Zstress: A Zebrafish High Throughput Phenotypic Screen And Validation In The Sod1 G93a Mouse Model

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Introduction:

Amyotrophic Lateral Sclerosis (ALS) is a rapid neurodegenerative disorder usually resulting in death 3-5 years from the onset of clinical symptoms. The disease progresses as motor neurons die causing muscle denervation and paralysis.

Currently Riluzole is the only FDA approved drug for the treatment of ALS and extends life marginally by 3-4months. The exact mode of action of Riluzole is unknown, but is hypothesised to function via glutamate inhibition, blocking voltage gated sodium channels with reduction of pre-synaptic glutamate release and inhibition of NMDA receptors.

Zebrafish are vertebrates, ideal for the study of early disease processes as they are transparent during early development, show high fecundity and are amenable for high throughput drug screening. Towards utilizing these beneficial attributes to better understand ALS pathophysiology and develop a tool for drug discovery, we developed a mutant sod1 transgenic zebrafish. Transgenic zebrafish with mutant sod1 develop classical hallmark symptoms of ALS seen in humans and rodents[1].

Background – Generation of mutant SOD1 zebrafish

Mutation: G93R-Glycine to arginine (GGT > CGT)

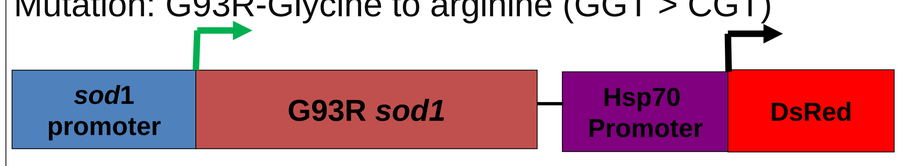


Figure 1: G93Ros10-Sh1 – High expressing mutant SOD1 line (4x SOD1 over expression). Hsp70 promoter tagged to DsRed fluorescent molecule as a marker of neuronal stress.

Interneurons are stressed in early development in the mutant SOD1 zebrafish[2,3]

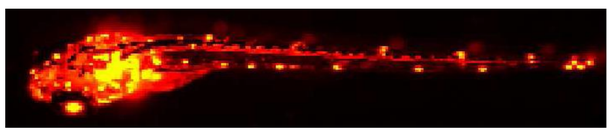


Figure 2: 24hpf mutant sod1 zebrafish activate the heatshock stress response (HSR) in the absence of heatshock in the spinal cord and hindbrain. This is caused by mutant SOD1 toxicity causing hsp70 upregulation as shown by DsRed fluorescence.

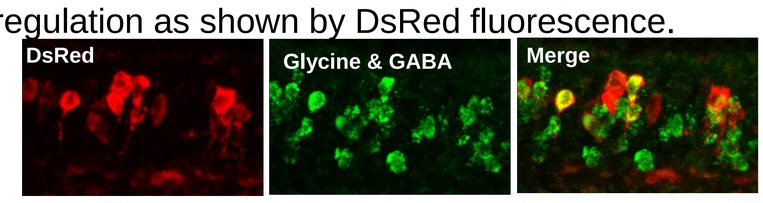


Figure 3: The stressed neurons are Glycine and GABA positive interneurons and show stress as early as 24hpf. This indicates that inhibitory interneuron dysfunction precedes the motor neuron loss seen at later stages.

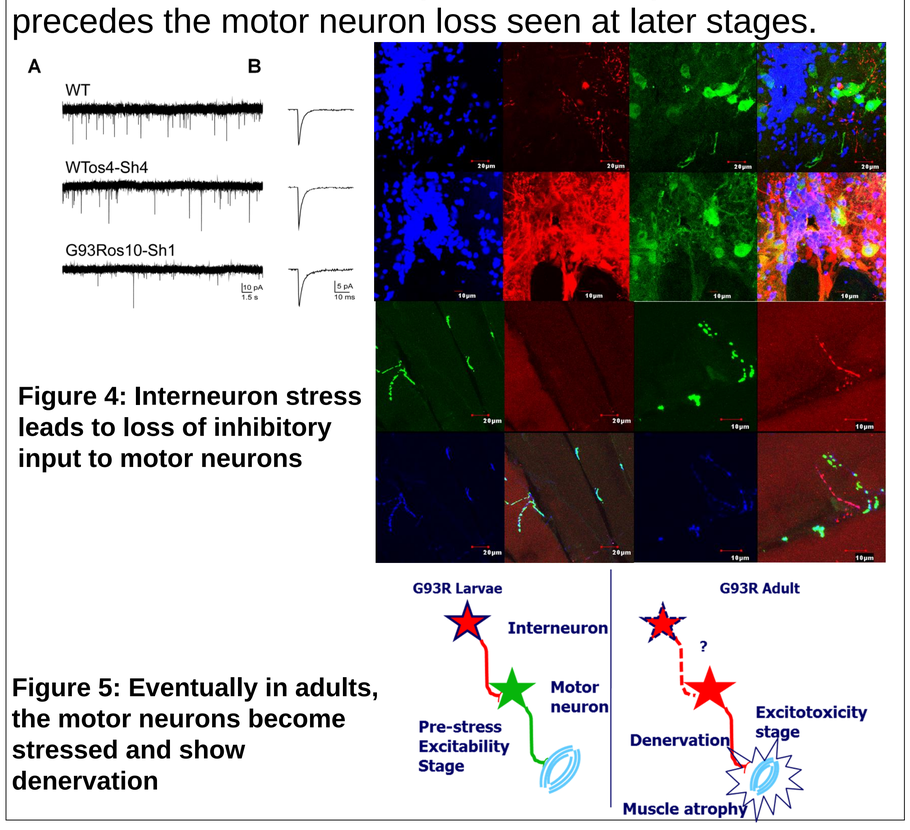


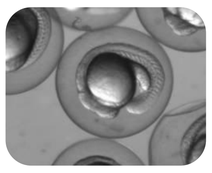
Figure 4: Interneuron stress leads to loss of inhibitory input to motor neurons

Figure 5: Eventually in adults, the motor neurons become stressed and show denervation

Drug screening protocol

Figure 6: Transgenic zebrafish are dosed with 2000 compounds from the spectrum library from 48hpf to 6dpf.

- G93ros10 x AB pair mating crosses
- Embryos kept at 28°C until 48hpf



- 24hpf – manual dechorination of embryos which are maintained in sterile embryo medium at 28°C

- Individual embryos are loaded into a 96 well plate in 50µl of embryo media

- Incell system is used to genotype the fish for DsRed fluorescence
- Brightfield imaging is undertaken to ensure embryos are not damaged

- Echo liquid handling system dispenses 2.5nl droplets from compound libraries
- All drugs dosed at 10µM

- At 6dpf the supernatant is then read for fluorescence on a microplate reader

Screen throughput

48 drugs per plate
4 plates per day = 192 drugs per day
2 runs per week = 384 drugs per week

The Spectrum library is 2000 compounds so a duplicate screen can be undertaken in 10 weeks.

Screen results and interpretation

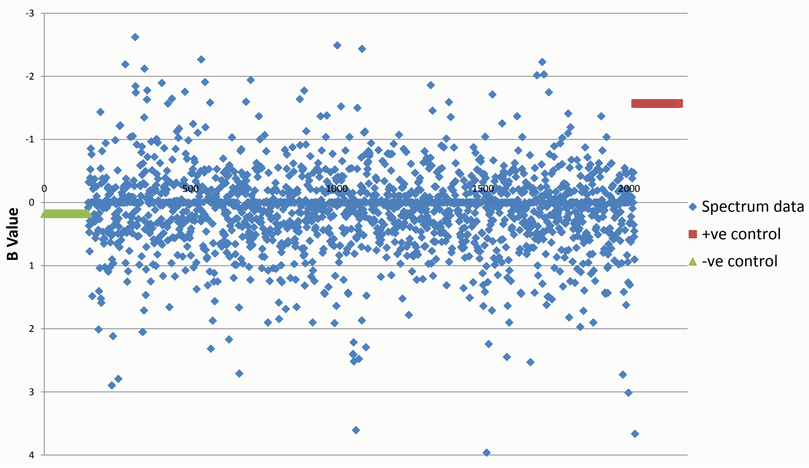


Figure 7: Data from a replicate of the primary screen. Data show both activators and inhibitors of fluorescence. The flow chart below highlights the possible outcomes from the screen.

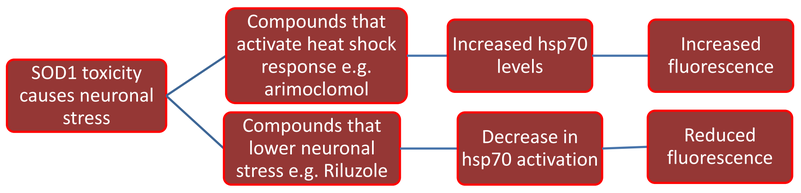
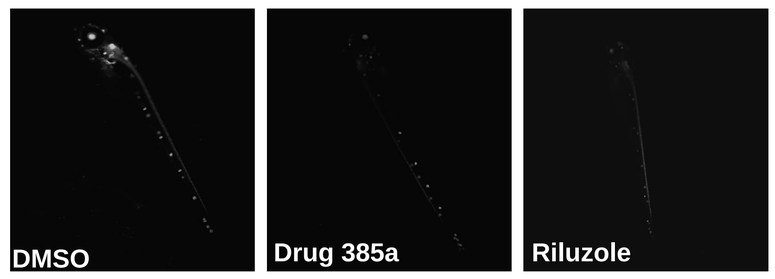


Figure 8: Compound 385a shows riluzole like efficacy in reducing neuronal stress



InCell images of the DsRed fluorescence in G93Ros10 zebrafish treated with the associated compounds from 48hpf-6dpf.
With Drug 385a and Riluzole a large reduction in fluorescence is seen in the hindbrain and spinal cord of the zebrafish. Both compounds have a sedative effect on the zebrafish and combination dosing is being investigated to identify any potential synergistic effect of the drugs at a non-sedative dose.

Figure 9: Compound 385a shows additive effect with riluzole in reducing neuronal stress

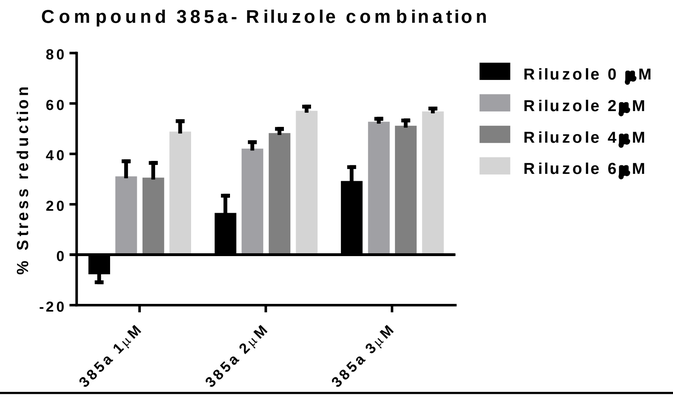
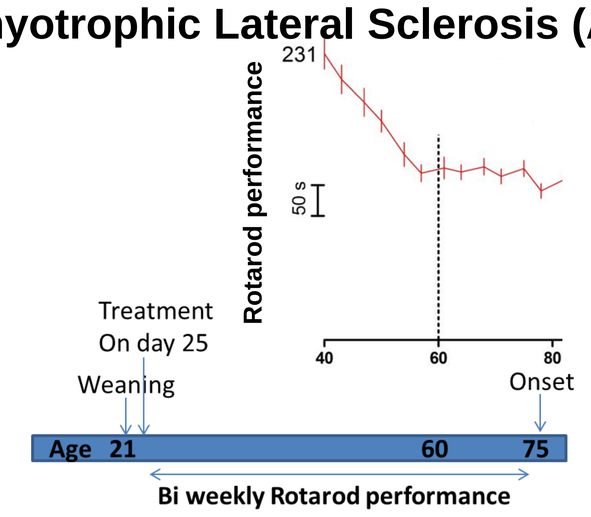


Figure 10: Rapid Pre-clinical Screening in the SOD1^{G93A} Transgenic Mouse Model of Amyotrophic Lateral Sclerosis (ALS)[4]



Study Design:
3 Arm study with DMSO and riluzole as negative and positive controls to assess efficacy of compound 385a as compared to riluzole.
Dosage:
Control arm: 20 female transgenics treated with drinking water and gavage with sesame oil.
Riluzole arm: 20 female transgenics treated with 240µg/ml riluzole in drinking water and gavaged with sesame oil.
Compound 385a arm: 20 female transgenics treated with drinking water and 12mg/Kg compound 385a in sesame oil.
Results:
1. Compound 385a is safe for chronic dosing . No untoward side-effects noted with over 120 days of dosing.
2. CNS levels of unmodified drug were modestly lower than required therapeutic levels after 5 day dosing.

3. Future Directions:
Investigate NMJ rescue at 60dpf in 385a dosed animals.
Perform combination dosing with riluzole in the mouse model

References:

1. Ramesh T et.al. 2010.DMM. 3(9-10):652.
2. McGown, A. *et al* (2013). Ann Neurol., 73: 246–258.
3. Ramesh T et.al. (2014). J Neurol Neurosurg Psychiatry.85(11):1288
4. Mead RJ et. al. (2011). Plos One. 6(8):e23244

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