

# Papel del intercambiador mitocondrial de sodio/calcio NCLX en la muerte por ferroptosis en isquemia-reperfusión cerebral

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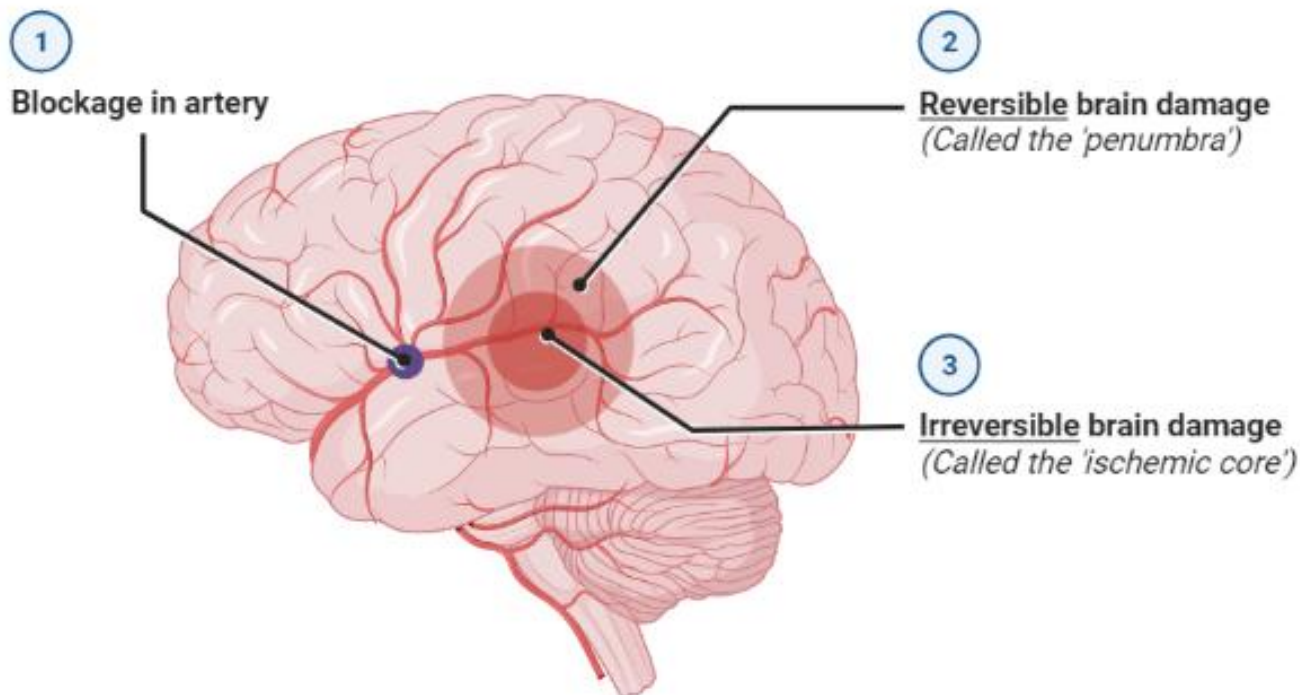
<sup>3</sup> *Instituto-Fundación Teófilo Hernando, Departamento de Farmacología y Terapéutica, Universidad Autónoma de Madrid, Spain.*

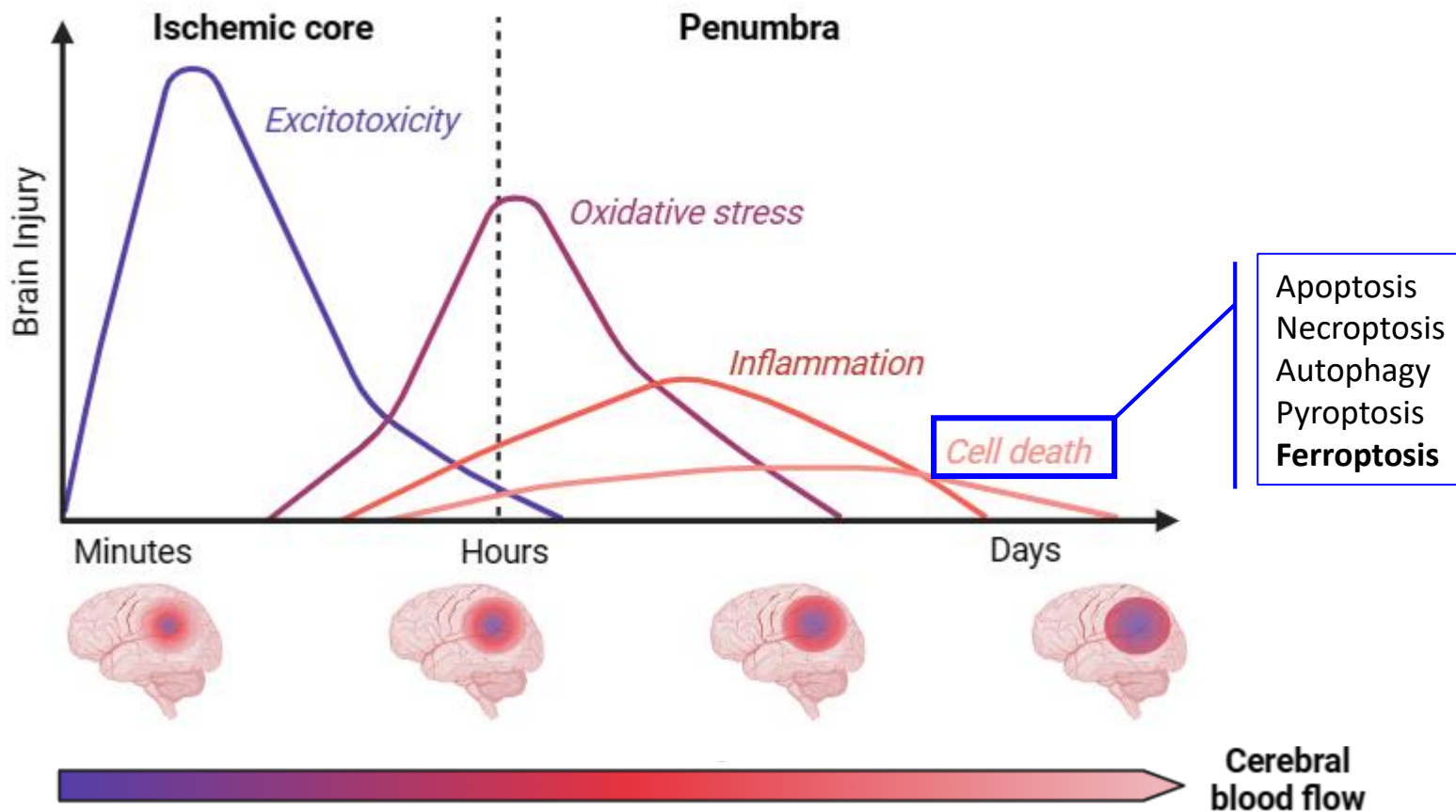
<sup>4</sup> *Department of Neuroimaging, Institute of Psychiatry, Psychology, and Neuroscience, King's College London, Londres, Reino Unido.*

<sup>5</sup> *Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad Complutense de Madrid, Spain.*

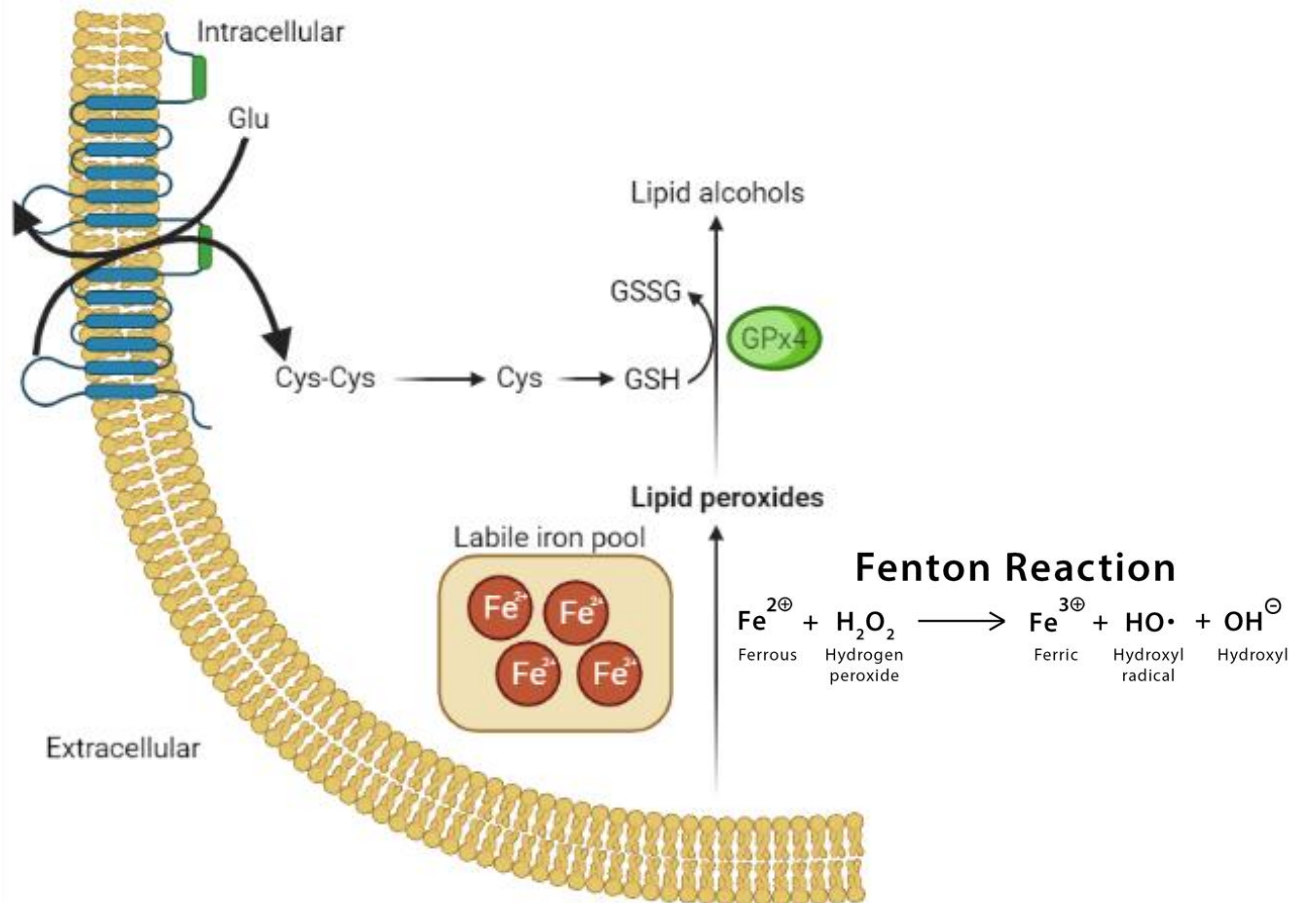
Email: [susadelg@ucm.es](mailto:susadelg@ucm.es)

- **Stroke** causes neuronal cell death and neurological dysfunction, and nearly 70% stroke cases are caused by cerebral ischemia.
- **Ischemia-reperfusion (I/R)** is a pathological condition characterized by a restriction of the blood supply to tissues, which leads to **hypoxia** and metabolic imbalance, followed by the restoration of blood flow and **reoxygenation**, which leads to excessive inflammatory responses and tissue damage, known as **I/R injury**.

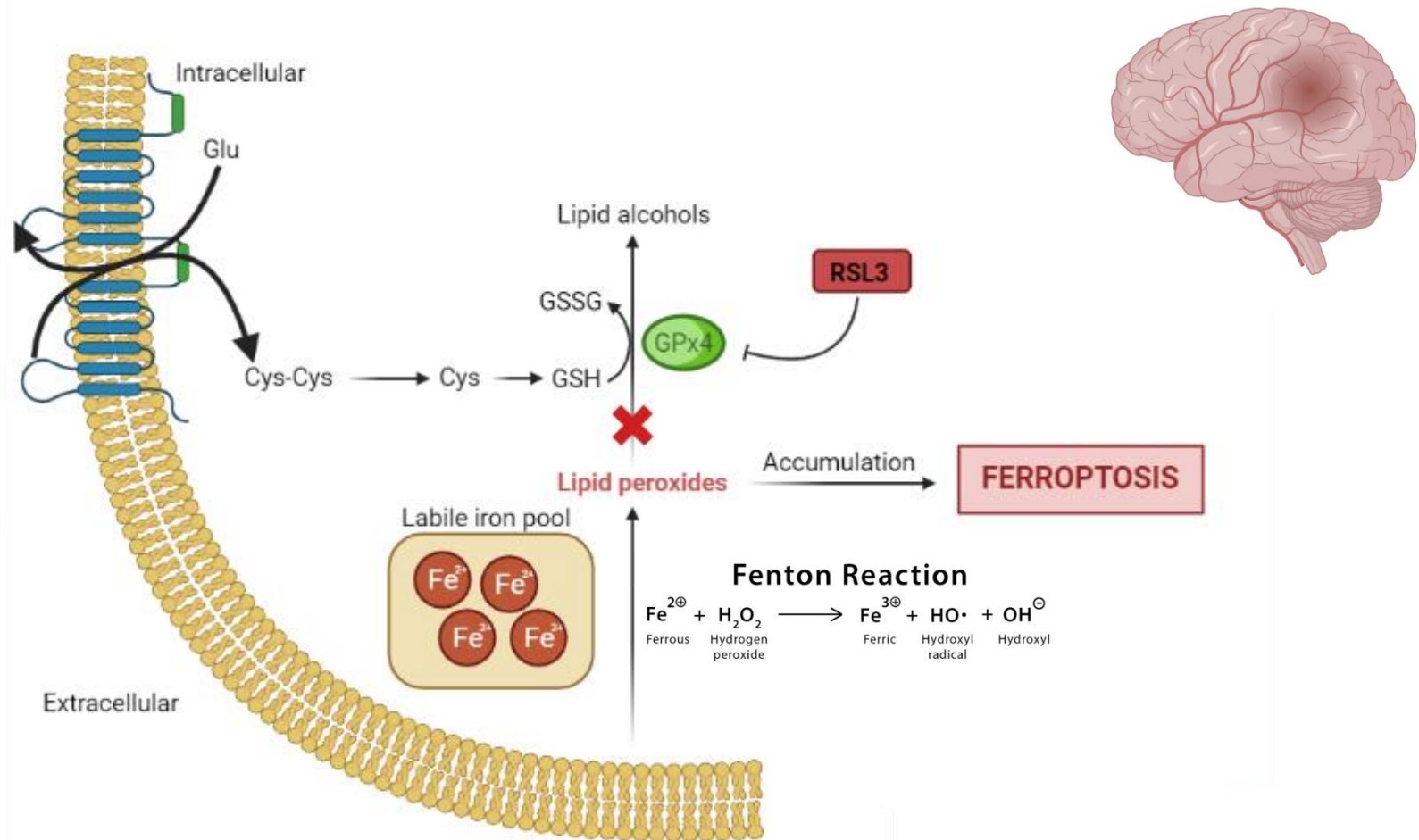


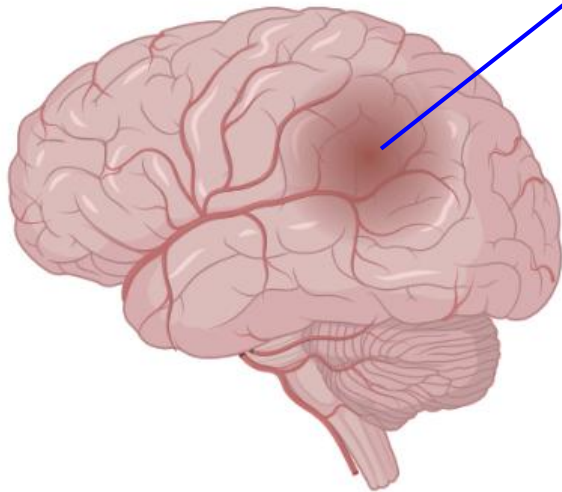


- Ferroptosis** is a unique form of cell death that is characterized by **iron-dependent oxidative damage to membrane phospholipids** and is involved in important pathological mechanisms of neurodegenerative disease.

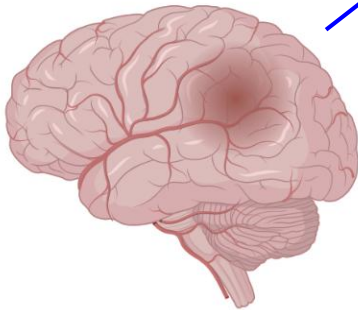


- **Excessive** reactive oxygen species (ROS) also target sensitive fatty acids and promote lipid peroxidation, which then impairs **the integrity of lipid membranes** and induces suicide signaling cascades.

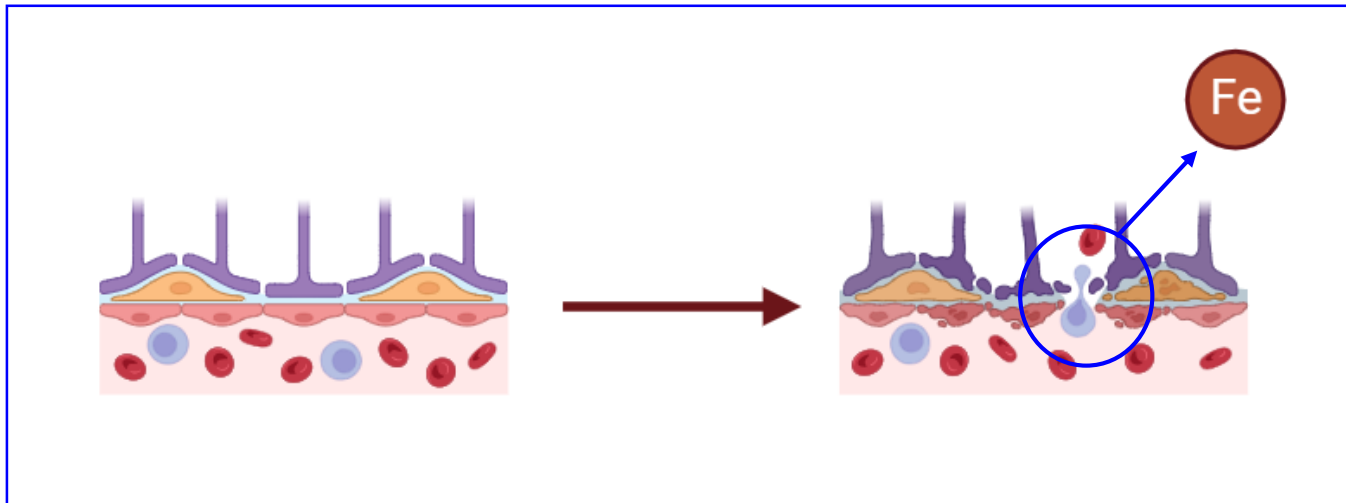


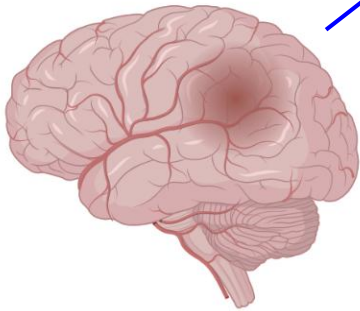


- Iron accumulation
- Oxidative damage to lipids
- Decrease of GSH and GPx4

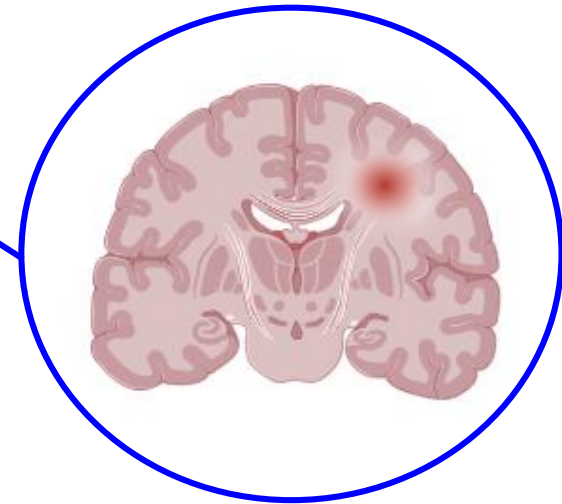
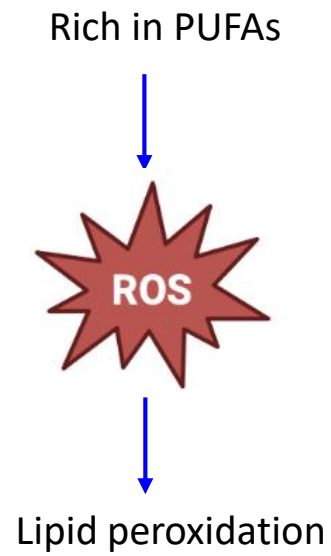


- **Iron accumulation**
- Oxidative damage to lipids
- Decrease of GSH and GPx4

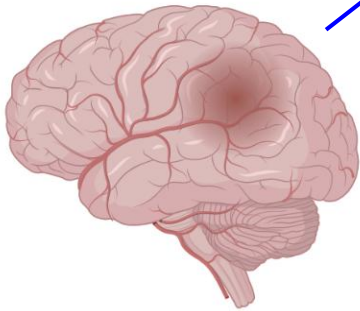




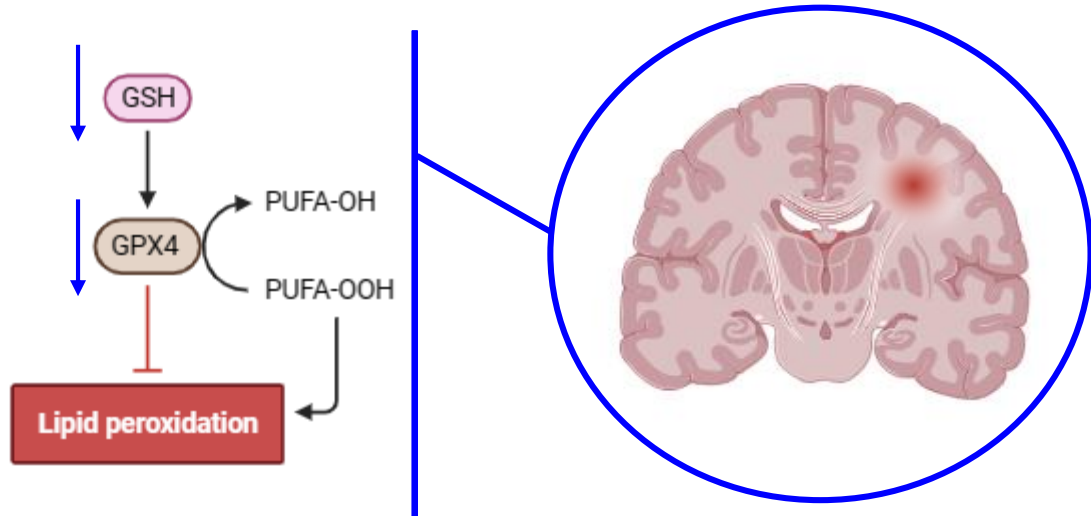
- Iron accumulation
- **Oxidative damage to lipids**
- Decrease of GSH and GPx4



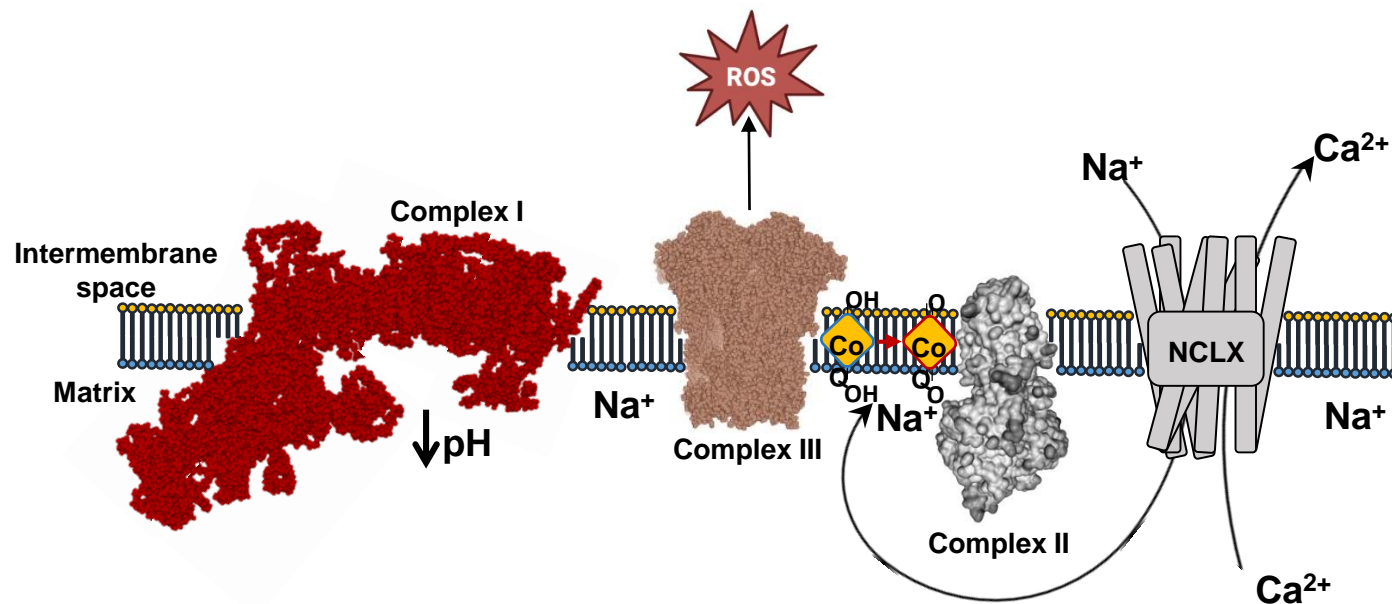




- Iron accumulation
- Oxidative damage to lipids
- **Decrease of GSH and GPx4**

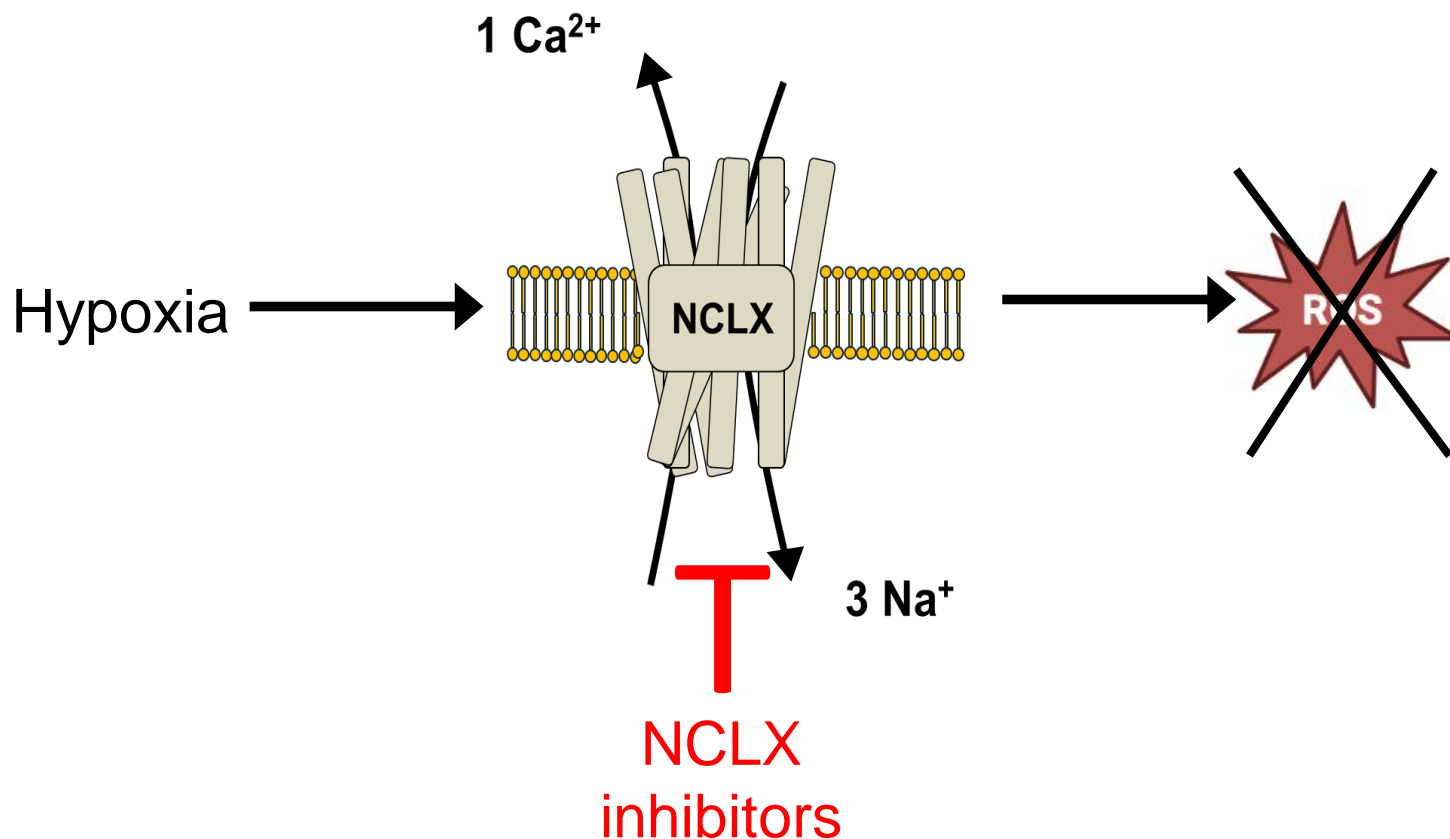


- Reactive oxygen species can be produced by the mitochondria during **hypoxia – reoxygenation**.
- The activation of the **mitochondrial sodium/calcium exchanger NCLX** during acute hypoxia drives superoxide production at complex III.
- The **inhibition of Na<sup>+</sup> import** through NCLX is enough to block this pathway (Hernansanz-Agustín et al., 2020).

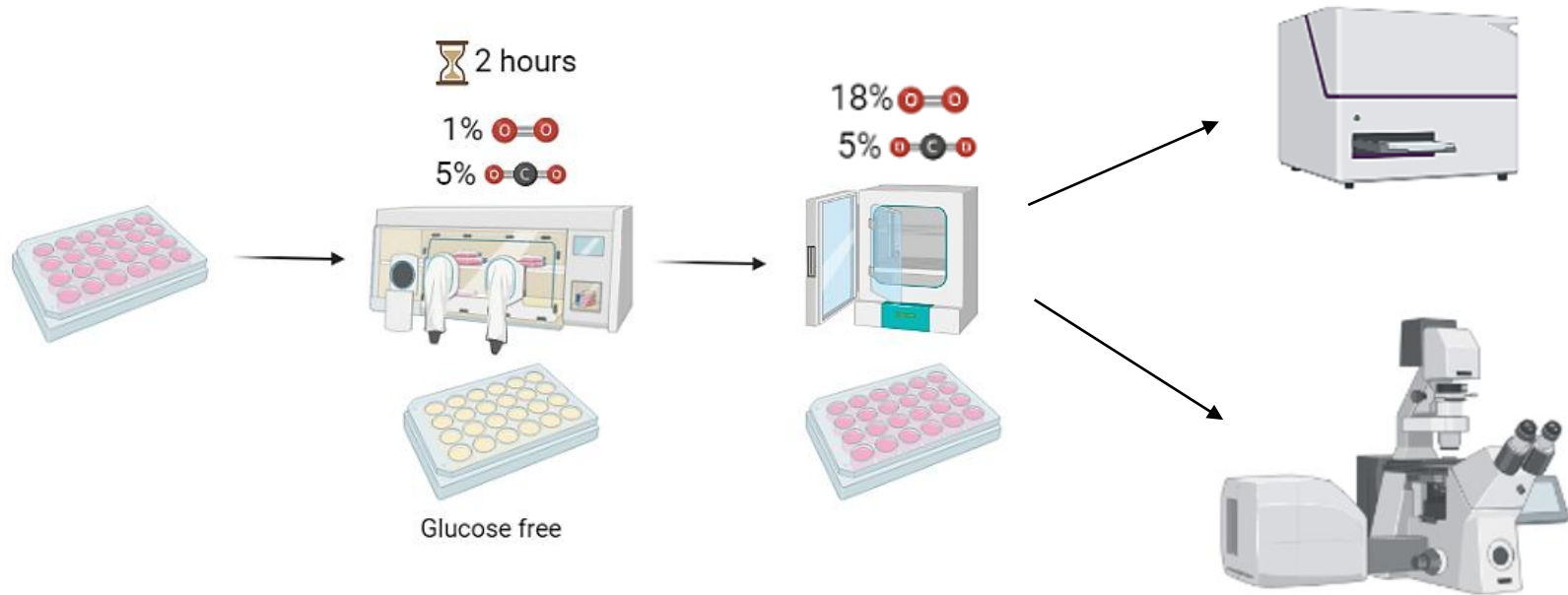


Our **hypothesis** is that, since **ROS** are produced in the **hypoxia-reoxygenation** process, these could oxidize lipids and trigger cell death by **ferroptosis**.

**Inhibition** of **NCLX** could **decrease mitochondrial ROS** production in response to hypoxia and, therefore, decrease lipid peroxidation and inhibit cell death by ferroptosis.



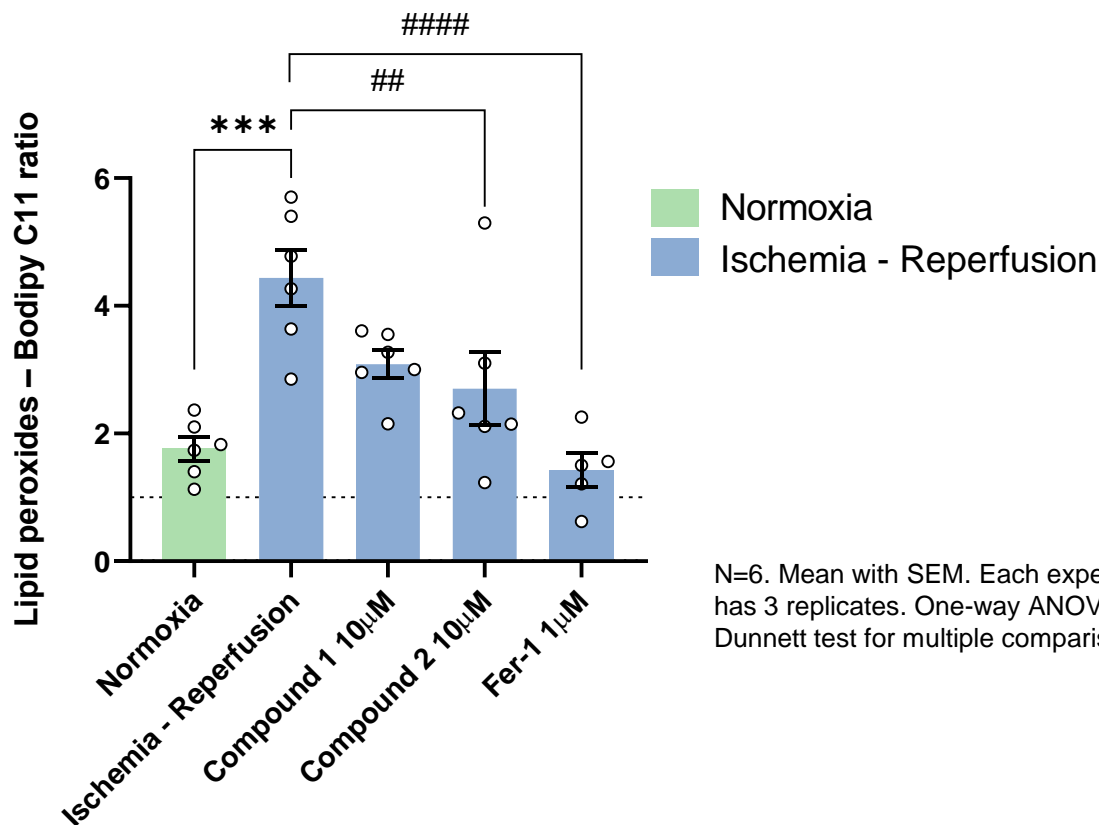
We measured **lipid peroxidation** in SK-N-DZ cells after 2 hours of ischemia and 24 hours of reperfusion, using the fluorescent probe **Bodipy 581/591 C-11**.





To detect **lipid peroxidation** cells were incubated with **Bodipy 581/591 C11**. Cells were treated with **NCLX inhibitors** (Compounds 1 and 2), alone or in combination with **Ferrostatin-1** during reperfusion.

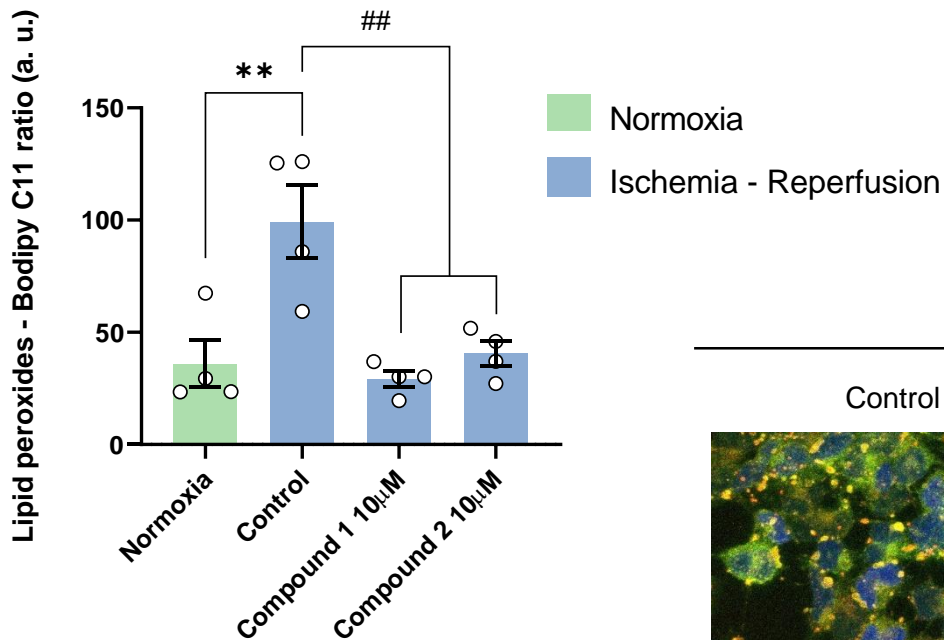
### Lipid peroxidation





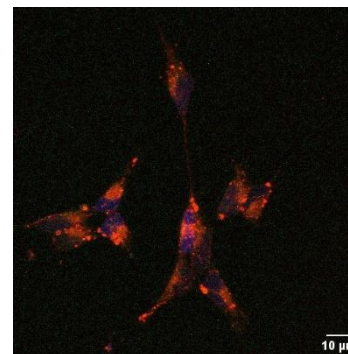
To detect **lipid peroxidation** cells were incubated with **Bodipy 581/591 C11**. Cells were treated with **NCLX inhibitors** (Compounds 1 and 2), alone or in combination with **Ferrostatin-1** during reperfusion.

Lipid peroxidation



N=4. Mean with SEM. Each experiment is normalized to its mean. One-way ANOVA with Dunnett test for multiple comparisons.

Normoxia

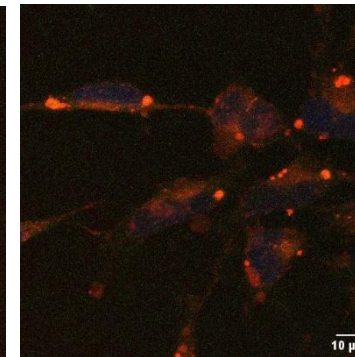
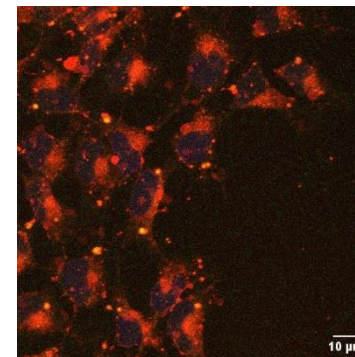
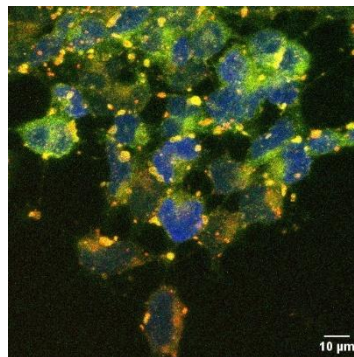


Ischemia - Reperfusion

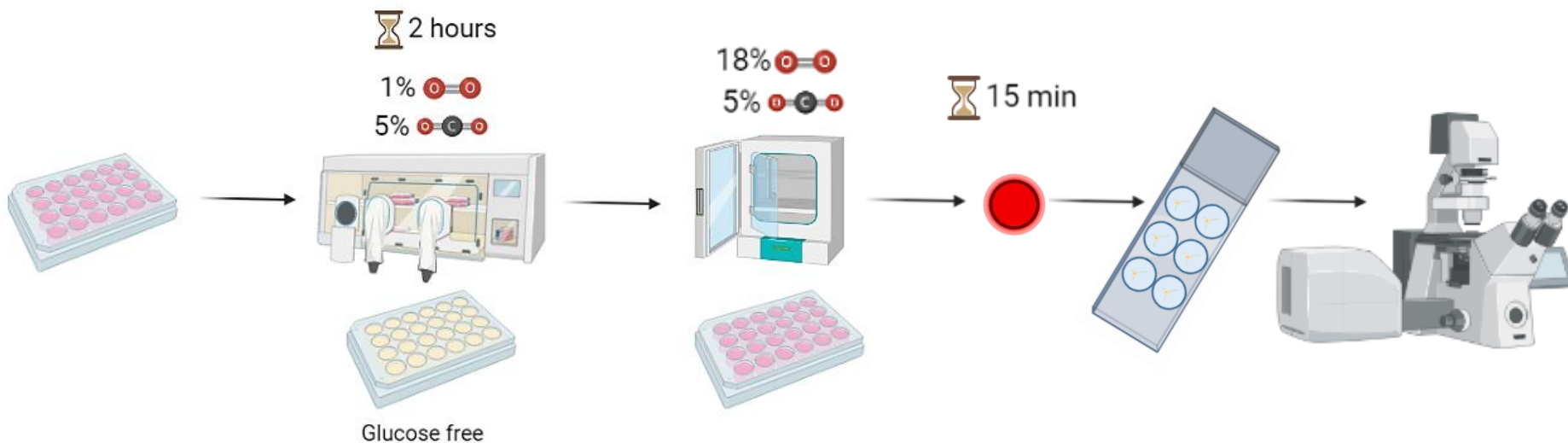
Control

Compound 1 10µM

Compound 2 10µM

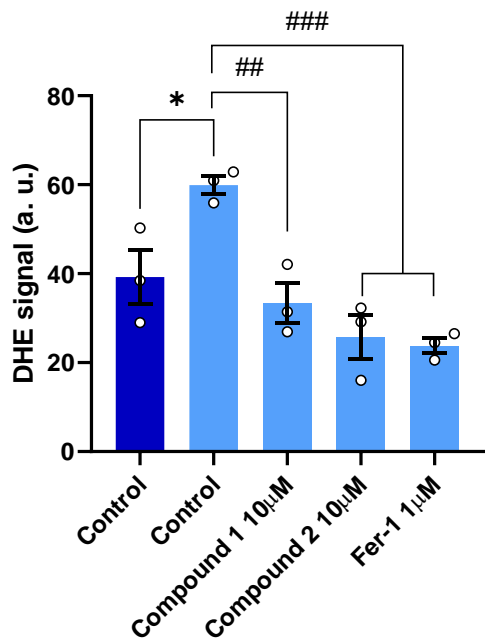


We determined **superoxide production** by cell imaging with a **confocal microscope** using the fluorescent probe **Dihydroethidium (DHE)**.



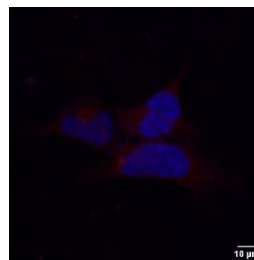


Superoxide production

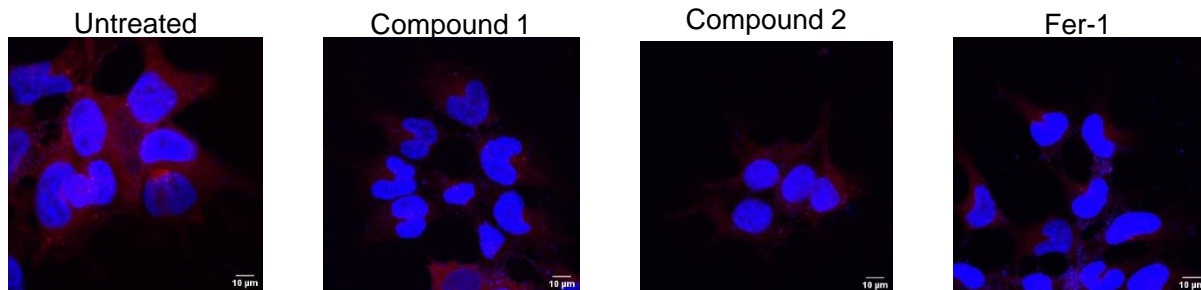


■ Reperfusion 0h  
 ■ Reperfusion 24h

0h reperfusion



24h reperfusion

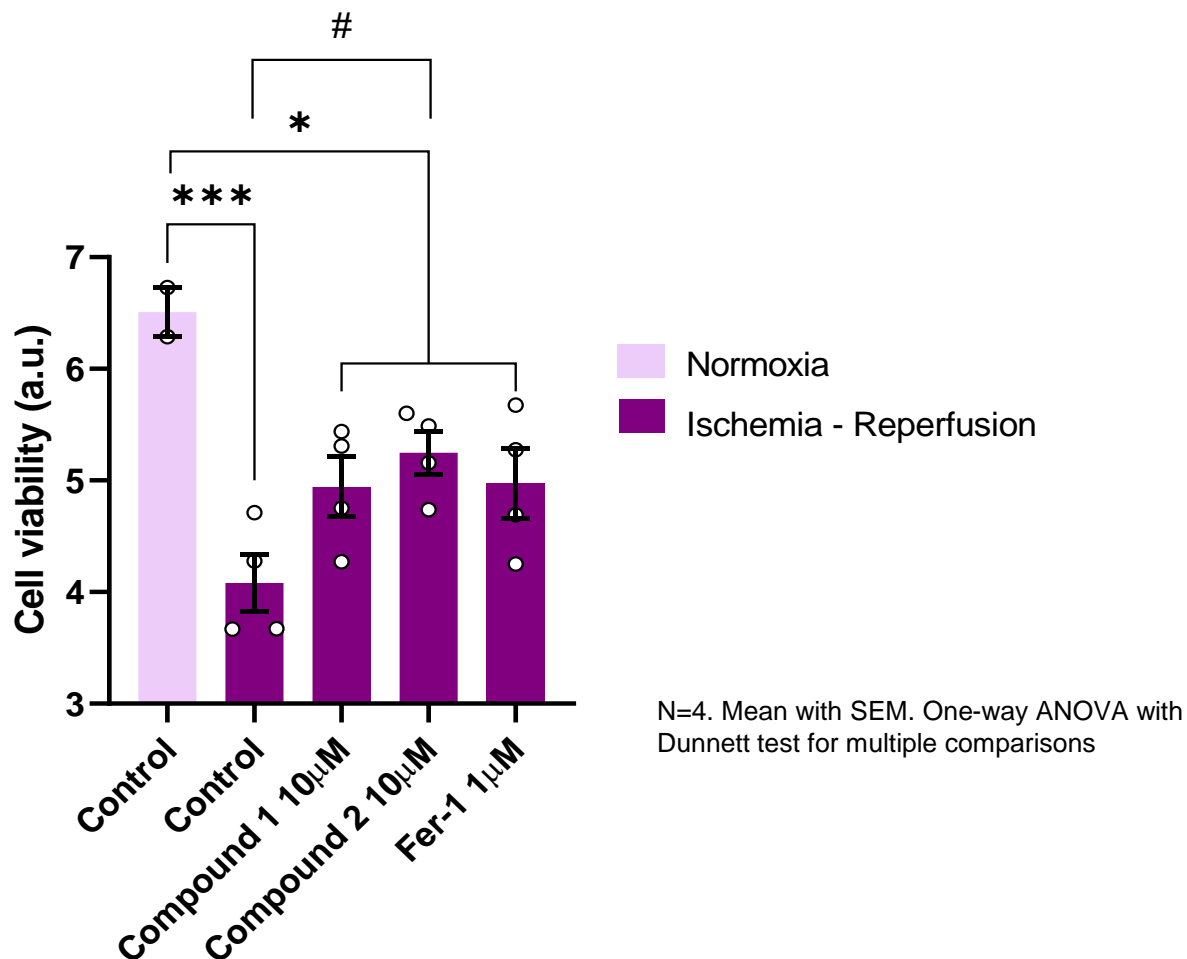




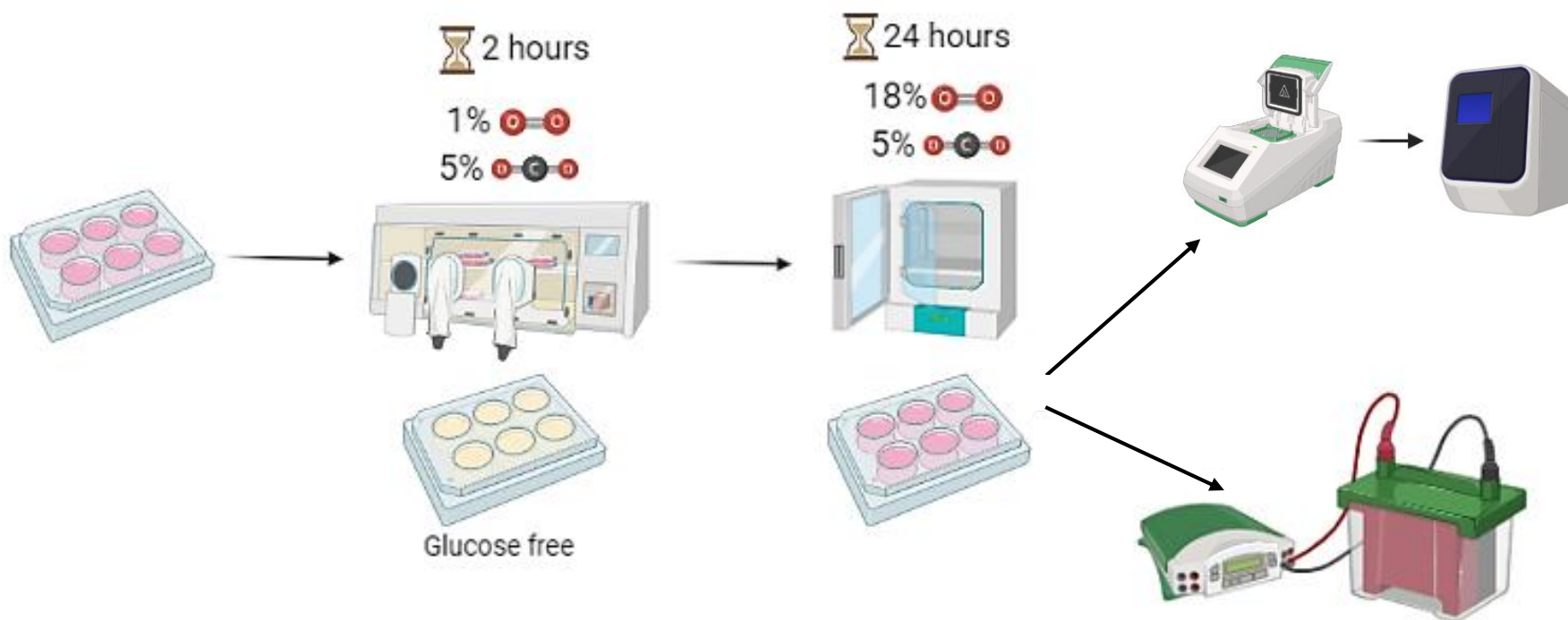


We measured **cell viability** in SK-N-DZ cells after 2 hours of ischemia and 24 hours of reperfusion, using the crystal violet assay.

Cells were treated with **NCLX inhibitors**, alone or in combination with **Ferrostatin-1**.



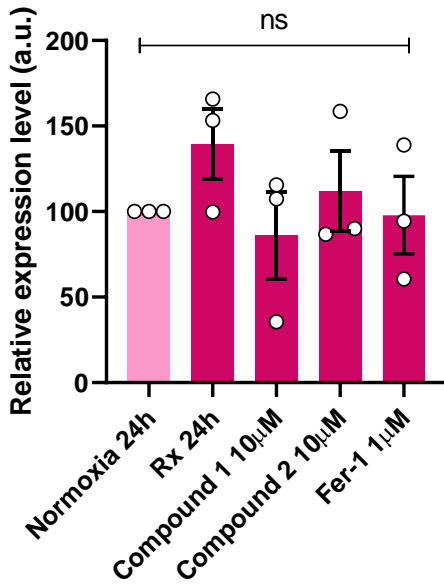
We also measured levels of different **ferroptosis-related proteins** in SK-N-DZ cells after 2 hours of ischemia and 24 hours of reperfusion by western blot and qPCR.





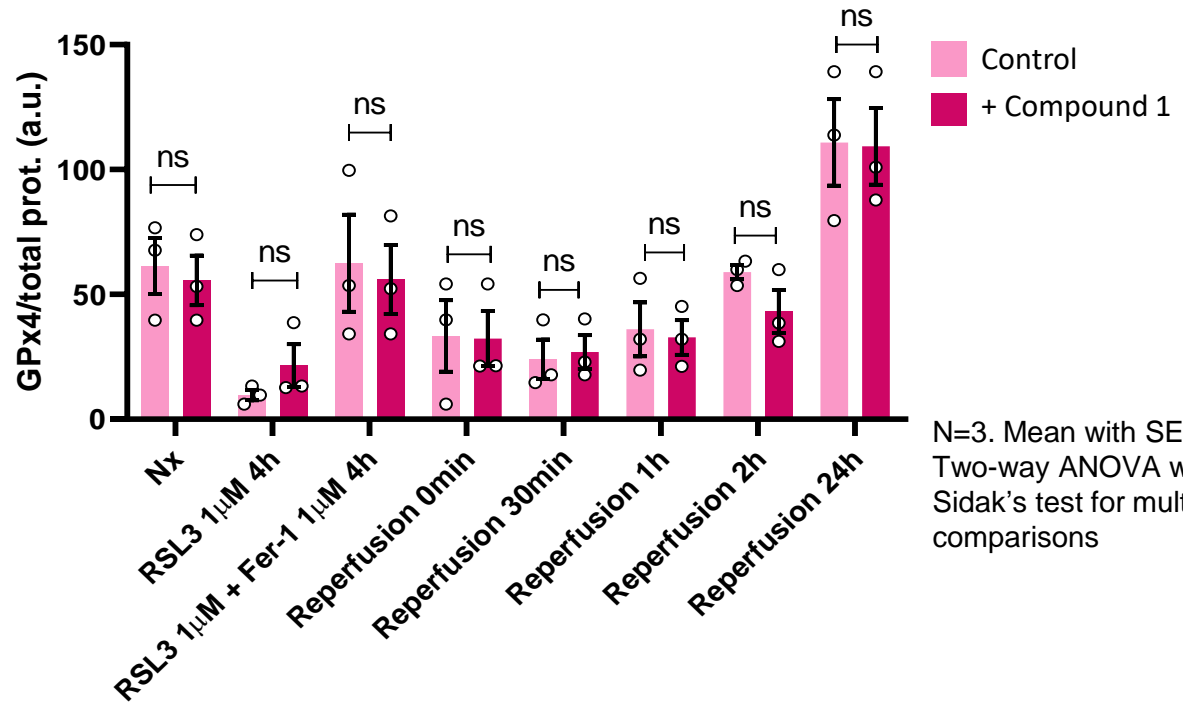
SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, with and without treatment with an **NCLX inhibitor**.

**gpx4 mRNA levels**



N=3. One-way ANOVA with Dunnett test for multiple comparisons

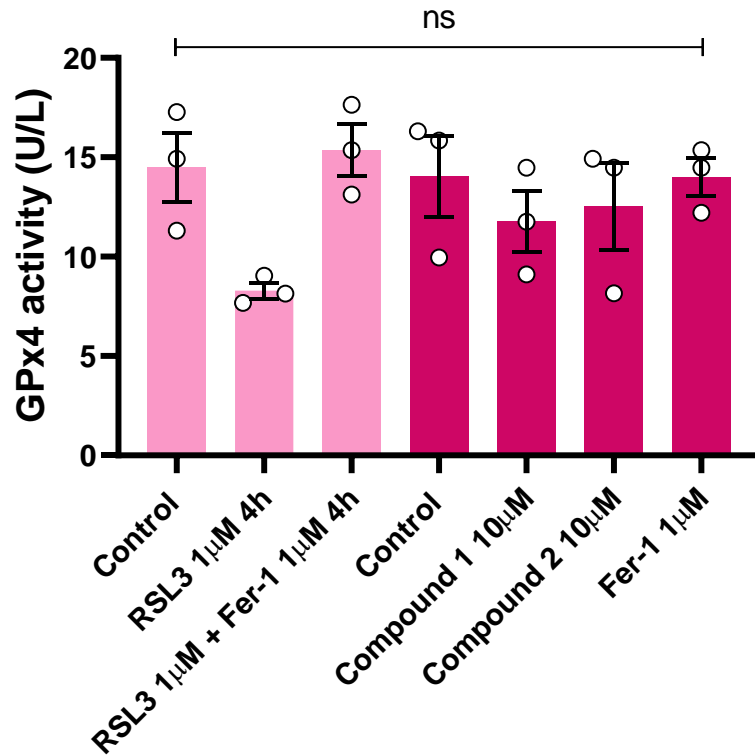
**GPx4 protein levels**



N=3. Mean with SEM. Two-way ANOVA with Sidak's test for multiple comparisons



We measured **GPx4 activity** in SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, by western blot, with and without treatment with an **NCLX inhibitor**.



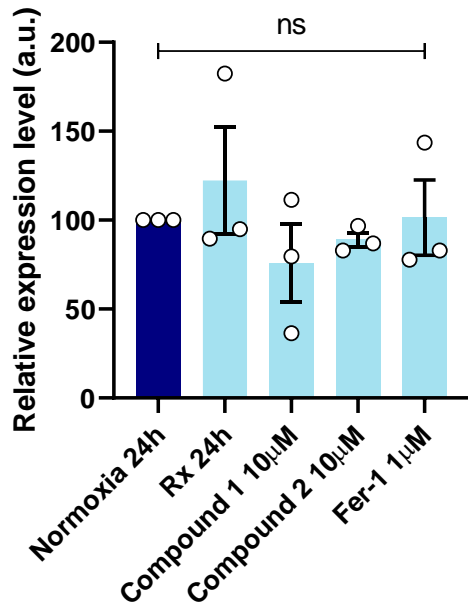
Normoxia  
Ischemia - Reperfusion

N=3. Mean with SEM. One-way ANOVA with Dunnett test for multiple comparisons



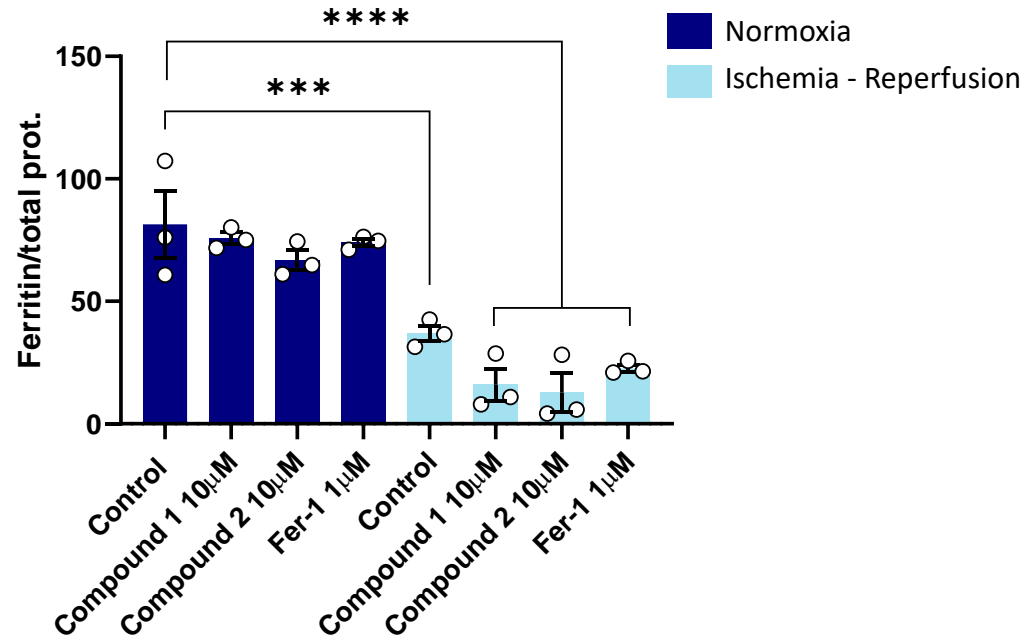
SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, with and without treatment with an **NCLX inhibitor**.

*fth1* mRNA levels



N=3. One-way ANOVA with Dunnett test for multiple comparisons

FTH1 protein levels



N=3. Mean with SEM. One-way ANOVA with Dunnett test for multiple comparisons

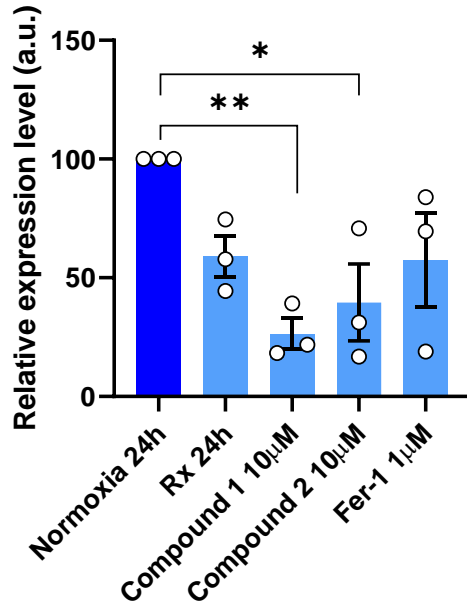
\*\*\*\* P value < 0.0001

\*\*\* P value = 0.0009



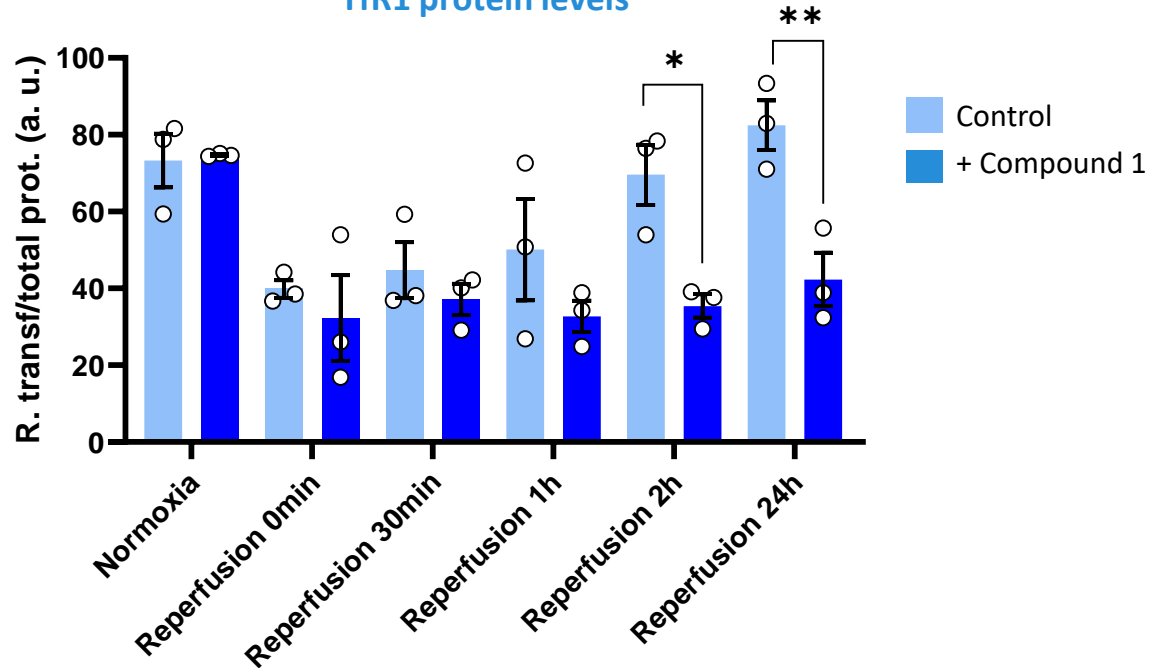
SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, with and without treatment with an **NCLX inhibitor**.

*tfr1* mRNA levels



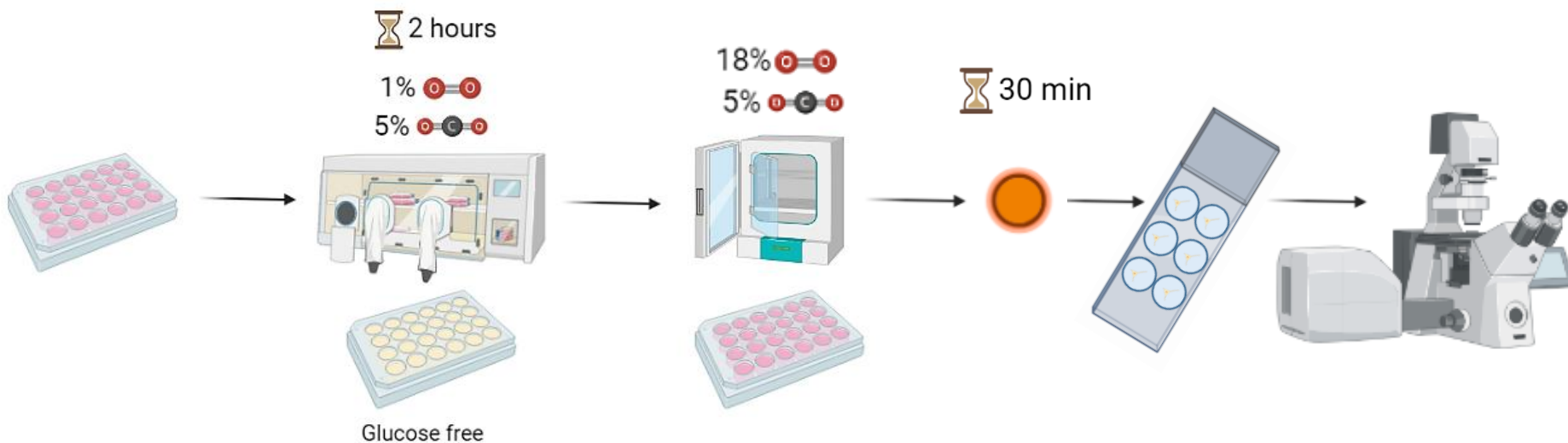
N=3. Mean with SEM. One-way ANOVA with Dunnett test for multiple comparisons  
 \* P value = 0.0201  
 \*\* P value = 0.0061

TfR1 protein levels

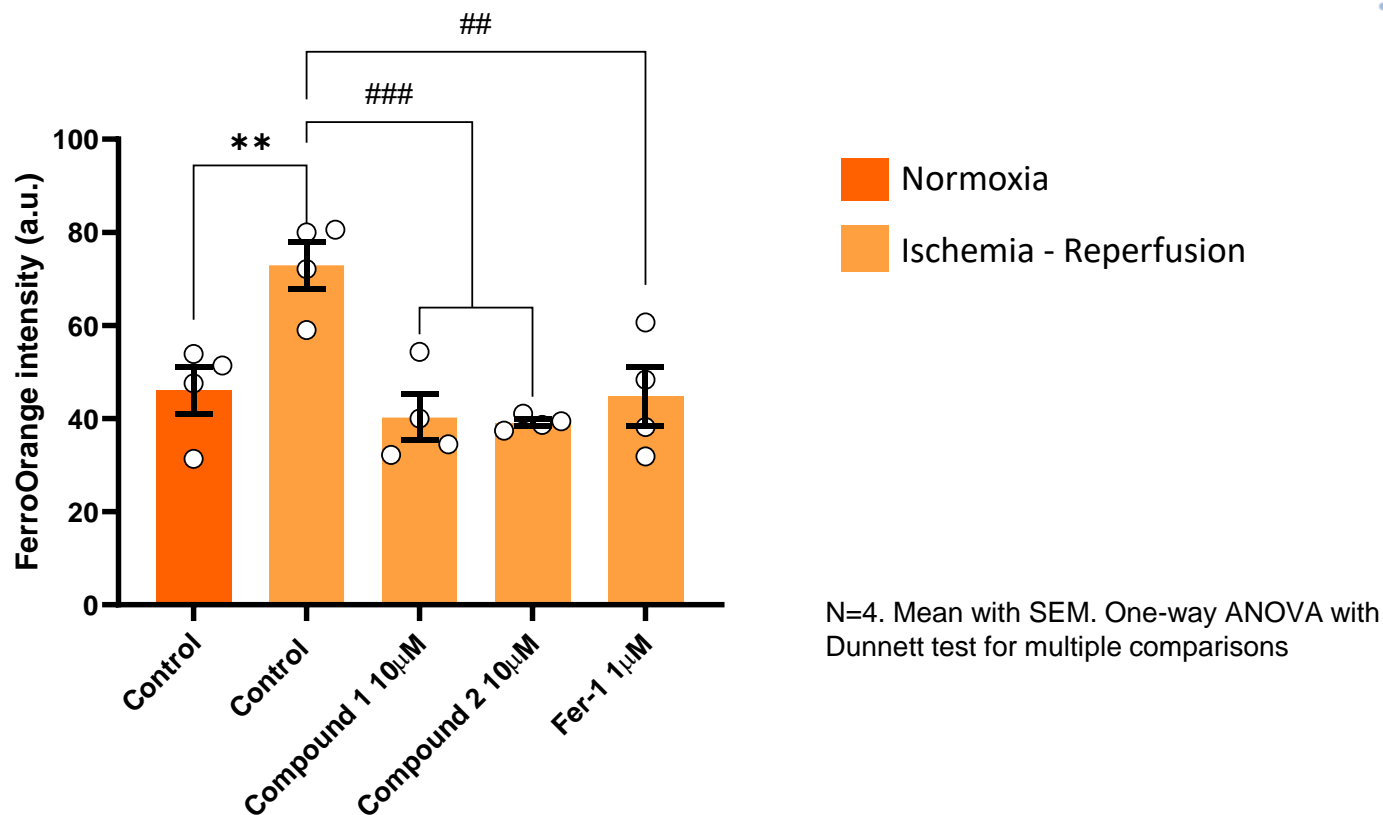


N=3. Mean with SEM. Two-way ANOVA with Sidak's test for multiple comparisons  
 \*\* P value = 0.0030  
 \* P value = 0.0135

We measured **labile Fe<sup>2+</sup> ions levels** by confocal imaging using the fluorescent probe **FerroOrange** in SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, with and without treatment with an **NCLX inhibitor**.

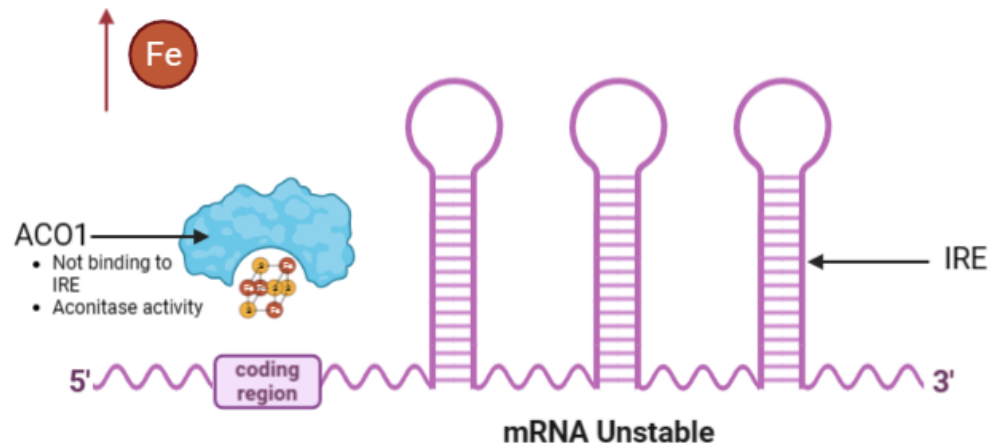
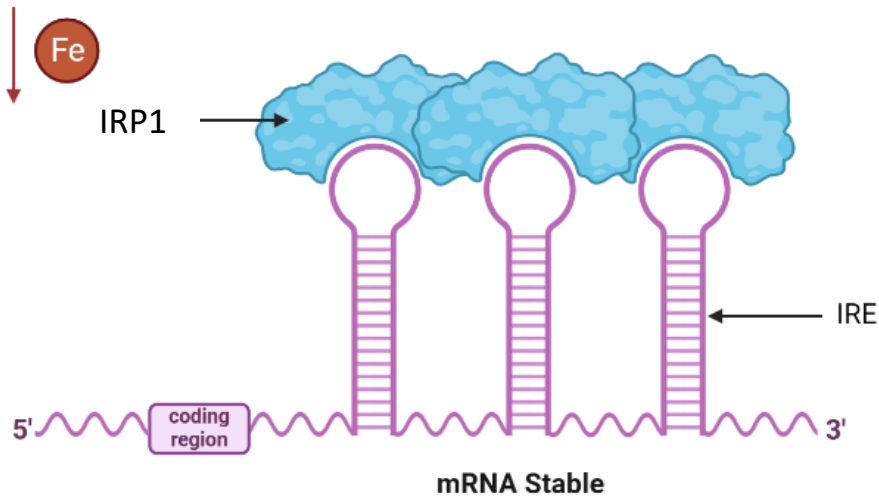


We measured **labile Fe<sup>2+</sup> ions levels** by confocal imaging using the fluorescent probe **FerroOrange** in SK-N-DZ cells after 2 hours of ischemia and 24 hours of reperfusion, with and without treatment with an **NCLX inhibitor**.





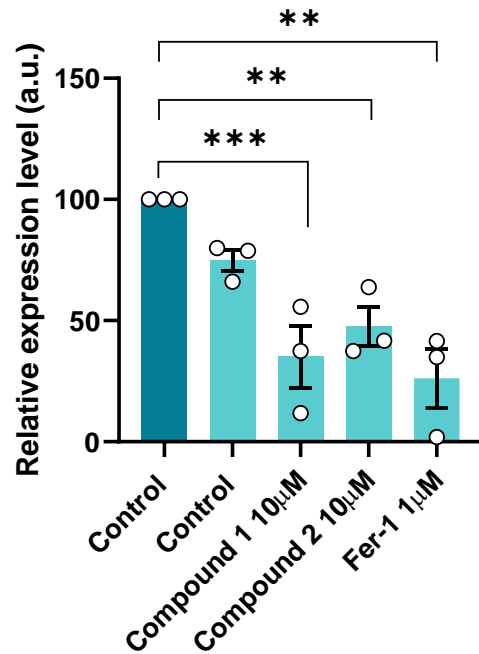
# Transferrin receptor





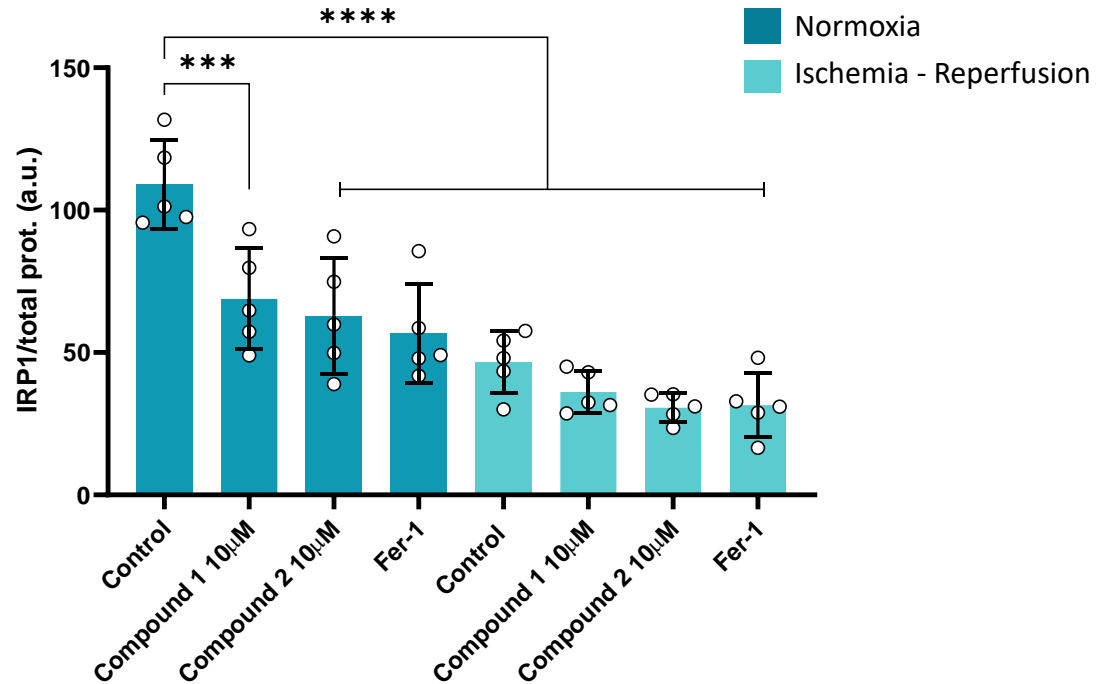
SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, with and without treatment with an **NCLX inhibitor**.

*aco1* mRNA levels



N=3. Mean with SEM. One-way ANOVA with Dunnett test for multiple comparisons  
 \*\* P value < 0.066  
 \*\*\* P value = 0.0006

IRP1 protein levels

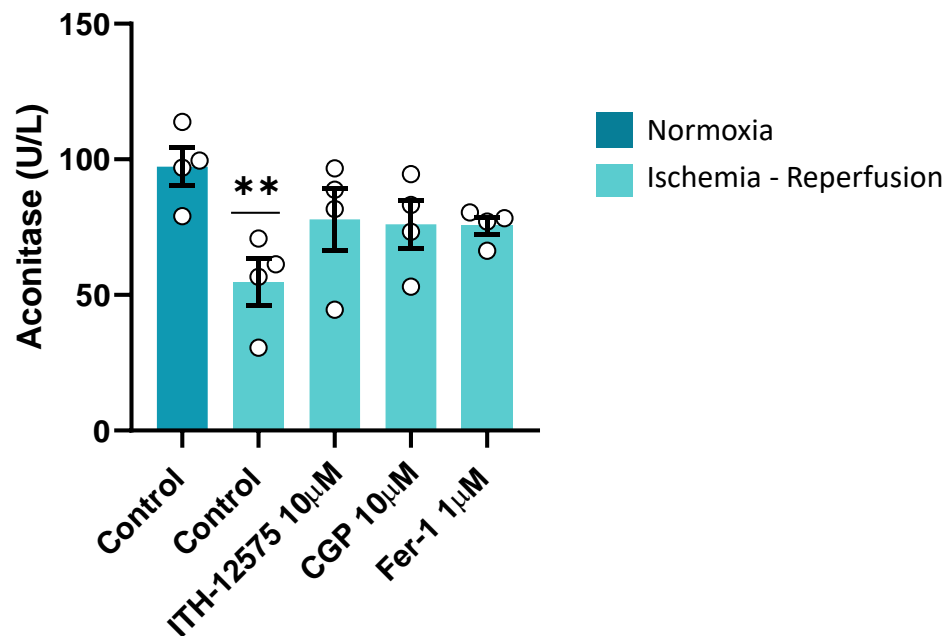


N=5. Mean with SEM. One-way ANOVA with Dunnett test for multiple comparisons.  
 \*\*\*\* P value < 0.0001  
 \*\*\* P value = 0.0007



SK-N-DZ cells after 2 hours of ischemia at different times of reperfusion, with and without treatment with an **NCLX inhibitor**.

### IRP1 aconitase activity

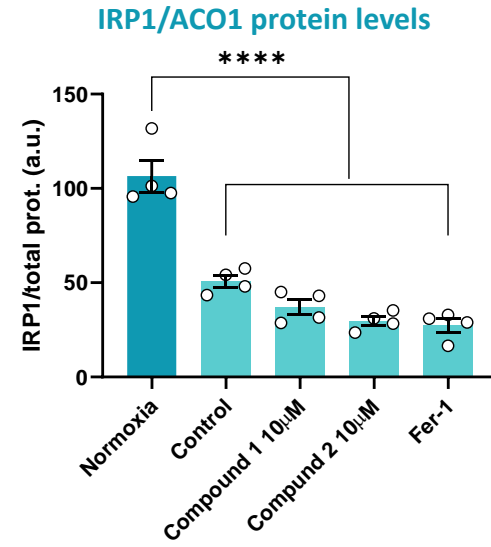
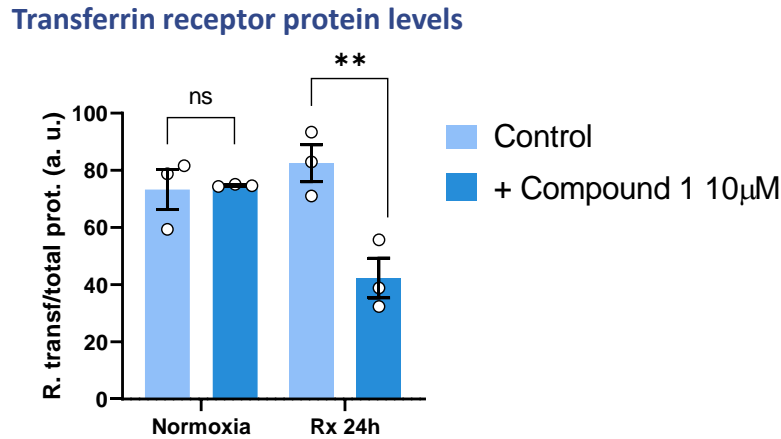
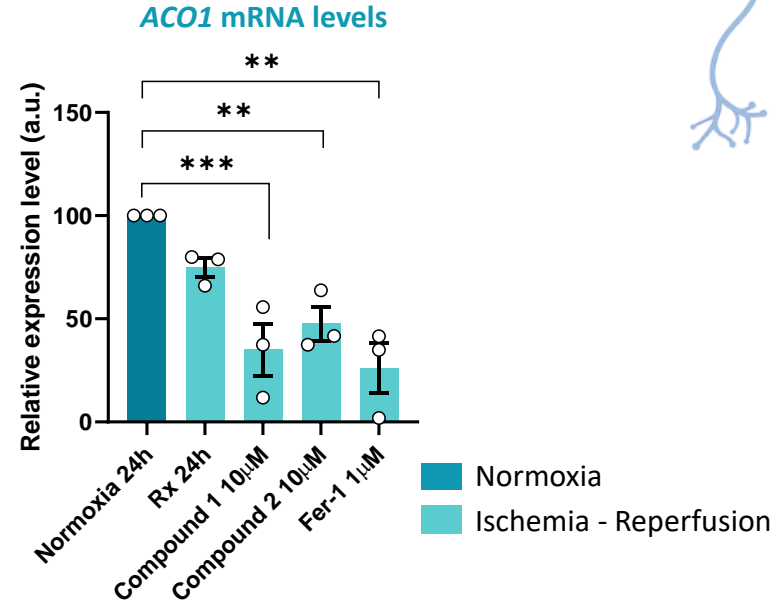
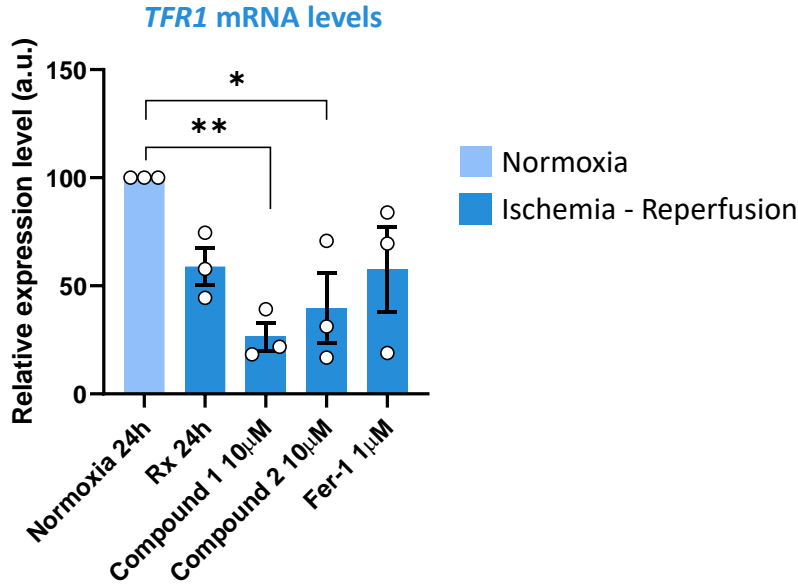


N=3. Mean with SEM. One-way ANOVA with Dunnett test for multiple comparisons

\*\* P value < 0.042

\*\*\* P value = 0.0003

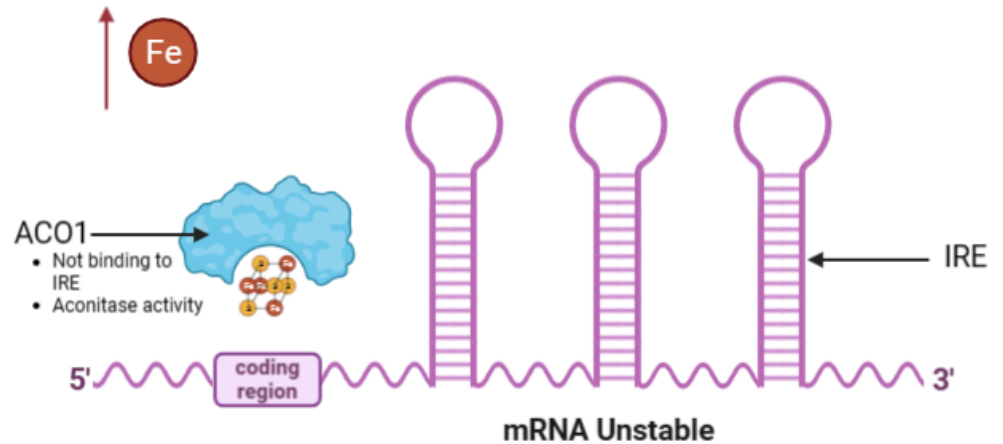
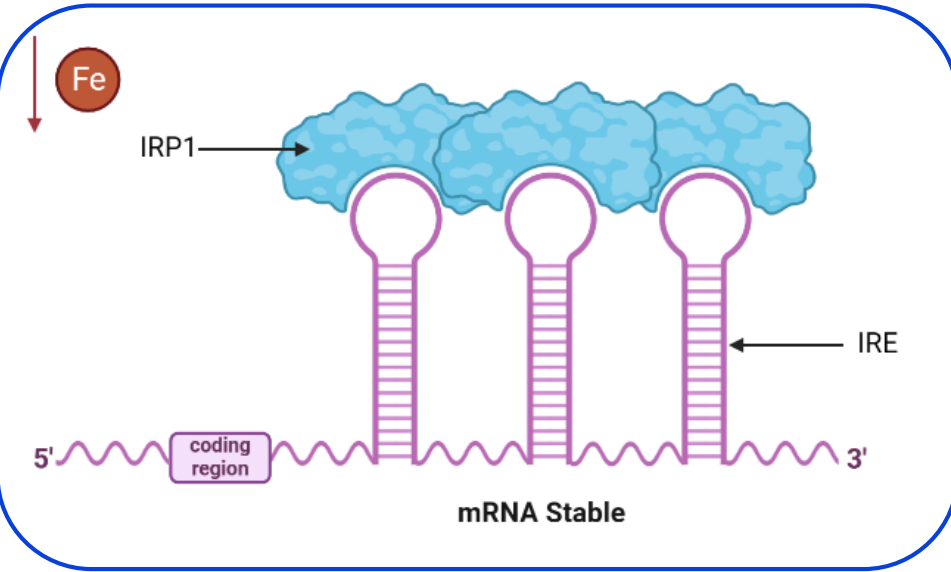
There is a **correlation** between **IRP1** and **transferrin receptor** levels at 24 hours of reperfusion.



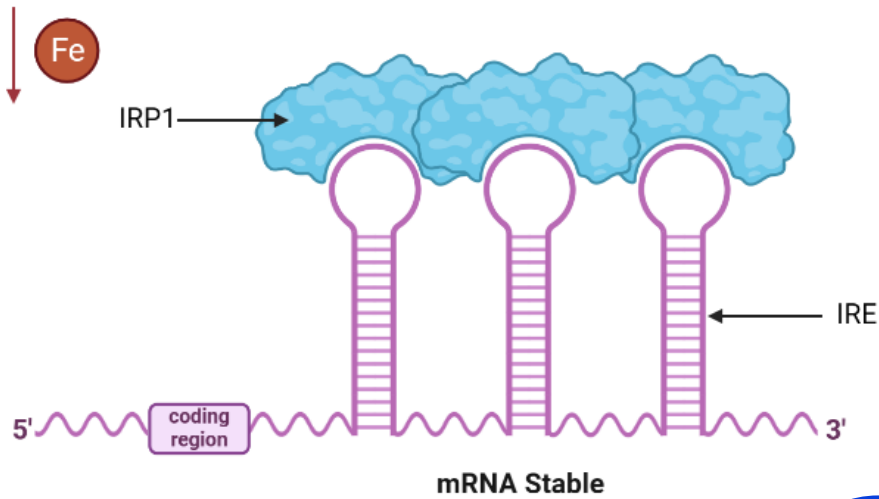
# Transferrin receptor

## Ischemia - Reperfusion

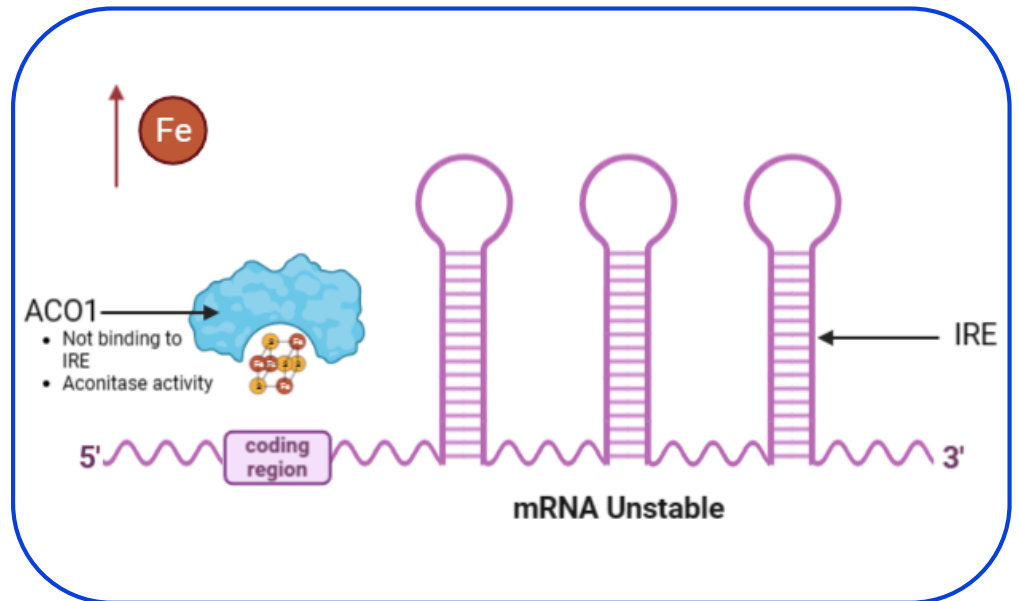
- Hydrogen peroxide increases IRP-binding activity
- Superoxide decreases IRP1 aconitase activity



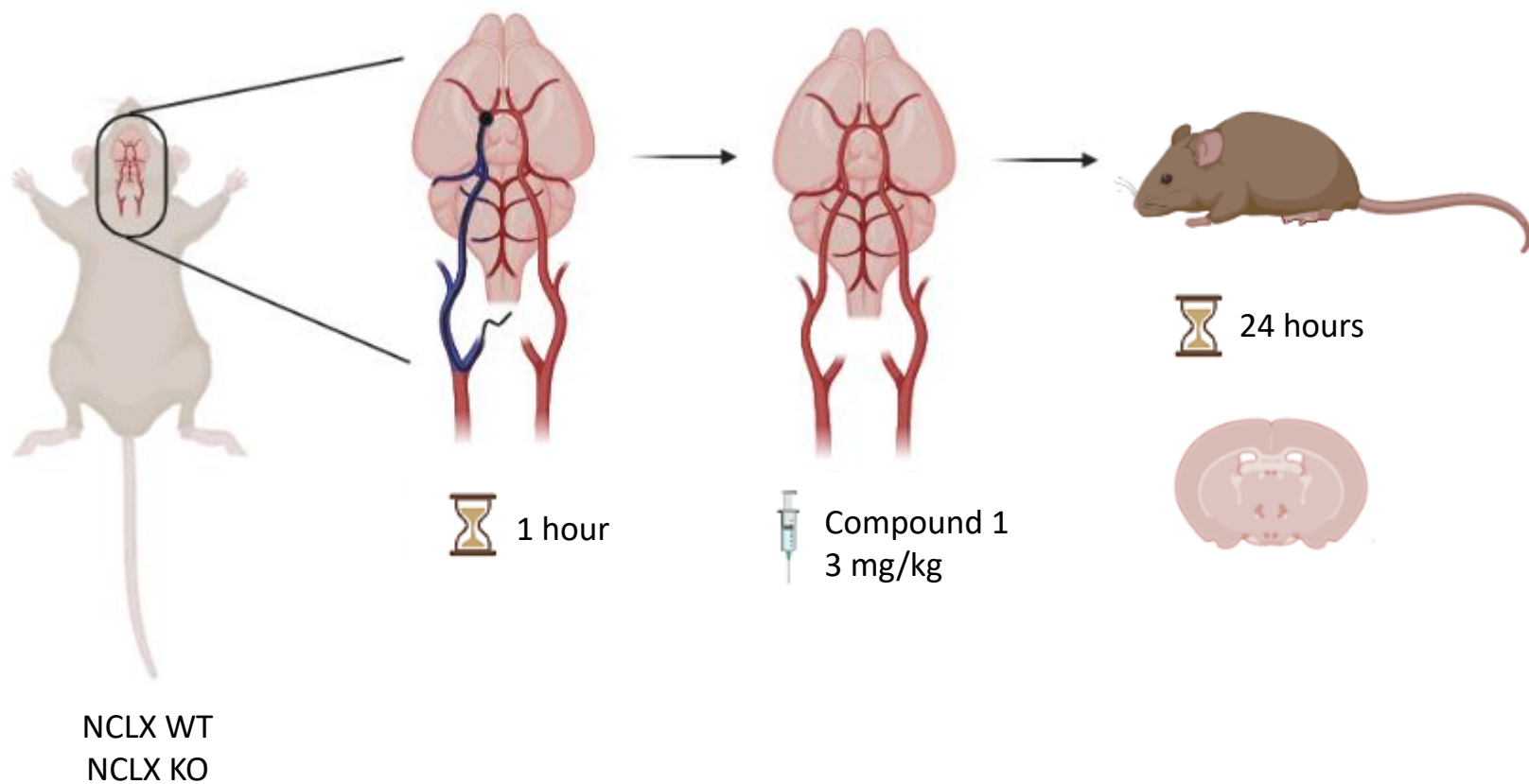
# Transferrin receptor

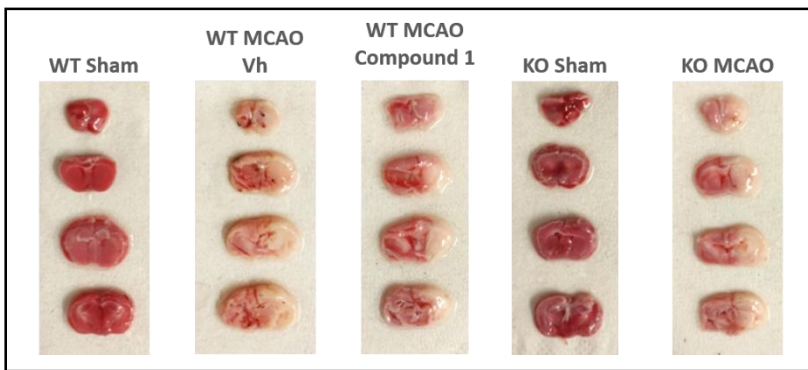


## Ischemia – Reperfusion with NCLX inhibitors

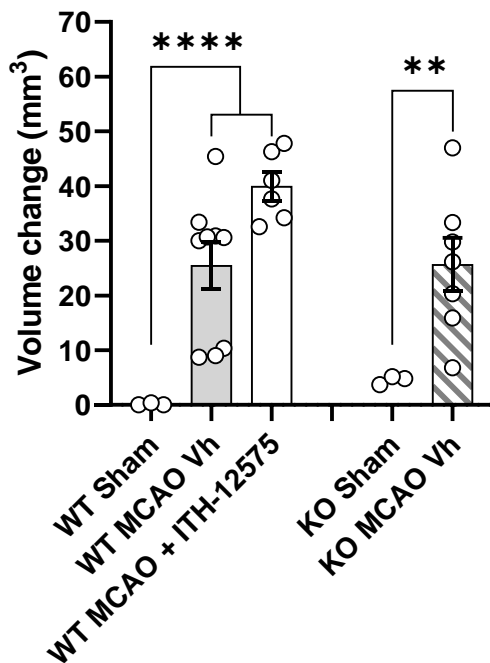


We used a **tMCAO mice model** to mimic **ischemic stroke**

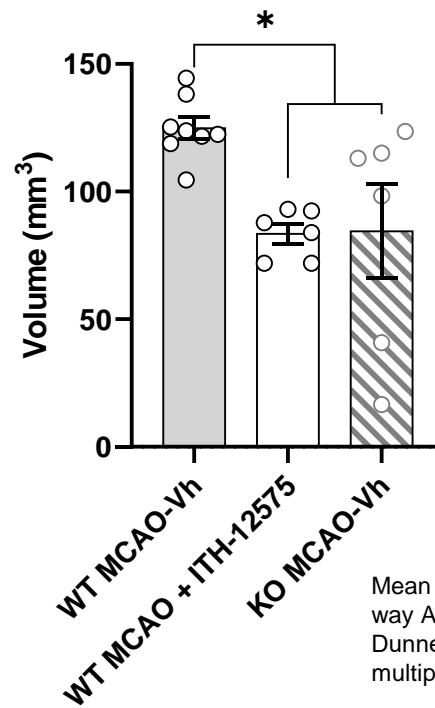




Edema

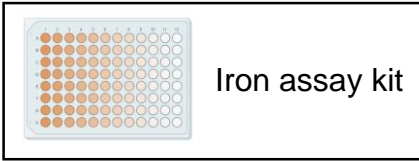


Infarct volume

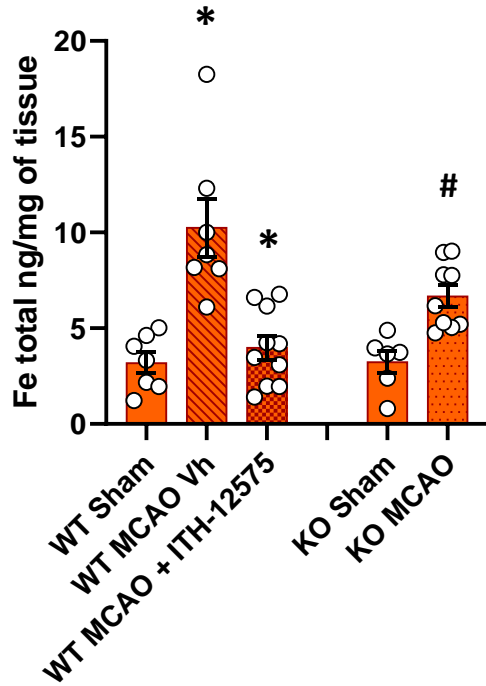


Mean with SEM. One-way ANOVA with Dunnett's test for multiple comparisons.



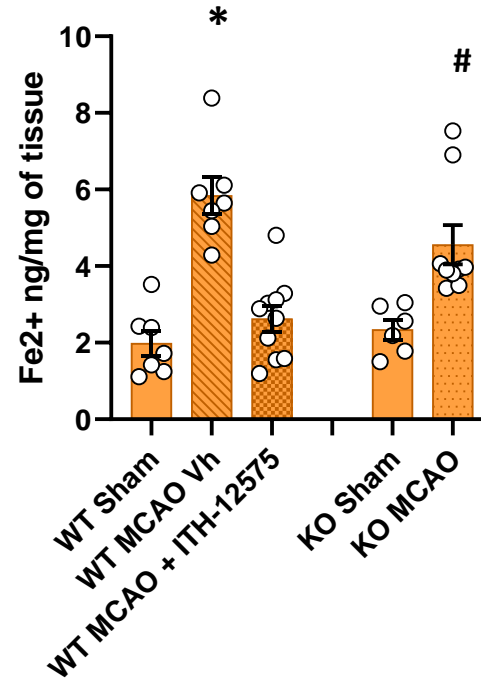


Total Fe levels



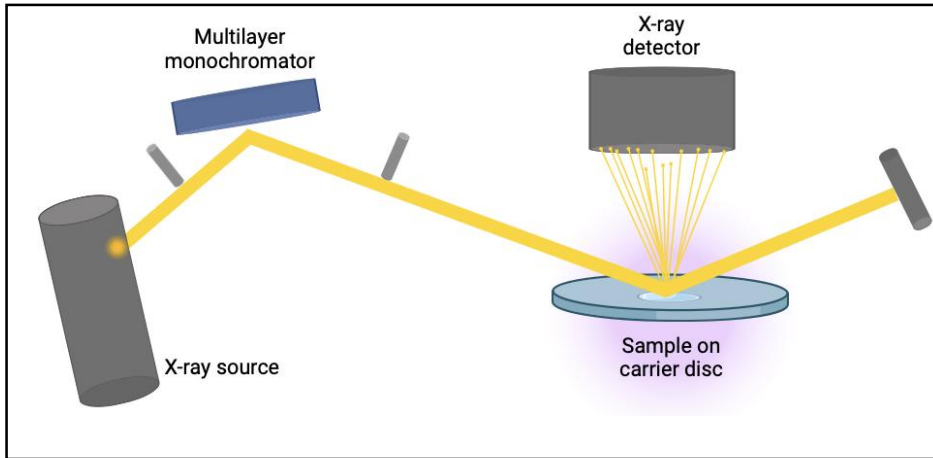
Mean with SEM. One-way ANOVA with Dunnett's test for multiple comparisons.

Fe<sup>2+</sup> levels

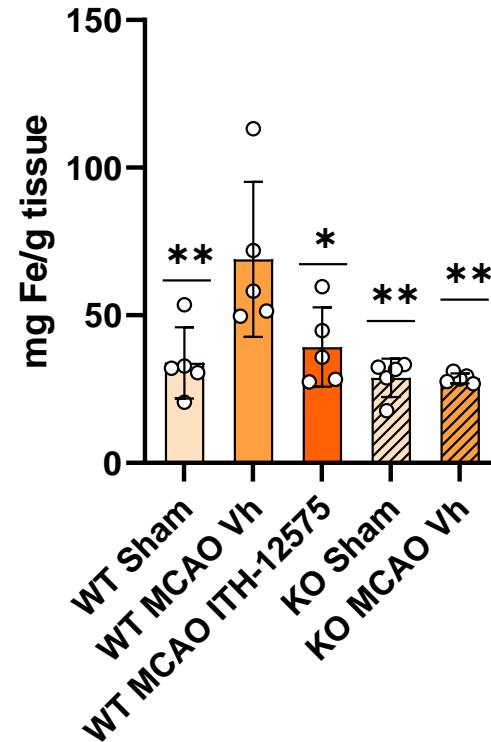


Mean with SEM. One-way ANOVA with Dunnett's test for multiple comparisons.

Total reflection X-ray fluorescence



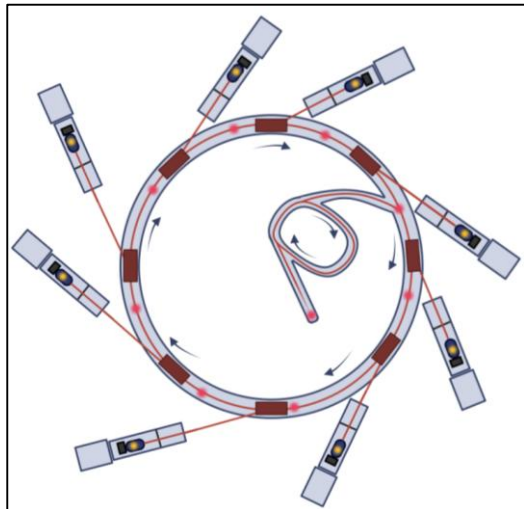
TXRF



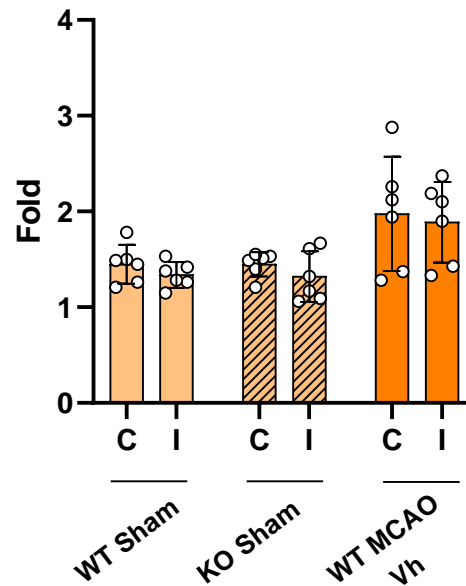
### Synchrotron X-ray fluorescence



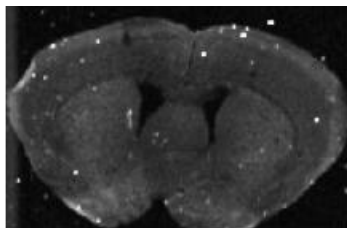
✦ Diamond Light Source, UK



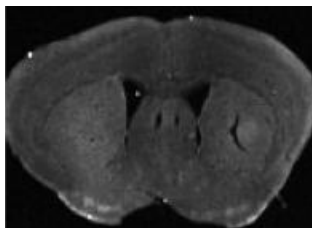
### SXRF



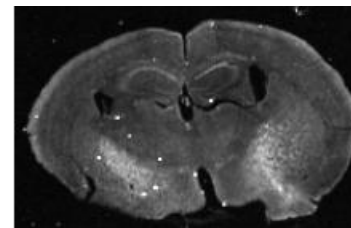
WT Sham



KO Sham

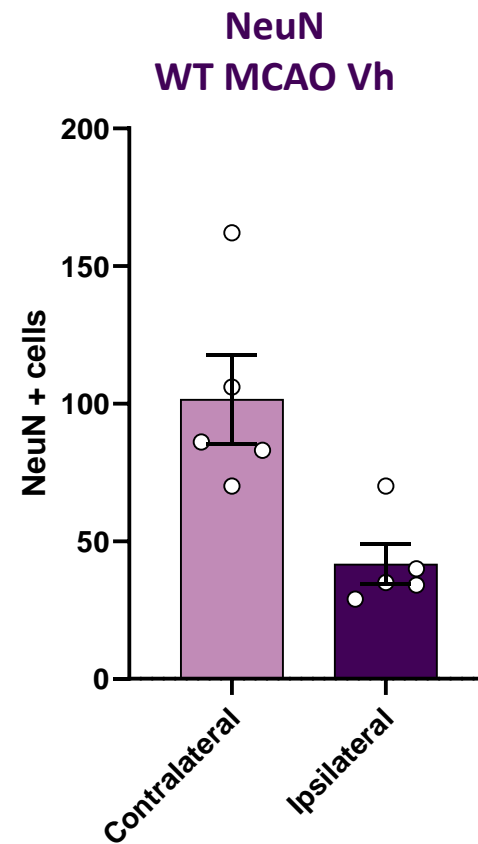
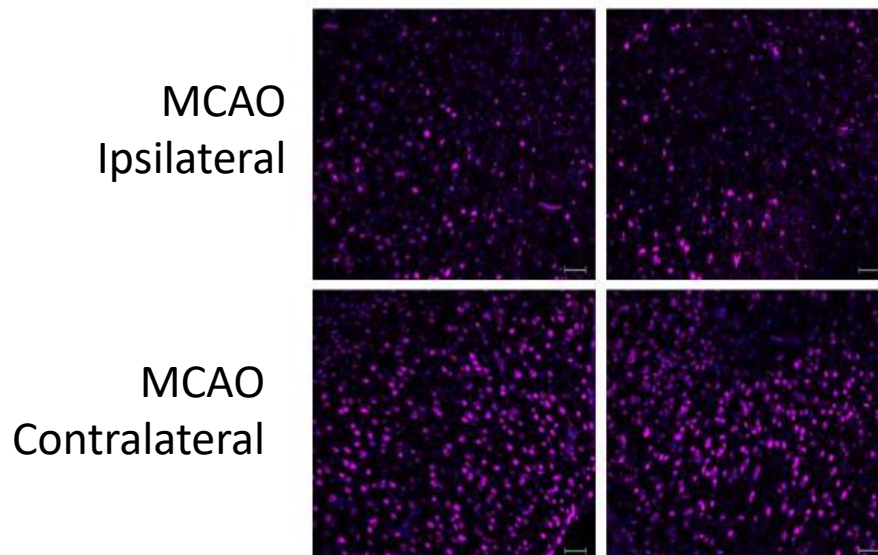


WT MCAO Vh



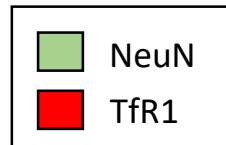
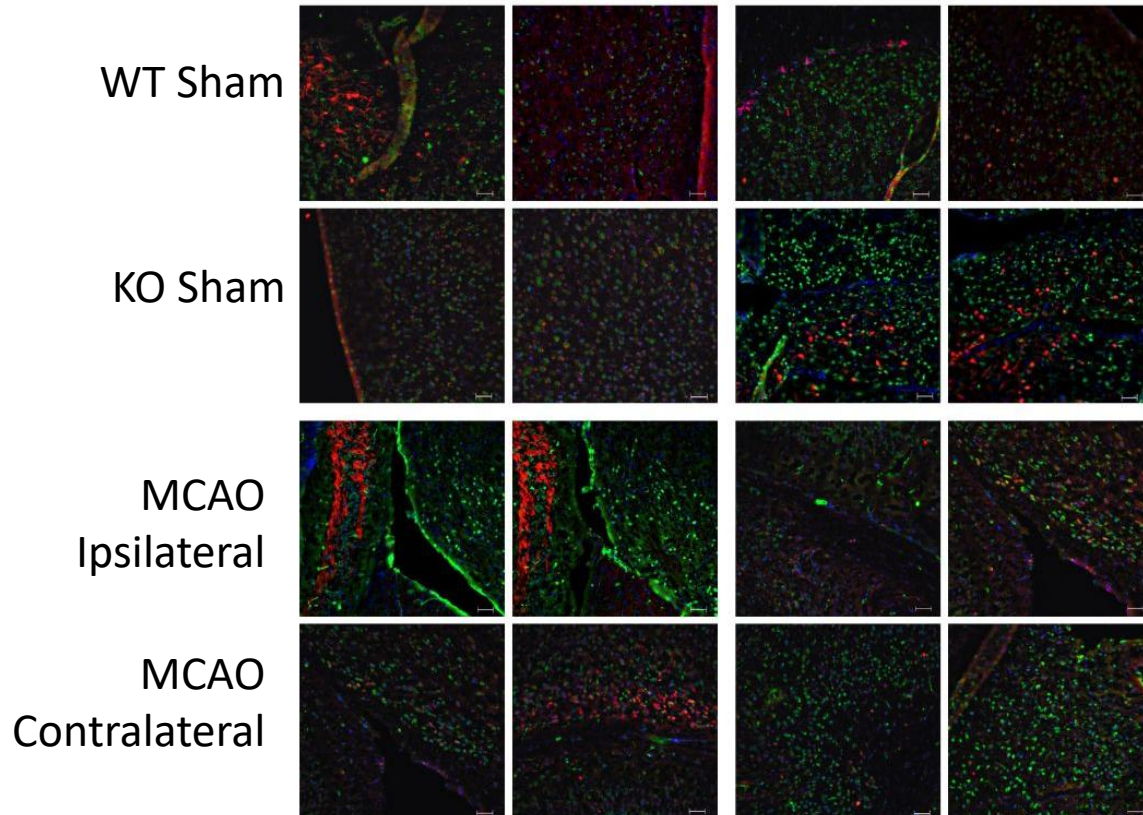


We measured ferroptosis markers by **immunofluorescence** and compared the **MCAO** mice hemispheres.



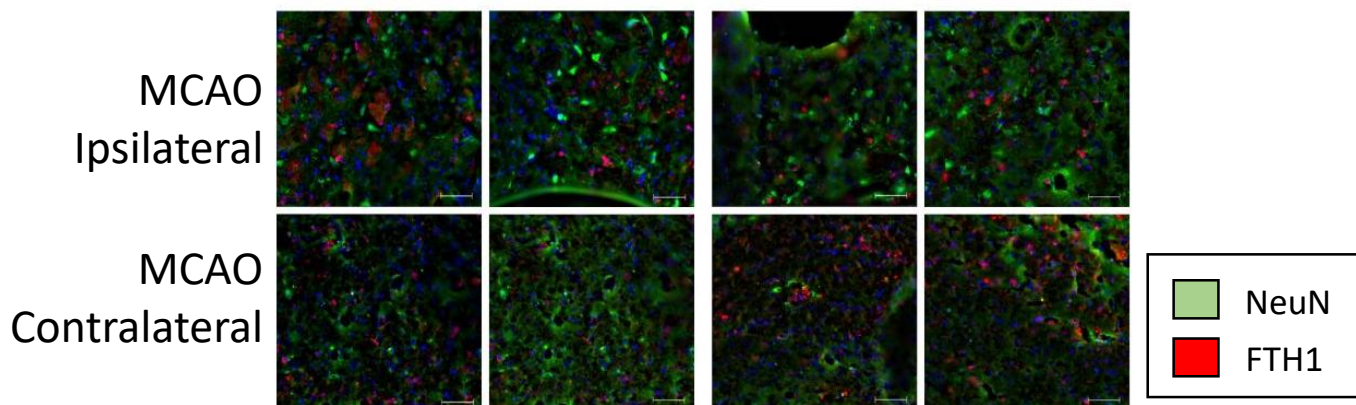


### TfR1

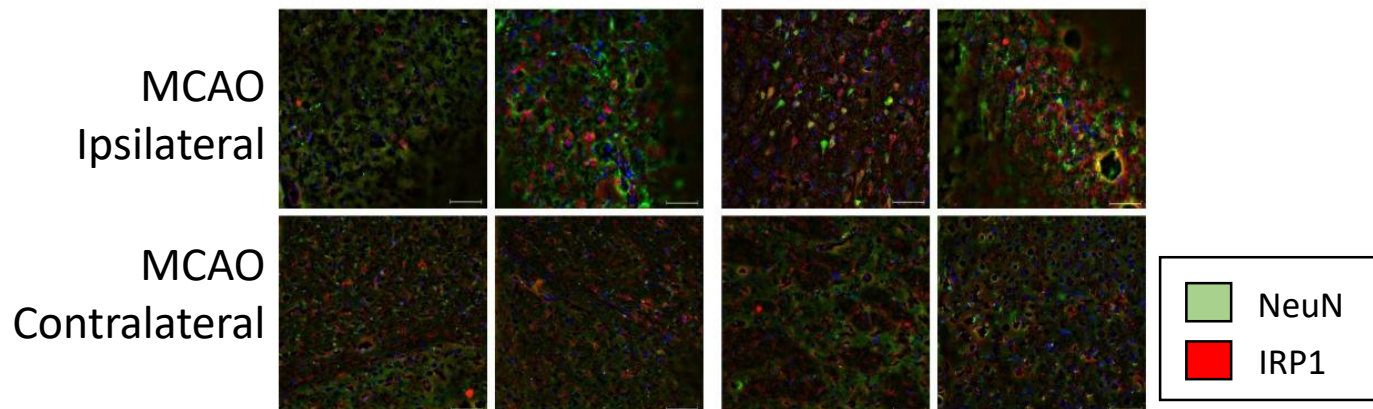




### FTH1



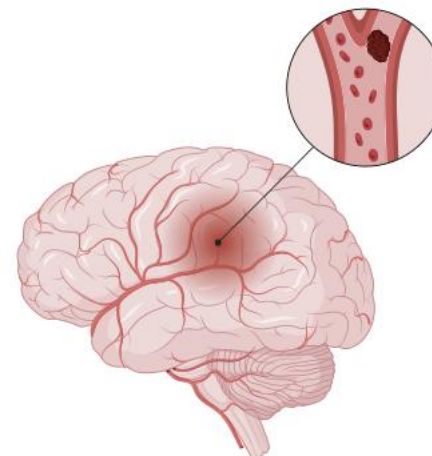
### IRP1



## Ischemia - Reperfusion

higher

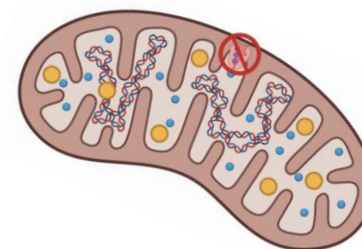
- Lipid peroxidation
- ROS production
- Cell death
- Transferrin receptor protein and mRNA levels
- Cell and brain iron content



## Ischemia – Reperfusion with NCLX inhibitors

lower

- Lipid peroxidation
- ROS production
- Cell death
- Transferrin receptor protein and mRNA levels
- IRP1 levels
- Cell and brain iron content



**NCLX** may play a role in **ferroptosis** during **ischemia-reperfusion**

# Acknowledgements

**Instituto de investigación  
sanitaria Hospital  
Universitario de La Princesa**

**Antonio Martínez**

Martín Hugo

Ana Belén López

Icía Polo

Javier Prieto

Ana María Pacheco

Esther Fuertes

**Javier Egea**

María Álvarez Rubal

**Cristóbal de los Ríos**

Lucía Viejo

**King's College London**

Po-Wah So

**Diamond Light Source**

Tina Geraki

**HI-STEM gGmbH**

Hamed Alborzinia



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