Mechanism of Fenfluramine in a Zebrafish Mutant Model of Dravet Syndrome?

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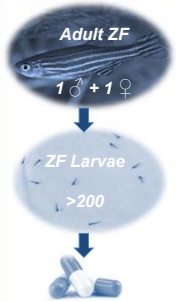
INTRODUCTION

Epilepsy and Dravet syndrome



- Epilepsy= common neurological disorder (up to 65 million people worldwide)
- 30% not responding to current anti-epileptic drugs (AEDs) (i.e. drug-resistant)
- Etiology: genetic, structural-metabolic or unknown
  - *SCN1A* (*neural sodium channel, type 1, subunit α*): most clinically relevant epilepsy gene
  - *SCN1A* mutation in 80% of Dravet syndrome patients[1]
- Dravet syndrome: **drug-resistant**, rare and severe epilepsy syndrome; starting in 1st year of life, accompanied by cognitive and behavioral impairments

➔ *need for* ***new, innovative AEDs***

Zebrafish (ZF) and drug discovery

- Small vertebrate model → ZF larvae: validated for fast, cost-efficient AED screening
- **Serotonin** receptors (=5-hydroxytryptamine, 5-HT-R): suggested, novel target of future AEDs[2]
- Experimental serotonergic drug in clinical trial for Dravet syndrome patients (**fenfluramine**)[1]:
  - Anti-epileptiform activity in *morpholino knockdown* ZF model of Dravet syndrome[3]
  - Severe, possible **side effects** due to stimulation of **5-HT$_{2B}$** receptor (valvulopathy, cardiotoxicity)
- Mechanism of action: involvement of 5-HT *or* other receptors?

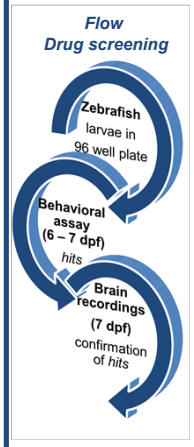
➔ ***from bed-side to bench and back to bed***

RATIONALE

How do *scn1Lab* mutant zebrafish larvae recapitulate Dravet syndrome and aid innovative anti-epileptiform drug discovery?

- Abnormal behavior and brain activity in *scn1Lab*$^{-/-}$ mutants (-/-) vs. wildtype *scn1Lab*$^{+/+}$ (+/+)[4]
- Drug screening of serotonergic analogs in locomotor assay leading to *hits*
- Confirmation of *hits* by measuring local field potentials and elucidating the mechanism of action

METHODS



1) Locomotor behavior and brain activity

***Locomotor behavior** 4 - 8 dpf*

- Distance travelled in large movements for 10 minutes (min) after 30 min habituation
- High throughput (**HT**) with an automated tracking device (ZebraBox™ apparatus; Viewpoint, Lyon, France)

-/-

+/+

***Brain activity** 7 dpf*

- Invasive forebrain open-field recordings for 10 min
- Low throughput testing (**LT**), ZF larva embedded in 2% low-melting-point agarose (Digidata 1440A digitizer; Axon instruments, USA)

2) Neurotransmission

***LC-ECD** 7 dpf*

Determination of the amount of neurotransmitters in heads of ZF larvae by Microbore Liquid Chromatography with Electrical Chemical Detection (LC-ECD)[5]

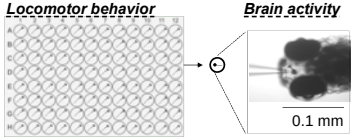
3) Drug screening

***Locomotor behavior** 6 - 7 dpf*

- Treatment of fenfluramine and serotonergic agonists at maximum tolerated concentration (MTC)
- Combined treatment of fenfluramine and serotonergic antagonists at MTC

***Brain activity** 7 dpf*

Confirmation of *hits* if a significant decrease in epileptiform brain activity

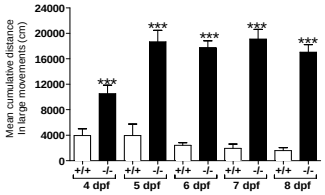


RESULTS

1) Behavior and brain activity

***Locomotor behavior** 4 - 8 dpf*

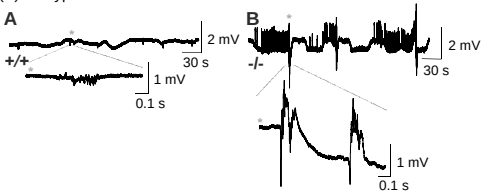
Higher activity of *scn1Lab*$^{-/-}$ mutants, compared to wildtype *scn1Lab*$^{+/+}$ = may reflect tonic-clonic onset of seizures in Dravet syndrome patients



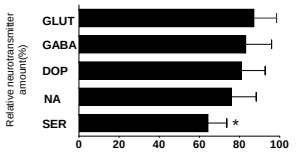
Quantification of distance in large movements (lardist) of ZF larvae. Black bars represent *scn1Lab*$^{-/-}$ mutants and white bars represent wildtype *scn1Lab*$^{+/+}$ (24 larvae on each day, experiment in triplicate)

***Brain activity** 7 dpf*

Epileptiform brain activity (representative 5 min recording) =amplitude >3 times the background noise and duration >50 milliseconds (ms) in (**B**) *scn1Lab*$^{-/-}$ mutants, compared to baseline in (**A**) wildtype *scn1Lab*$^{+/+}$



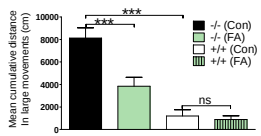
2) Neurotransmission



Decrease in serotonin in heads of *scn1Lab*$^{-/-}$ mutants. Comparison to wildtype *scn1Lab*$^{+/+}$ (Normalized against these age-matched controls and expressed in percentage (%) (54 head homogenates for each condition)

3) Drug screening

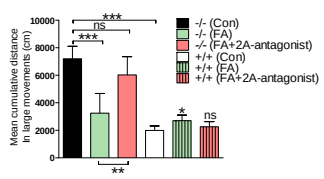
***Locomotor behavior** 6 - 7 dpf (single treatment)*



Fenfluramine specifically lowers locomotor behavior in *scn1Lab*$^{-/-}$ mutants. (10 larvae for each condition, experiment in triplicate)

➔ **Fenfluramine and the 5-HT$_{1D}$-, 5-HT$_{1E}$-, 5-HT$_{2A}$-, 5-HT$_{2C}$-, and 5-HT$_7$-agonist decrease epileptiform locomotor behavior in *scn1Lab*$^{-/-}$ mutants**

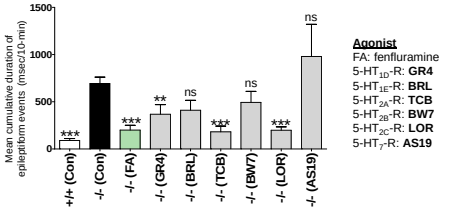
***Locomotor behavior** 6 - 7 dpf (combined treatment)*



Combined treatment experiment. (10 larvae for each condition, experiment in triplicate)

➔ **Counteraction of fenfluramine-induced decrease by the 5-HT$_{1D}$-, 5-HT$_{2A}$-, 5-HT$_{2C}$-antagonist**

***Brain activity** 7 dpf*



Agonist
FA: fenfluramine
5-HT$_{1D}$-R: **GR4**
5-HT$_{1E}$-R: **BRL**
5-HT$_{2A}$-R: **TCB**
5-HT$_{2B}$-R: **BW7**
5-HT$_{2C}$-R: **LOR**
5-HT$_7$-R: **AS19**

Decrease in epileptiform events. Comparison to VHC-treated *scn1Lab*$^{-/-}$ mutants (-/-(Con)), represented by a black bar (at least 10 larvae per condition)

➔ **Fenfluramine and the 5-HT$_{1D}$-, 5-HT$_{2A}$- and 5-HT$_{2C}$-agonist decrease epileptiform brain activity in *scn1Lab*$^{-/-}$ mutants**

CONCLUSIONS

- *scn1Lab*$^{-/-}$ mutant zebrafish larvae mimic Dravet syndrome, in line with the findings of Baraban *et al.*[4]
- Reduction of **serotonin** in DS ZF larvae = highlights role of serotonin → also in animal and human studies of **drug-resistant epilepsies**[6]
- Fenfluramine, the 5-HT$_{1D}$-, 5-HT$_{2A}$, and 5-HT$_{2C}$-agonist reduced epileptiform locomotor and brain activity → **5-HT$_{2B}$-receptor not involved!**
- 5-HT$_{1D}$-, 5-HT$_{2A}$, and 5-HT$_{2C}$-antagonists counteracted the decrease by fenfluramine in locomotor assays → **5-HT$_{2B}$-receptor not involved!**

➔ ***Contributes to elucidation mechanism of fenfluramine*** ➔ ***Highlights 3 serotonin receptor subtypes (5-HT$_{1D}$-, 5-HT$_{2A}$- and 5-HT$_{2C}$) as interesting targets for future innovative AEDs***

1. Ceulemans, B. et al. Epilepsia 53, 1131–9 (2012).
2. Löscher, W., Klitgaard, H., Twyman, R. E. & Schmidt, D. Nat. Rev. Drug Discov. 12, 757–76 (2013).
3. Zhang, Y. et al. PLoS One 10, e0125898 (2015).
4. Baraban SC, Dinday MT, Hortopan G A. Nat. Commun. 4: 2410 (2013).
5. Sarre S. et al. Methods Mol. Biol. 72: 185–96 (1997).
6. Fonseca NC. et al. Epilepsy Res.111: 18–25 (2015).

STATISTICS**: Locomotor behavioral analyses: One-way ANOVA followed by Dunnett's multiple comparison tests; Brain activity analyses: Mann-Whitney U test; Neurotransmission: Student's t-test (passed D'Agostino & Pearson omnibus normality test).* ***Statistically significant differences represented by (*) if p<0.05, (**) if p<0.001 and (***) if p<0.0001