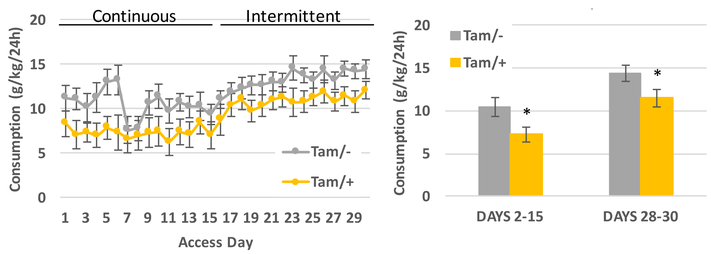


Figure 1: Forebrain deletion of Gsk3b in Camk2a+ neurons decreases ethanol consumption in mice
Gsk3b deletion in Camk2a+ neurons resulted in a consistent decrease in daily consumption of ethanol on a combined continuous and intermittent access paradigm (**Left**). Average ethanol consumption (**Right**) with constant access (days 2-15) and in the last week of intermittent access (days 28-30) were significantly lower in the Cre+ animals (*p<0.05).

Double-floxed inverted Orientation (DiO) Constructs

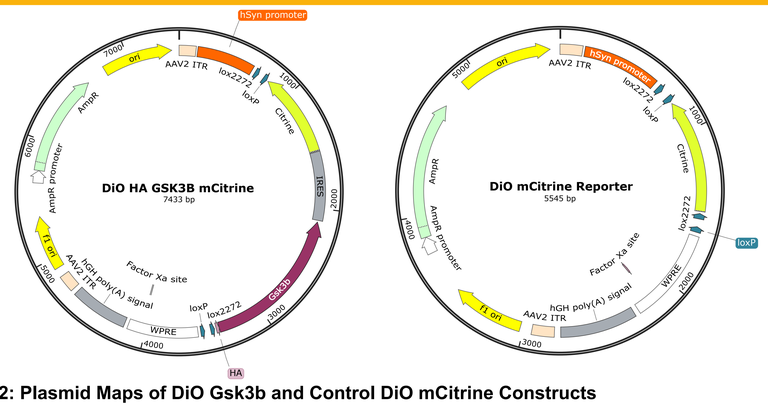


Figure 2: Plasmid Maps of DiO Gsk3b and Control DiO mCitrine Constructs
Expression vectors are driven by an hSyn promoter and contain both an expression enhancing post translational response element (WPRE) and a poly A signal. Within the double-floxed region in DiO Gsk3b (**Left**) contains an HA-fused Gsk3b ORF and an mCitrine reporter. The control DiO construct (**Right**) contains the same vector but with only the reporter gene inside the double-floxed region. In the presence of Cre recombinase, the double-floxed region will invert its orientation, allowing for expression of the DiO cassette.

Validation of Construct Sequences

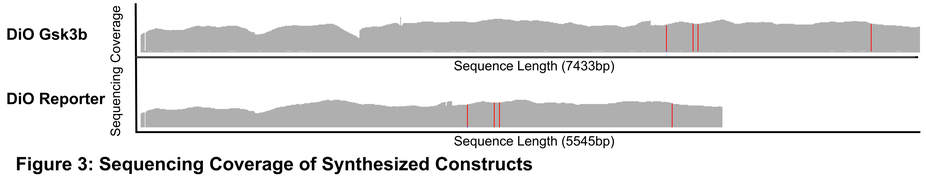


Figure 3: Sequencing Coverage of Synthesized Constructs
MiSeq sequencing reads for DiO Gsk3b (**Top**) and DiO Reporter (**Bottom**) plasmids were aligned against their reference sequences. Gray shows perfect matches while red indicates mismatched nucleotides.

Validation of DiO Gsk3b Protein Expression

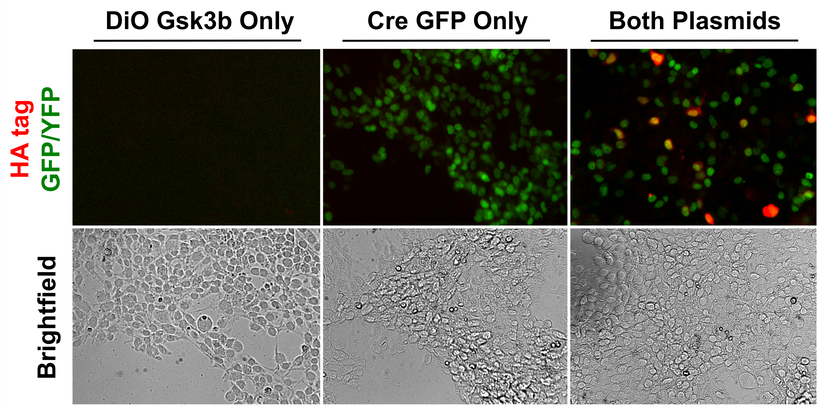


Figure 4: Immunocytochemistry for HA-GSK3B
HEK 293FT Cells were transfected with DiO Gsk3b (**Left**), Cre GFP (**Middle**), or both plasmids (**Right**). Red indicates staining for hemagluttanin (HA), while green shows fluorescence from GFP and YFP. Brightfield microscopy is also shown for the position of all plated cells.

Summary and Future Directions

- Deletion of *Gsk3b* in the forebrain Camk2a+ neurons of B6 mice decreased ethanol intake
- We synthesized an overexpression plasmid for Cre-dependent expression of *Gsk3b* in neurons, and a control plasmid for Cre-dependent expression of a reporter gene, mCitrine
- HA-tag was present only when cells were co-transfected with DiO Gsk3b and Cre GFP, indicating a Cre-dependent expression of HA-GSK3B

Additional Validation

- DiO mCitrine plasmid will need to be validated for Cre-dependent synthesis of YFP
- A western blot staining for HA can be done to validate the molecular weight of the synthesized HA-fused protein, to further confirm the overexpression of GSK3B

Future Directions

- Package the plasmid constructs into viral vectors for stereotaxic injections in Camk2a-Cre mouse PFC to induce overexpression of GSK3B in the forebrain neurons in the PFC
- Use the viral vectors with mouse models of different Cre-drivers to induce *Gsk3b* overexpression in other neuron types
- Study the behavioral and molecular response to ethanol in these mouse models to dissect the role of *Gsk3b* in different neuron types

References

Nelson CD, Kim MJ, Hsin H, Chen Y, Sheng M (2013) Phosphorylation of threonine-19 of PSD-95 by GSK-3β is required for PSD-95 mobilization and long-term depression. J Neurosci 33:12122–12135

van der Vaart A, Meng X, Bowers MS, Batman AM, Aliev F... Miles MF (2018) Glycogen synthase kinase 3 beta regulates ethanol consumption and is a risk factor for alcohol dependence. Neuropsychopharmacology 43:2521–2531

Wolen AR, Phillips CA, Langston MA, Putman AH, Vorster PJ... Miles MF (2012) Genetic dissection of acute ethanol responsive gene networks in prefrontal cortex: functional and mechanistic implications. PLoS One 7:e33575

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