Report of research project

“Effect of neuroprotective molecules dietary supplementation on ARSACS zebrafish model development”

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Summary: ARSACS is considered the second most common form of autosomal recessive hereditary ataxia in Europe and Canada, and it remains an incurable condition, affecting a significant number of patients worldwide. Although mouse models mimic largely the disease progression seen in humans, their use in the validation of effective therapies has not yet been proposed. Recently, we generated a zebrafish *sacs* knock-out mutant line that replicates main features of ARSACS and demonstrated that both acetyl-DL leucine (ADLL) and tauroursodeoxycholic acid (TUDCA) improved locomotor and biochemical phenotypes in treated mutant larvae in short term treatments. In this work we included these neuroprotective agents as feed supplement in the fish diet (with inclusion levels equal to 100 mg/L). The experimental diets was provided to fish from the beginning of their larval stage (when they start feeding independently) until adult life (12 months). During development, fish we monitored growth parameters, locomotor performances, social and cognitive behavior. Through this preclinical study, we evaluated the effectiveness of these functional ingredients to ameliorate the phenotype observed in *sacs* deficient zebrafish model during adult lifespan. We found that both ADLL/Tanganil™ and TUDCA are able to improve locomotor performance during the "novel tank" and "open field test". Adult knock-out *sacs* fish showed an enhancement in social and cognitive behaviour during the T-maze test and improved, respectively, spatial and retention memory, as assessed by the novel object recognition test. These findings suggest that the pretreatment with ADLL/Tanganil™ or TUDCA could play an important role to ameliorate locomotor and cognitive impairment observed in ARSACS zebrafish model supporting further optimization of these neuroprotective molecules in other preclinical and clinical settings.

1. Introduction

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is an early-onset, neurodevelopmental and neurodegenerative condition presenting with ataxia, spasticity, and peripheral neuropathy. In addition to the classic triad of symptoms many affected individuals (though not all) exhibit mild intellectual disability [1]. In the absence of FDA-approved medications for the treatment of degenerative ataxia [2], there are few options available to counteract the progressive degeneration of Purkinje cells (PC) seen in patients [3]. Repurposing of FDA-approved drugs likely acting on multiple molecular targets seems a good avenue to be explored at a preclinical level. Growing evidence supports the potential of zebrafish as an alternative model in preclinical research of neurodegenerative disorders, complementing existing murine models because lower ethical impact in agreement with the 3R rules. Recently, we developed a zebrafish *sacs* deficient mutant line that replicates main features of ARSACS [4] and we showed the potential role of ADLL/Tanganil™ and TUDCA to ameliorate the phenotype observed in mutant larvae [4]. ADLL/Tanganil™ is able to modulate glutamate neurotransmission in the cerebellum through the branched-chain amino acid transferase, which is important both for glutamate release during excitation and for the activation of metabotropic glutamate receptors required for cerebellar plasticity [5]. Mouse model of Sandhoff disease (*Hexb*$^{-/-}$) treated with ADLL (0.1 g/kg/day) from 3 weeks of age showed a modest but significant increase in life span, accompanied by improved motor function [5]. TUDCA showed neuroprotective effects by acting as a mitochondrial stabilizer and antiapoptotic agent in several models of neurodegeneration [6]. Recently it has been demonstrated that TUDCA-treated aged mice (300 mg/kg) displayed increased energy expenditure and metabolic flexibility, as well as a better cognitive ability [7]. Moreover, the pretreatment of TUDCA (500 mg/kg, ip) in a chronic model of Parkinson's disease (PD) with severe dopaminergic degeneration was able to protect against dopaminergic neuronal damage, prevented the microglial and astroglia activation [8]. In this study, we explored the use of adult fish model of ARSACS to test the effectiveness of ADLL/Tanganil™ or TUDCA diet supplementations in ameliorating the locomotor and cognitive impairment observed in ARSACS zebrafish model.

2. Results

In this work, single purified drug have been added on self-made experimental zebrafish feeds according to the indications published in [9]. In total, 4 different diets have been prepared: 2 control diets and 2 experimental diets, containing ADLL or TUDCA with inclusion levels equal to 0,1 %. We provided the experimental diets from five days post fertilization until the adult stage (12 months) 2 times a day. Control fish were kept under the same conditions used for the treated fish, but without exposure to any treatment. Firstly, during the first month of feeding we evaluated the survival (%) figure 1A. During the experimental period, no marked abnormalities or major differences were observed in feeding behavior between the diet groups. Both experimental diets did not induce significant mortality (figure 1A) increasing the survival rate of sacs deficient larvae during the first period of development. Next, we measured the body weight at 3 months old of adult male and female zebrafish observing that both experimental diets lead to an improvement in growth parameter.

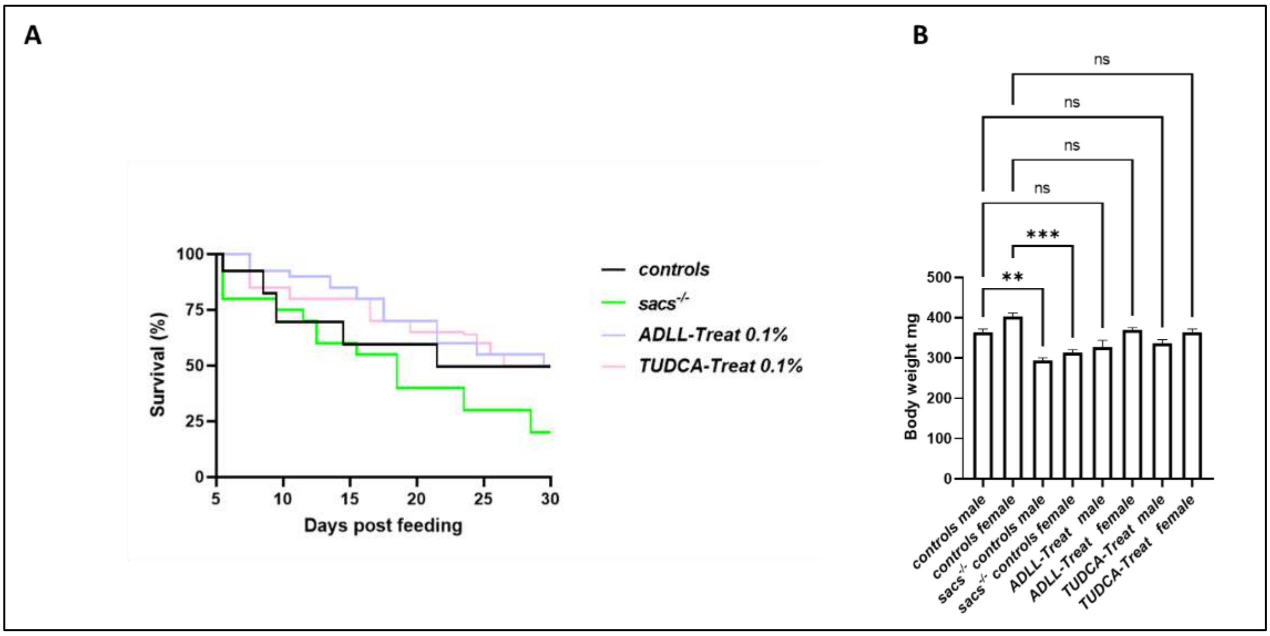

Figure 1: Effects of ADLL or TUDCA diets treatment on the survival rate and growth of zebrafish juvenile and adult ARSACS model. N.S, no significant difference, $^{**}P < 0.01$, $^{***}P < 0.001$, by one-way ANOVA and LSD post-hoc test.

To explore the effects of both ADLL/Tanganil and TUDCA diet supplementation on our ARSACS model we used validated protocols. The following read-out have been evaluated: a) **locomotion behavior and anxiety** b) **sociability** c) **cognition** and ability to cope with changing environments. All the experiments of locomotion, social and cognitive behavior have been performed and analysed using the automated tracking system software "Ethovision XT12" (Noldus Information Technology, Wageningen, The Netherlands).

A) Locomotion behavior and anxiety:

The locomotor activity of adult *sacs*$^{-/-}$ zebrafish was examined performing novel tank diving test (figure 2A). We observed that adult *sacs*$^{-/-}$ showed reduced locomotor activity in terms of both velocity and distance covered compared with control siblings (figure 2B). Then, we measured the effect of experimental diets on locomotor performances recording the spontaneous motor activity (distance travelled and average velocity). We observed a significant improvement of locomotor performances in both experimental diets of *sacs*$^{-/-}$*-TUDCA/ADLL* treated fish (figure 2A-B). Although the novel tank diving test is commonly used for analysing exploratory and locomotor profiles, this task is often used to analyses anxiety-like behaviour [10]. The normal response to novel environment is an active response associated with the natural drive to explore unfamiliar places [10]. Typically zebrafish behavior is to settle at the bottom of the apparatus for the first few minutes of testing, followed by top exploration [10]. In this experiments, behavioural activity was recorded in front of the tank using a webcam for 5 min to analyse diving response. The tank was virtually divided in two areas (bottom and top) and time at the bottom was used to assess anxiety-like phenotypes. *Sacs*$^{-/-}$ adults showed anxiety, a passive response (time spent to bottom half) associated with low levels of exploratory activity (figure 2C). Surprisingly, the pretreatment with the experimental diets lead to an overall behavioural improvement in *sacs*$^{-/-}$*-TUDCA/ADLL* treated fish compared to *sacs*$^{-/-}$ -no treated fish, as shown in figure (2A-C).

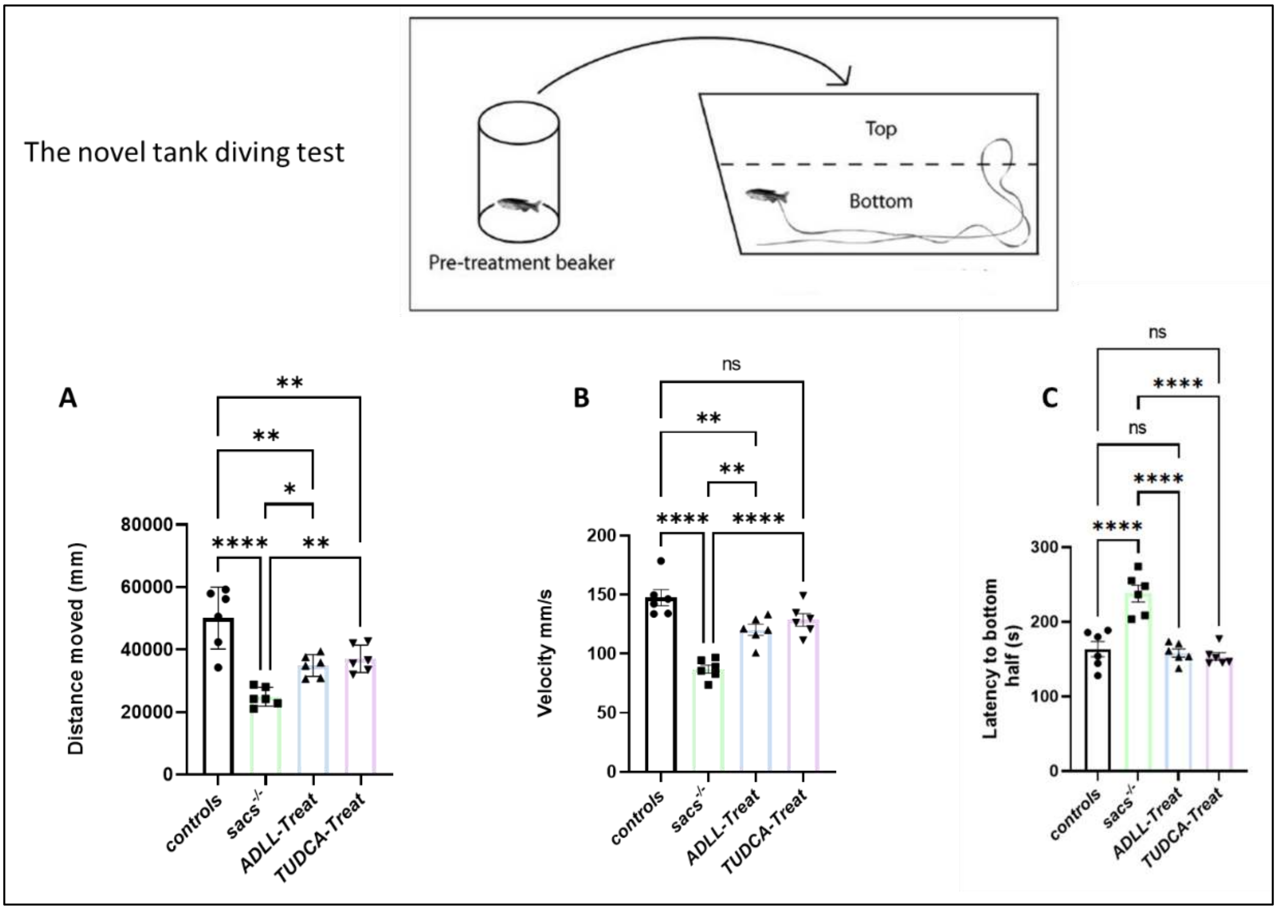

Figure 2. Zebrafish locomotor activity and anxiety-like behaviour in **Novel test** during 5 min. A) Total distance travelled and B) average speed while moving (mm/s). C) Time in bottom area (s). Data are expressed as mean ± S.E.M (N=6 per group). Each circle indicates individuals used for each treatment and the asterisk above bars indicates significance. N.S, no significant difference, * $P < 0.05$,**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ was calculated by one-way ANOVA and LSD post-hoc test. Image modified from [11].

One of the most commonly used behavioural endpoints measured in preclinical studies is thigmotaxis (or "wall-hugging") [12]. Animals that are engaged in thigmotaxic behaviour strongly avoid the center of an arena and stay or move in close proximity to the boundaries of a novel environment. This behaviour is evolutionarily conserved and displayed by a wide range of species [12]. To determine whether absence of *sacs* alters thigmotaxis, the 4 groups of adult zebrafish (controls, *sacs*$^{-/-}$, *sacs*$^{-/-}$-*ADLL treated* and *sacs*$^{-/-}$-*TUDCA treated*) were assessed considering their exploration behaviour (distance moved and velocity) and for the percentage of time spent in the inner zone (center of the tank). Compared with controls$^{+/+}$ zebrafish, *sacs*$^{-/-}$ zebrafish travelled less distances in the arena and spent considerably more time in the peripheral area than in inner zone (center of the tank) (figure 3A-D). Controls$^{+/+}$ individuals habituated faster and showed decreased thigmotaxis (less anxiety), while *sacs* deficient adult zebrafish ones are less prone to leave the security of the side areas of the open tank and presented higher anxiety (figure 3A-D). Albeit in a moderate way, *sacs*$^{-/-}$-*ADLL treated* and *sacs*$^{-/-}$-*TUDCA* treated fish showed a more intense, exploratory and locomotor behavioural response than *sacs*$^{-/-}$ *no treated* fish (figure 3B-D).

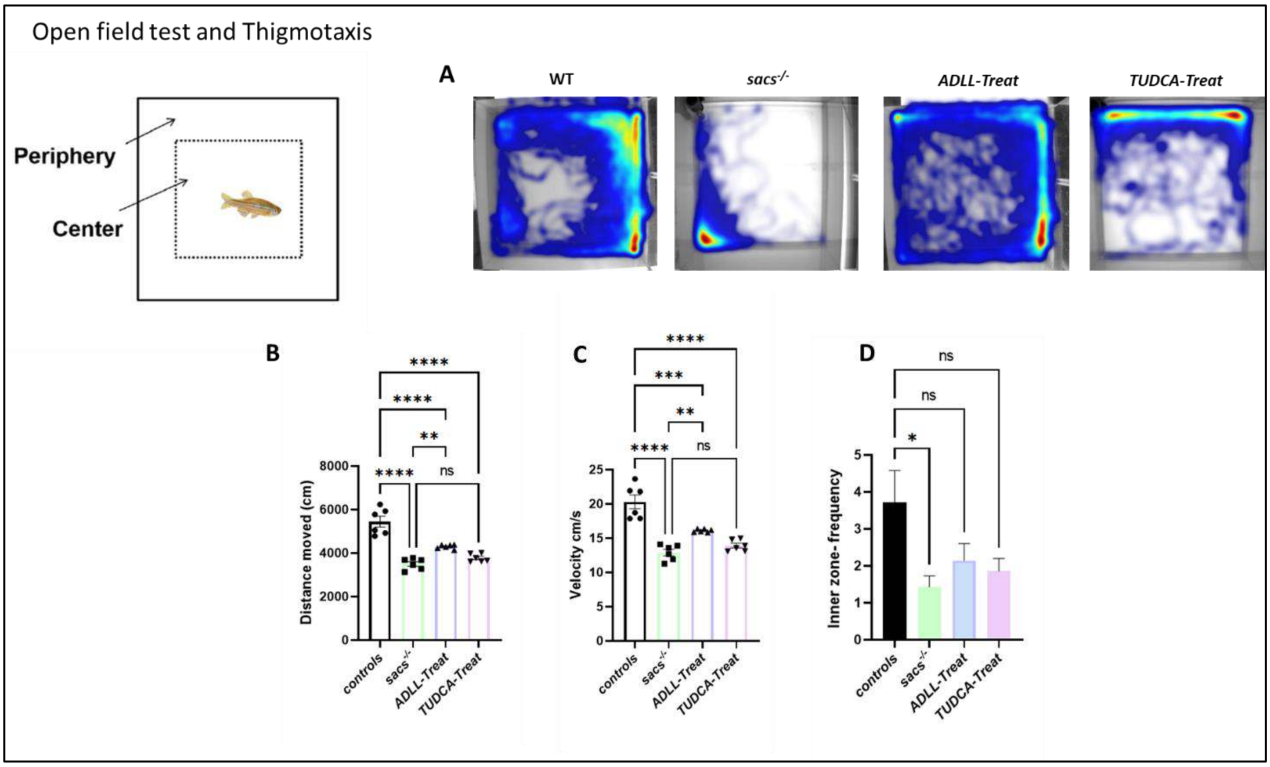

Figure 3. Schematic diagram of the **Open-field test** and **Thigmotaxis** test of adult zebrafish. In the analysis of thigmotaxis test, the area of the peripheral zone is equal to the center zone (dotted line). Data are presented as mean ± S.E.M (N=6 per group). A) Heat maps. Single adult fish was exposed to an open field arena for 10 min, assessing the amount and temporal patterning of their exploration. B) Total distance travelled (cm) and C) average speed while moving (cm/s). D) Frequency in the inner zone. Data are expressed as the mean± S.E.M. Each circle indicates individuals used for each treatment and the asterisk above bars indicates significance. N.S, no significant difference, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ was calculated by one-way ANOVA and LSD post-hoc test. Image modified from [13].

B) Sociability

As a shoaling species, zebrafish prefer to spend time in proximity to conspecifics [14]. Shoaling serves several adaptive functions, including protection from predators, as well as increasing foraging efficiency and mating success [15]. However, into a novel tank, stressed fish tend to swim closer together, with smaller inter-fish distance, than non- stressed fish [16,17]. Indeed tighter shoals are reflective of higher anxiety [18]. We therefore used the shoaling test [15] to assess the social cohesion among homogeneous groups of zebrafish (figure 4). In this assay, the four experimental group (controls, *sacs$^{-/-}$, sacs$^{-/-}$-ADLL treated* and *sacs$^{-/-}$-TUDCA* treated) zebrafish were placed in the testing tank. The average inter-fish distance was measured. As shown in figure 4A , *sacs$^{-/-}$* group appeared more stressed swimming closer together (distance between subject < 6 cm) than controls with a reduced exploration behavior. Again *sacs$^{-/-}$-ADLL treated* and *sacs$^{-/-}$-TUDCA treated* fish showed a more inter-fish distance than *sacs$^{-/-}$ no treated* fish (figure 4A). The social preference and interaction tests were subsequently performed as described in [19]. During the habituation phase, we continued to observe an upgrading of exploratory behaviour in *sacs$^{-/-}$-ADLL treated* and *sacs$^{-/-}$-TUDCA treated* fish comparing to *sacs$^{-/-}$-no treated* fish. In the test phase, a group of 4 conspecific zebrafish was placed in the right side, and a single fish for each experimental group (controls, *sacs$^{-/-}$*, *sacs$^{-/-}$-ADLL treated* and *sacs$^{-/-}$-TUDCA treated*) was placed on the left side (figure 4B). *Control* $^{+/+}$ zebrafish generally contacted the group on the right side and spent more time in the conspecific sector rather than the empty sector, showing a strong group tendency (figure4 B-C). In contrast, *sacs$^{-/-}$* zebrafish spent their time evenly throughout the region and exhibited reduced time ratio of social contacts with the peer group and distance ratio in the conspecific sector (figure4 B-C). Both supplemental diets improved the behaviour sociability of *sacs* deficient fish compared to *sacs*$^{-/-}$ no-treated mutant fish as show in figure 4.

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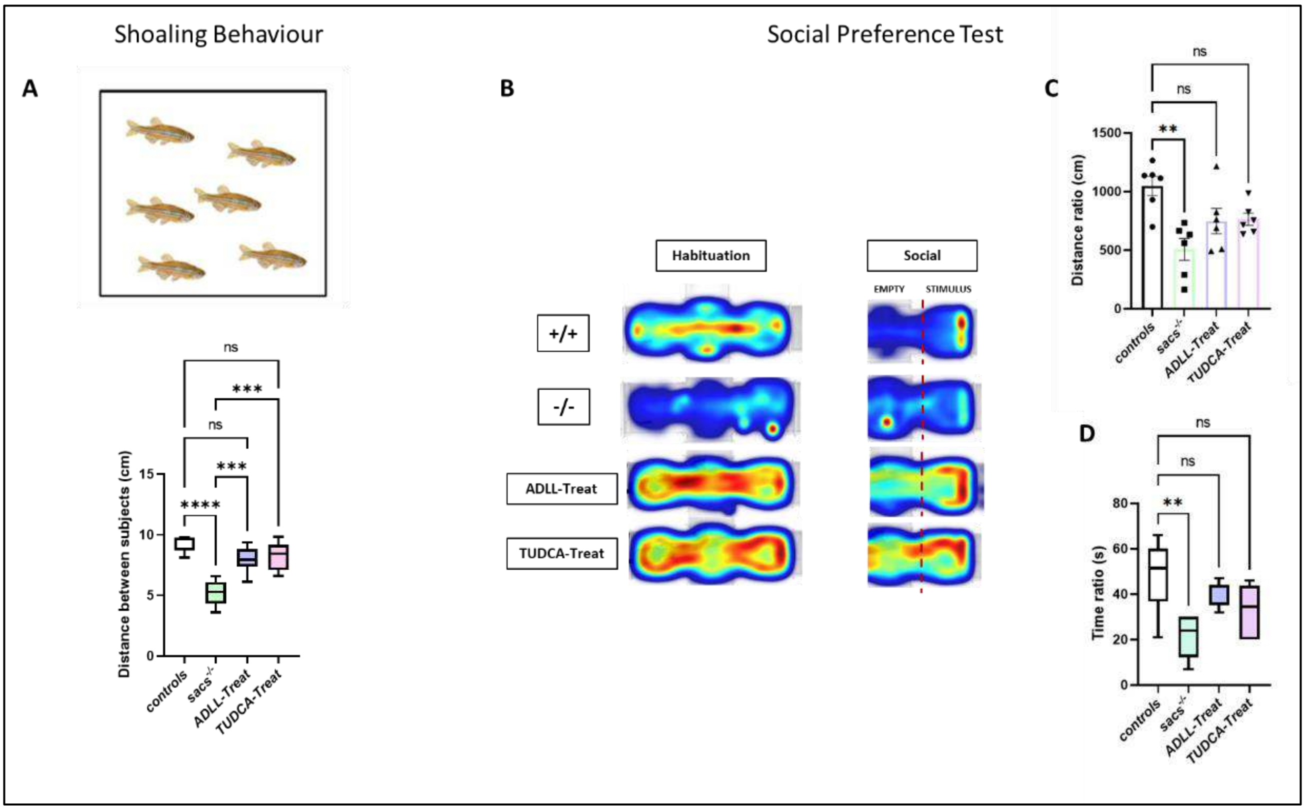

Figure 4. A) **Shoaling test**. Data are presented as mean ± S.E.M (N=6 per group). The test consists in placing a group of conspecific fish into a novel tank for 5 min, after an acclimatization period. The shoal is video-recorded for behavioral analysis, and quantifying social cohesion in the group of fish, which is measured by the average mean distance among members. B) **Schematic of social preference test** of zebrafish (B) Heat map shows that controls zebrafish displayed significant higher frequency near a group of zebrafish than *sacs*$^{-/-}$ zebrafish and the pretreatment with ADLL or TUDCA improved the behaviour sociability of *sacs* deficient fish compared to no treated mutant fish. Distance ratio (C) Time ratio (D). N = 6 for each group. N.S, no significant difference, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ were calculated by one-way ANOVA and LSD post-hoc test.

C) Cognition

Intellectual disability and behavioural abnormalities have been reported in some cases of ARSACS [20,21]. Zebrafish can been used to model complex human behavioural traits such as reward responsiveness, learning and memory [22]. We evaluated novel the object recognition task (NORT) memory and analysed the exploration time of the objects. NOR is very useful for studying both short-term and long-term memory. Both tasks require an open-field-testing arena. The object location memory task (OLT) is an important aspect of spatial memory, allowing us to remember the position of objects in our environment [22]. Time spent exploring the novel object, over the familiar one, is characterized as an index of memory [23]. Pinheiro-da-Silva et al. (2017) demonstrated that the control animals explored more the new object than the familiar object [24]. To perform novel object recognition task before training, each animal was habituated to experimental apparatus in the absence of objects for 5 min. In the training phase, animals were exposed to two identical cubes (with the same color) for 10 min. In the test, a new object (with different color) replaced one of the copies of the familiar object and the exploration time of each object was evaluated for 6 minutes. To avoid thigmotaxis influence, the distances between the objects and the walls were kept the same. In the object recognition task, the 4 experimental groups did not have a preference between objects A and B (data not shown). We evaluated the exploration time of the new object (%). Figure 5 shows that control animals had a preference in exploring the new object rather than the new object ($p < 0.0001$). Similarly, the *sacs* knock out animal fed with TUDCA or TANGANIL also presented a significant preference in exploring the new object in comparison to the new object ($p <0.0001$). Our findings demonstrated a preference for the novel object in the object recognition memory task, which are in agreement with the previous studies using simpler forms and repeated habituation periods. Unlike, evaluating the *sacs*$^{-/-}$ *no treated*-group we observed a significant preference in spent their time close to the familiar object (figure 5B-C).

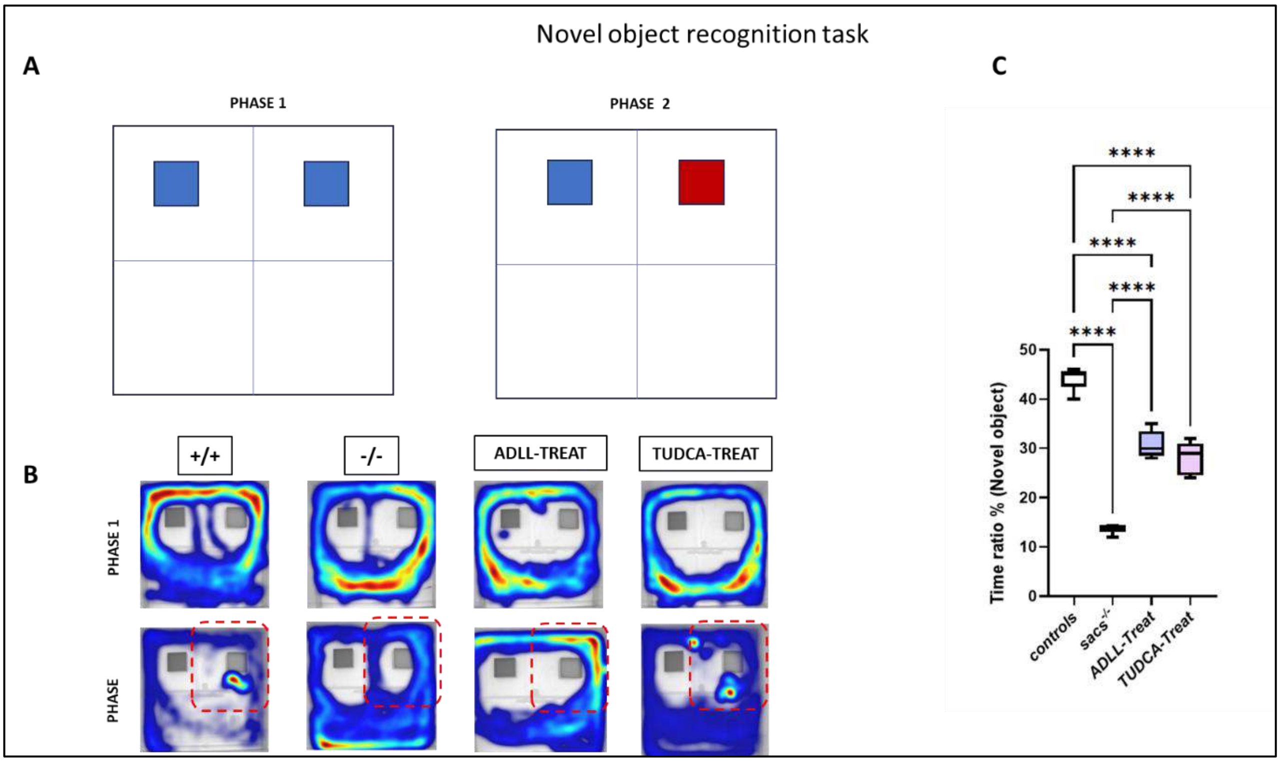

Figure 5. A) Schematic diagram of the **Object recognition memory test.** B) Heat map shows that *sacs* knock out animal fed with TUDCA or TANGANIL presented a significant preference in exploring the new object in comparison to the familiar object similarly to controls zebrafish. Unlike *sacs* mutant no-treated spent their time close to the familiar object. C) The exploration time (Time ratio %) of each object was analysed in test session. Data are presented as mean ± S.E.M (N=6 per group). NS, no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 was calculated by one-way ANOVA and LSD post-hoc test. Image modified from [22].

Then, we performed Object location task. In the test, one object remained in the initial location and another was moved to a novel location and evaluated for 6 minutes and evaluating the exploration time of each object (%). In the training session, the 4 experimental groups did not show preference between objects A and B (figure 6C). In the test session, we observed no preference of *sacs*$^{-/-}$ *no-treated* animal to explore the object in the new location in comparison to the familiar location during the test compering to control group ($p < 0.01$; Figure 6B). However the pretreatment with TUDCA or Tanganil of ARSACS zebrafish model leads to an improvement in their behavioural performance (figure 6B-C). These data demonstrated that *sacs* knock out adult zebrafish show an impairment in the object location memory and in the preference pattern in object recognition and that the pretreatment with ADLL/Tanganil™ or TUDCA could play an important role to ameliorate this cognitive impairment.

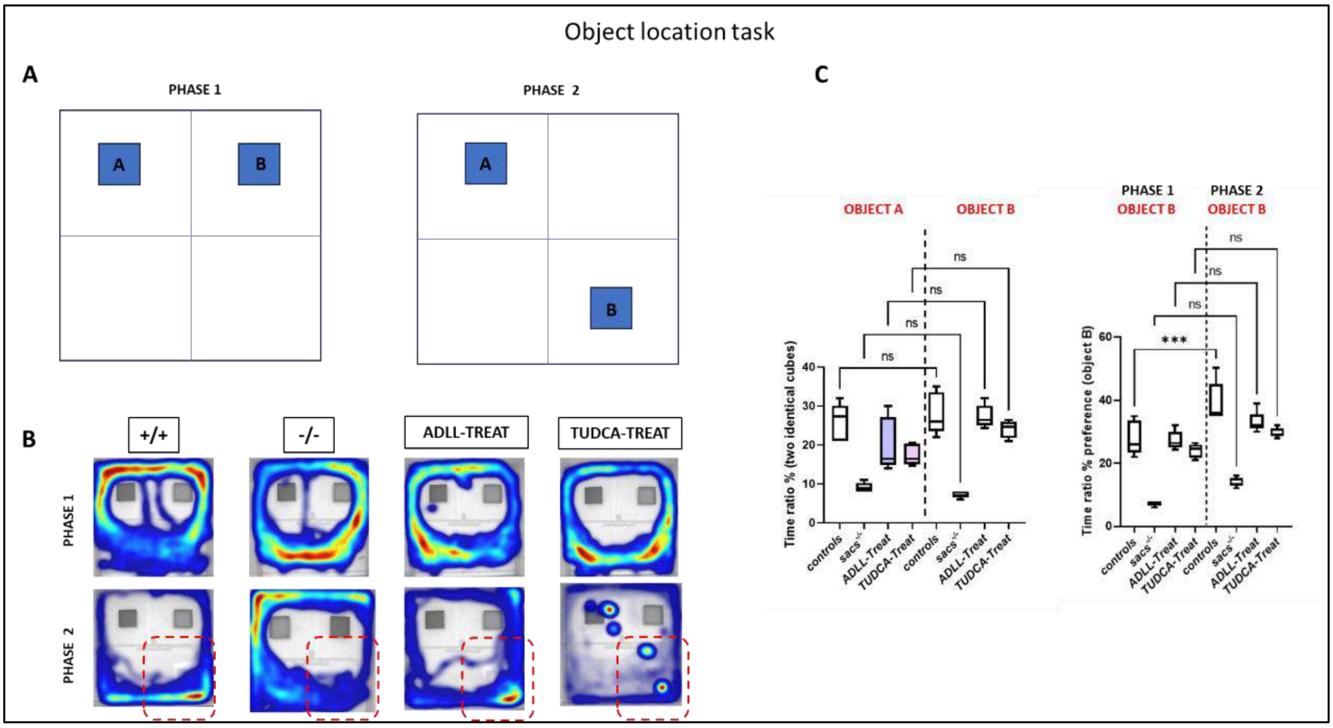

Figure 6. A) Schematic diagram of the **Object location test.** The exploration time of each object (Time ratio %) was analysed during training between A and B objects in the initial location and between the object in the novel location (A-B) in the familiar location in the test session. The image B) and C) shows the task during training (phase 1) and test (phase 2). Data are presented as mean ± S.E.M (N=6 per group). NS, no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were calculated by one-way ANOVA and LSD post-hoc test. Image modified from [22].

3. Conclusions

The development of new drugs is costly and time-consuming, limiting the number of new drugs introduced into clinical practice. Drug repurposing - the application of an existing therapeutic to a new disease indication - may allow faster and more effective clinical translation. ARSACS is incurable disorder and there is an urgent need to define new therapies. *Sacs* homozygous adult fish displays autism-like behavioral characteristics, locomotor deficit and cognitive impairment. In this work, for the first time, we successfully demonstrated that the pretreatment with ADLL/Tanganil™ or TUDCA could play an important role to ameliorate locomotor and cognitive impairment observed in ARSACS zebrafish model. Our study supports the view that zebrafish represent a valuable preclinical model for drug discovery demonstrating the potentialities of the system for future high-throughput screening studies paths before embarking on more costly studies in mice.

4. Ongoing and future investigations

As described in the project financed, to evaluate the effect of Tanganil™ and TUDCA supplementation on RNA and proteomic brain regulation profile, we dissected brains on adult fish for each experimental group (controls, *sacs*$^{-/-}$, *sacs*$^{-/-}$*-ADLL treated* and *sacs*$^{-/-}$-TUDCA). GENARTIS srl (https://genartis.it/) company is working on RNA-Sequencing analysis while our collaborators of University of Genova (Ospedale San Martino- UOS Proteomica e Spettrometria di Massa) is working on proteomic brains profile of our samples. we will receive these results in the coming weeks. These analyses result very useful in order to identify the mechanism(s) by which TUDCA or TANGANIL are able to ameliorate the phenotype observed in our zebrafish ARSACS model allowing us to discover potentially new ARSACS-related biomarkers.

5. METHODS

Animals. Adults (12 months) wild-type zebrafish; *sacs*$^{-/-}$; *sacs*$^{-/-}$*-ADLL treated* and *sacs*$^{-/-}$-TUDCA were used (N=6 for each group). Animals were kept in automated re-circulating systems (Zebtec, Tecniplast, Italy) with reverse osmosis filtered water equilibrated to reach the species recommended temperature (28° C ± 2° C), pH (7.0 and 7.5), conductivity and ammonia, nitrite, nitrate and chloride levels. Animals were subjected to a light/dark cycle of 14/10 hours, respectively. All experiments were conducted in accordance with the European Union (EU) Directive 2010/63/EU for animal experiments, and under the supervision of the Institutional Animal Care and Use Committee (IACUC) of the University of Pisa and following the 3Rs principles.

Novel tank. Zebrafish are exposed to the experimental challenge in a pre-treatment beaker before being transferred (via net) into the novel tank for behavioural observation and phenotyping as reported in [11]. After pre-treatment, zebrafish are placed individually in a 1.5-Ltrapezoidal tank maximally filled with aquarium treated water. In this experiments, behavioural activity was recorded in front of the tank using a webcam for 5 min to analyse diving response. The tank was virtually divided in two areas (bottom and top) and time at the bottom was used to assess anxiety-like phenotypes.

Open-field test and Thigmotaxis: Behavioural experiments were conducted between 10 a.m. and 4 p.m. The tank was 30 × 30 × 30 cm, with walls made of opaque partitions, and a video camera was suspended above the tank. Adult zebrafish were allowed to freely swim inside the tank, and videos were recorded for 10 min. Thigmotaxis was evaluated as reported in [13].

Shoaling test: Adult zebrafish were acclimated to the novel tank apparatus for 2 min before the test. Videos were recorded for 5 min. The shoaling assessment was performed by measuring the inter-fish distance that represents the average of all distance between each zebrafish in a shoal.

Social preference test. Social preference testing was performed ad described in [25] with three chambers. Social behaviour is assessed by observing how an individual responds to, or interacts with, a social stimulus. Social preference tests are composed of two operational phases. The first is the habituation phase, during which the tested zebrafish is left alone in a chamber of the test tank to explore the novel environment. The second is the interaction phase, which starts with the introduction of the social stimulus consisting of one, or usually two, small groups of live conspecifics [25]. The zebrafish behaviours were quantified as a distance distribution or as presence in a zone adjacent to the group or conspecifics. The time ratio was the time spent in the conspecific sector divided by the total time. The distance ratio was the distance travelled in the conspecific sector divided by the total distance travelled. The zebrafish tested were all adult males.

Novel object recognition task. Before training, each animal was habituated to experimental apparatus (Open Field) in the absence of objects for 5 min. In the training phase, animals were exposed to two identical cubes (with the same color) for 10 min. After the training, the animals were submitted to a retention interval of 1 h**.** In the test, a new object (with different color) replaced one of the copies of the familiar object and the exploration time of each object was evaluated for 6 minutes. To avoid thigmotaxis influence, the distances between the objects and the walls were kept the same.

We evaluated the exploration time of each object (%). The exploration area was defined as 8 × 8 cm area centered on the object and preference percentages were calculated as: [time of exploration of novel object / time of exploration of familiar object + time of exploration of novel object ×100] [22].

Object location task. Before training, each zebrafish was habituated to experimental apparatus in the absence of objects for 6 min. In the training phase, animals were exposed to two identical cubes for 10 min in the same sector (initial location). After the training, the animals were submitted to a retention interval of 1h. In the test, one object remained in the initial location and another was moved to a novel location and evaluated for 6 minutes. To avoid thigmotaxis influence, the distances between the objects and the walls were kept the same. We evaluated the exploration time of each object (%) [22].

Statistical analysis. Statistical analyses were performed using GraphPad Prism software. Analysis of variance (ANOVA) tests were used to compare four group (controls zebrafish; *sacs*$^{-/-}$; *sacs*$^{-/-}$*-ADLL treated* and *sacs*$^{-/-}$-TUDCA. P values < 0.05 were considered as statistically significant. Values are presented as mean ± SEM.

6. References

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