Investigating the mechanistic and temporal regulation of inhibitory synapse elimination during cerebral ischemia

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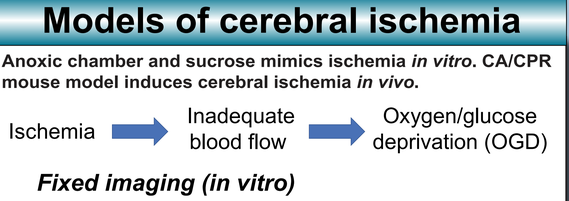
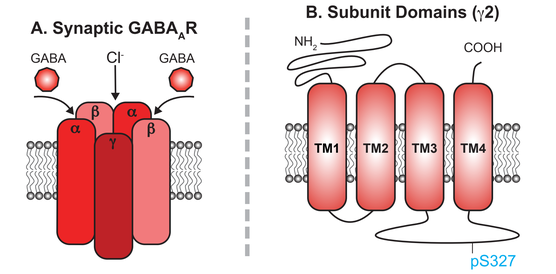
Introduction

Inhibitory synapses are crucial for maintaining correct neuronal excitability, which is important for efficient circuitry and proper brain function. Inhibitory $GABA_A$ receptors (**$GABA_A$Rs**) mediate the majority of fast synaptic inhibition in the brain. Thus, the number of postsynaptic $GABA_A$Rs influences inhibitory strength. Shifts in neuronal excitability have been implicated in a variety of neurological disorders, including ischemia. The oxygen and glucose deprivation (**OGD**) observed during ischemic insult in hippocampal regions leads to synaptic depression through $GABA_A$R and gephyrin loss from synaptic sites. *However, mechanisms that regulate $GABA_A$R declustering and gephyrin elimination following an ischemic insult remain undefined.* In this project, I propose that $GABA_A$R declustering is mediated by calcineurin activity and this is the first step in facilitating synapse elimination. Furthermore, I speculate a role of the cystine protease, ***Calpain***, in mediating gephyrin loss during OGD. Based on this, I plan to investigate (i) mechanisms of synaptic $GABA_A$R declustering and gephyrin elimination in hippocampal pyramidal neurons following OGD (ii) probe the temporal regulation to determine the sequential flow of events promoting $GABA_A$R and gephyrin loss and (iii) use an *in vivo* model of cerebral ischemia to compare cell-type specific mechanisms in the CA1 hippocampus.



Synaptic $GABA_A$Rs mediate inhibitory transmission

Subunit composition and PTMs determines both $GABA_A$R localization and function.



Synaptic $GABA_A$Rs mediate phasic inhibition in the adult CNS. $GABA_A$Rs are heteropentameric structures, with each subunit containing 4 transmembrane domains and an intracellular loop. The intracellular loop is where majority of post translational modifications of the receptor occurs.

Models of cerebral ischemia

Anoxic chamber and sucrose mimics ischemia *in vitro*. CA/CPR mouse model induces cerebral ischemia *in vivo*.

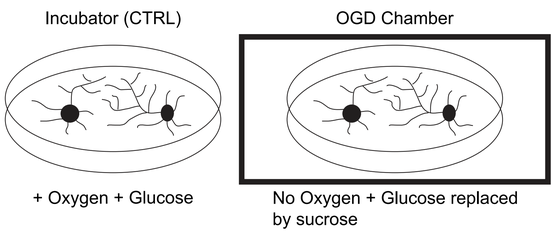
Ischemia → Inadequate blood flow → Oxygen/glucose deprivation (OGD)

Fixed imaging (in vitro)

DIV 16-18: OGD 30 min → PFA Fixation → ICC: Surface γ2 Total geph/vGat → Image

Biochemistry (in vitro)

DIV 16-18: OGD Timecourse → Collection: 1% triton → Solubilize: 30 minutes → Western Blot Analysis

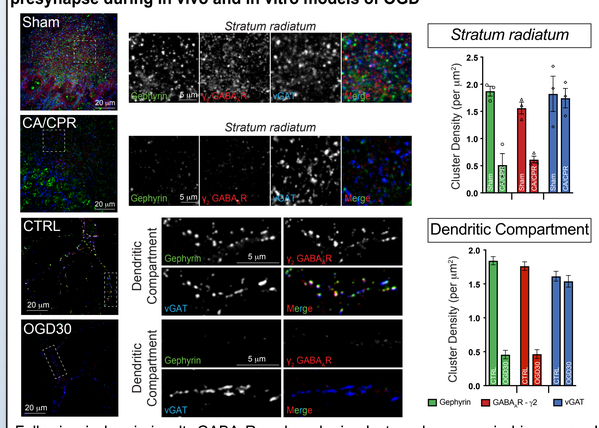


CA/CPR mouse model (in vivo)

Cardiac Arrest: 6 min (KCl) → Resuscitation (2 min) → PFA Fixation (Perfusion) → Staining/Image (30 micron slices)

In vivo and *in vitro* OGD models alter inhibitory synapse morphology

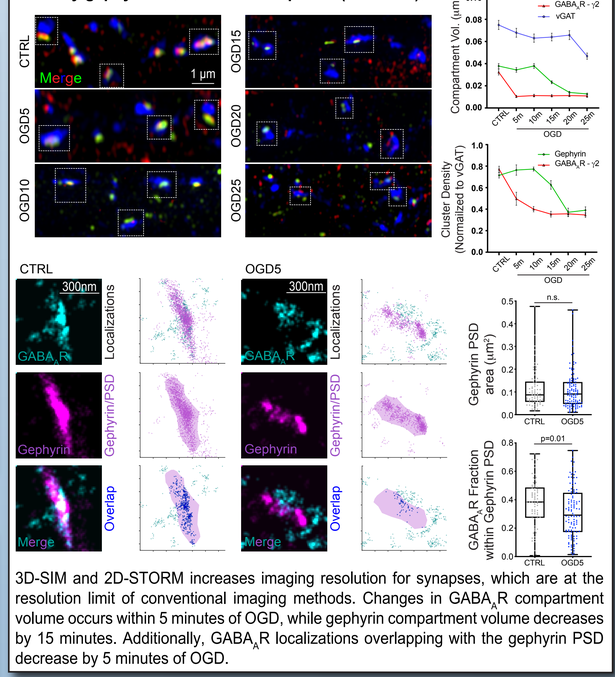
Postsynaptic components of the inhibitory synapse are removed before the presynapse during in vivo and in vitro models of OGD



Following ischemic insult, $GABA_A$R and gephyrin clusters decreases in hippocampal pyramidal neurons. This decrease is not observed for vGAT suggesting that an OGD insult initially targets postsynaptic components of the inhibitory synapse.

Super resolution imaging reaveals nanoscale alterations to postsynapse

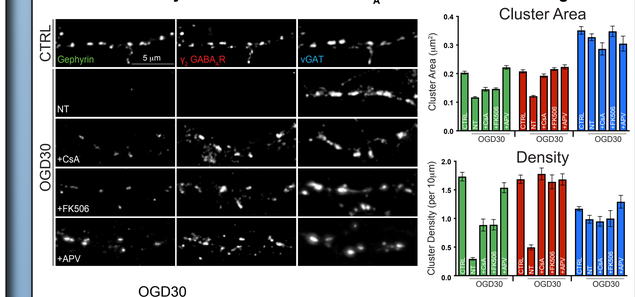
SIM and STORM imaging show initial loss (5min) of $GABA_A$Rs, which is followed by gephyrin loss at later time points (10-15min).



3D-SIM and 2D-STORM increases imaging resolution for synapses, which are at the resolution limit of conventional imaging methods. Changes in $GABA_A$R compartment volume occurs within 5 minutes of OGD, while gephyrin compartment volume decreases by 15 minutes. Additionally, $GABA_A$R localizations overlapping with the gephyrin PSD decrease by 5 minutes of OGD.

Calcineurin activation promotes $GABA_A$R loss during OGD

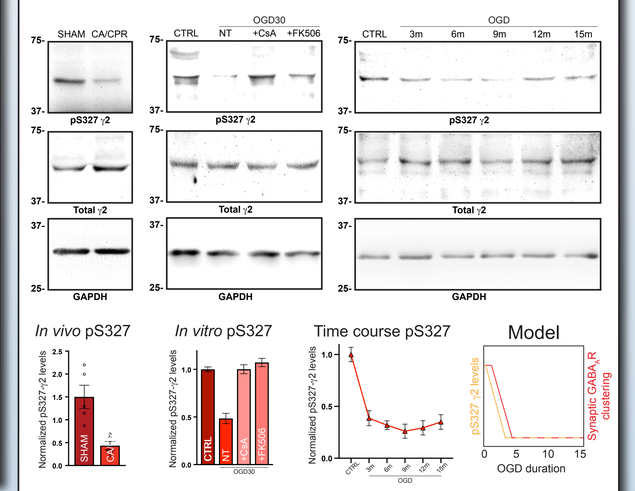
Calcineurin activity facilitates surface $GABA_A$R cluster removal during OGD.



Inhibiting calcineurin (CaN) activity using Cyclosporin A (CsA, 5 μm) or FK506 (5 μm) restores cluster area for $GABA_A$Rs fully and gephyrin partially. $GABA_A$R surface accumulation is also restored in the presence of CsA inhibitors. This suggests that CaN activation promotes $GABA_A$R and gephyrin downregulation is independent of CaN activity.

OGD onset promotes CaN-mediated dephosphorylation of $GABA_A$Rs (S327)

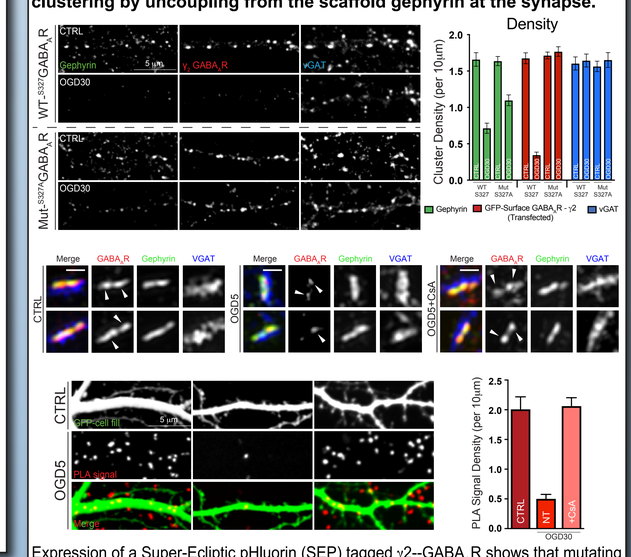
Serine 327 (S327) dephosphorylation on the γ2 subunit of $GABA_A$Rs occurs immediately following OGD via CaN activation.



Synaptic declustering of $GABA_A$Rs from postsynaptic sites is mediated through CaN-dependent dephosphorylation of serine 327 (S327) on the γ2 subunit during inhibitory synaptic depression. This site is dephosphorylated both *in vivo* and *in vitro* following OGD. S327 dephosphorylation is facilitated by CaN during OGD and occurs immediately following an insult.

Blocking dephosphorylation maintains $GABA_A$R clustering with gephyrin

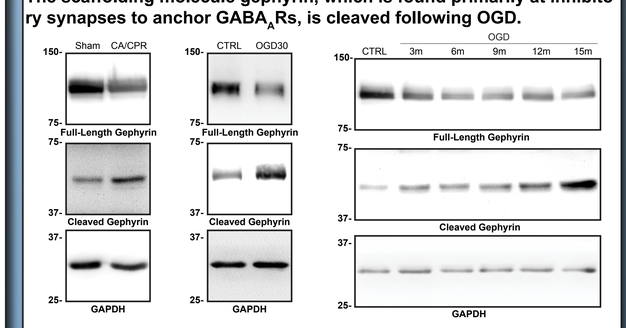
CaN dependent dephosphorylation of S327 reduces synaptic $GABA_A$R clustering by uncoupling from the scaffold gephyrin at the synapse.



Expression of a Super-Ecliptic pHluorin (SEP) tagged γ2--$GABA_A$R shows that mutating serine 327 to alanine prevents the loss of surface $GABA_A$R clustering during OGD and partially restores gephyrin clustering. Using proximity ligation assays (PLA), which detects interactions under 40nm, the physical interaction between $GABA_A$Rs and the scaffold gephyrin is lost by 5 minutes following OGD. This interaction is maintained during OGD when CaN activity is blocked. Together, this data shows an important role for S327 dephosphorylation in synaptic declustering of $GABA_A$Rs from gephyrin during OGD.

Gephyrin is cleaved during OGD

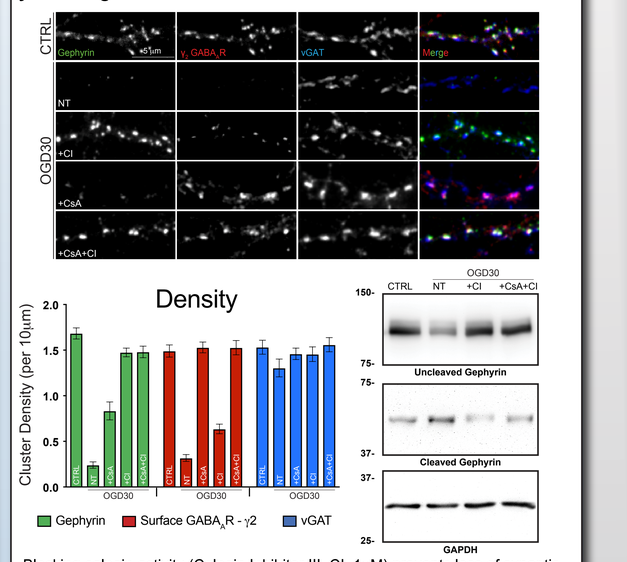
The scaffolding molecule gephyrin, which is found primarily at inhibitory synapses to anchor $GABA_A$Rs, is cleaved following OGD.



Gephyrin cleavage is one mechanism that facilitates turnover of the scaffold found mainly at inhibitory synapses. The scaffold is cleaved both in vivo and in vitro following OGD. Gephyrin cleavage follows S327 dephosphorylation and $GABA_A$R declustering.

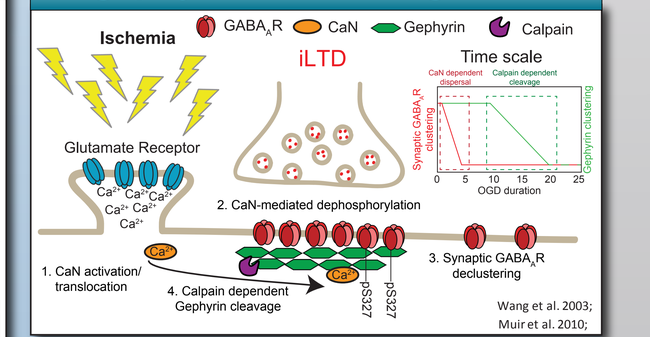
Calpain activity leads to gephyrin cleavage during OGD

Activation of the cystine protease calpain cleaves full-length gephyrin during OGD.



Blocking calpain activity (Calpain Inhibitor III, CI, 1μM) prevents loss of synaptic gephyrin and partial loss of $GABA_A$Rs. Use of both CsA and CI together restores clustering of both $GABA_A$Rs and gephyrin.

Model of $GABA_A$R and gephyrin loss during OGD



Wang et al. 2003; Muir et al. 2010;

Conclusions

- Post synaptic components of the inhibitory synapse are lost initially during an ischemic insult both *in vitro* and *in vivo*.
- SIM imaging reveals $GABA_A$R clustering is lost first (5min) followed by gephyrin loss (15min).
- STORM imaging shows $GABA_A$R outside of gephyrin PSD following OGD.
- CaN mediates synaptic declustering of $GABA_A$Rs through S327 dephosphorylation.
- Blocking S327 dephosphorylation prevents $GABA_A$R cluster loss and restores gephyrin interaction.
- Gephyrin is cleaved by calpain during OGD.
- Blocking both CaN and calpain activity restores both the $GABA_A$R and gephyrin

Future Directions

- Investigate the role of $GABA_A$R endocytosis following declustering.
- Examine if inhibitory synapse loss is persistent following reperfusion.
- Investigate GABAAR sorting to late endosomes or lysosomes for receptor degradation.
- Explore neuroprotection to cell-death when $GABA_A$R declustering or gephyrin cleavage is blocked.