A zebrafish model for calcineurin-dependent brain function

Sara Tucker Edmister, Rahma Ibrahim, Rohit Kakodkar, Jill A. Kreiling, Robbert Creton

ARTICLE INFO

Keywords:
Zebrafish
Behavior
Calcineurin signaling
Down syndrome
Alzheimer’s disease

ABSTRACT

Small-molecule modulators of calcineurin signaling have been proposed as potential therapeutics in Down syndrome and Alzheimer’s disease. Models predict that in Down syndrome, suppressed calcineurin-NFAT signaling may be mitigated by proINDY, which activates NFAT, the nuclear factor of activated T-cells. Conversely, elevated calcineurin signaling in Alzheimer’s disease may be suppressed with the calcineurin inhibitors cyclosporine and tacrolimus. Such small-molecule treatments may have both beneficial and adverse effects. The current study examines the effects of proINDY, cyclosporine and tacrolimus on behavior, using zebrafish larvae as a model system. To suppress calcineurin signaling, larvae were treated with cyclosporine and tacrolimus. We found that these calcineurin inhibitors induced hyperactivity, suppressed visually-guided behaviors, acoustic hyperexcitability and reduced habituation to acoustic stimuli. To activate calcineurin-NFAT signaling, larvae were treated with proINDY. ProINDY treatment reduced activity and stimulated visually-guided behaviors, opposite to the behavioral changes induced by calcineurin inhibitors. The opposing effects suggest that activity and visually-guided behaviors are regulated by the calcineurin-NFAT signaling pathway. A central role of calcineurin-NFAT signaling is further supported by co-treatments of calcineurin inhibitors and proINDY, which had therapeutic effects on activity and visually-guided behaviors. However, these co-treatments adversely increased excitability, suggesting that some behaviors are regulated by other calcineurin signaling pathways. Overall, the developed methodologies provide an efficient high-throughput platform for the evaluation of modulators of calcineurin signaling that restore neural function, while avoiding adverse side effects, in a complex neural system.

1. Introduction

Calcineurin is a calcium-dependent serine-threonine phosphatase with a well-described signaling pathway (Fig. 1) and broad clinical significance [1–3]. Calcineurin inhibitors are used as immunosuppressants to prevent rejection of organ transplants [1]. Additionally, modulated calcineurin signaling is associated with neural dysfunction in Down syndrome, Alzheimer’s disease, schizophrenia, epilepsy, neuro inflammation, and traumatic brain injury [3]. In Down syndrome (trisomy 21), the extra copy of chromosome 21 leads to a suppression of calcineurin signaling [4]. In contrast, various studies have shown that calcineurin signaling is elevated in Alzheimer’s disease and suggest that the inhibition of calcineurin may serve as a viable therapeutic strategy for treating early stage Alzheimer’s disease [5–8]. This concept is supported by a study showing that Alzheimer’s disease rarely develops in transplant patients treated with the calcineurin inhibitors cyclosporine (CsA) or tacrolimus (FK506), in all age groups above 65 [9]. While some neural degeneration may be beyond repair, modulators of calcineurin signaling have the potential to prevent progressive neural degeneration in various disorders.

Little is known about the risks and potential benefits of treatments that aim to restore calcineurin signaling pathways in the brain. Clinical or population studies are limited to a few potential treatments [9]. Animal model systems are available, but subtle morphological changes in specific neurons are easily missed when studying an organ as complex as the brain. The analysis of behavior offers a potential solution, since subtle changes in neural structure and function can be detected.

Zebrafish are well suited for large-scale analyses of behavior [10–13]. Zebrafish have a prototype vertebrate brain with conserved calcineurin signaling pathways and Alzheimer’s-related proteins such as the amyloid precursor protein and the microtubule-associated protein tau [14]. At 5 days post-fertilization, the developing zebrafish larvae have inflated swim bladders, hunt for food and display avoidance behaviors [15]. The larvae are only 4 mm long at this time and are well

* Corresponding author.
E-mail address: Robbert_Creton@brown.edu (R. Creton).
suited for automated analyses of behavior in 96-well plates [10].

Using the zebrafish model, the present study shows that modulators of calcineurin signaling have therapeutic effects on activity and visually-guided behaviors, but adversely affect acoustic excitability. The developed methodologies provide an efficient platform for the evaluation of modulators of calcineurin signaling that restore neural function, while avoiding adverse side effects, in developmental and neurodegenerative disorders.

2. Materials and methods

2.1. Zebrafish

The research project has been conducted in accordance with local and federal guidelines for ethical and humane use of animals and has been reviewed and approved by the Brown University Institutional Animal Care and Use Committee. Zebrafish (Danio rerio) were collected and grown to larval stages as previously described [10,16]. Adult wild-type zebrafish are maintained at Brown University as a genetically-diverse outbred strain in a mixed male and female population. Zebrafish embryos from 0 to 3 days post-fertilization (dpf) and zebrafish larvae from 3 to 5 dpf were maintained at 28.5 °C in 2L culture trays with egg water, containing 60 mg/L sea salt (Instant Ocean) and 0.25 mg/L methylene blue in deionized water. Embryos and larvae were kept on a 12 h light/12 h dark cycle and were randomly assigned to different experimental groups prior to experimental manipulation. The sex of embryos and larvae cannot be determined at such early stages because zebrafish use elusive polygenic factors for sex determination, and both males and females have juvenile ovaries between 2.5 and 4 weeks of development [17]. Zebrafish larvae were imaged at 5 dpf when the larvae display a range of locomotor behaviors and consume nutrients available in the yolk sac [18]. Larvae are approximately 4 mm long at the 5 dpf stage.

2.2. Pharmacological treatments

Cyclosporine (cyclosporin A, Enzo Life Sciences), FK506 (tacrolimus, Enzo Life Sciences), rapamycin (Santa Cruz Biotechnology) and proINDY (Tocris Bioscience) were diluted in egg water from 1000× stocks dissolved in dimethyl sulfoxide (DMSO). DMSO (1 μL/mL DMSO) was added to the single treatments and the corresponding DMSO concentration (2 μL/mL) was used as a vehicle control. Larvae were exposed at 5 dpf to treatment solutions or DMSO for a total of 6 h. Larvae were first treated in a Petri dish for 2 h, transferred with the treatment solution to white 96-well ProxiPlates (PerkinElmer, 6006290) for 1 h, and then imaged in the treatment solution for 3 h. Immediately after exposure, larvae from each treatment group were washed in egg water and transferred to Petri dishes with 50 mL egg water. Larvae that were imaged again at 6 and 7 dpf were given food twice prior to each re-imaging session.

2.3. Imaging system

Zebrafish larvae were imaged in an imaging system that holds four 96-well plates for automated analysis of behavior in a 384-well format as previously described [10]. Briefly, the imaging system is housed in a 28.5 °C temperature-controlled cabinet where larvae in white 96-well ProxiPlates are placed onto a glass stage. Above the stage, a high-resolution camera (18-megapixel Canon EOS Rebel T6 with an EF-S 55–250 mm f/4.0–5.6 IS zoom lens) captures an image of the larvae in the four 96-well plates every 6 s. The camera is connected to a continuous power supply (Canon ACK-E10 AC Adapter) and controlled by a laptop computer using Canon’s Remote Capture software (EOS Utility, version 3), which is included with the camera. Unlike previous descriptions of this imaging system, two small speakers (OfficeTec USB Computer Speakers Compact 2.0 System) were attached speaker-side down to the glass stage. Speakers were connected by USB to the laptop computer and set to maximum volume, reaching 85 dBA on the stage. Below the glass stage, a M5 LED pico projector (Aaxa Technologies) with a 900 lumens LED light source displays Microsoft PowerPoint presentations through the opaque bottom of the 96-well plates.

2.4. Behavioral assay

Visual and acoustic stimuli are controlled by an automated 3-h PowerPoint presentation that is shown to the larvae. The entire 3-h presentation has a light gray background and starts with a 1-h period without visual or acoustic stimuli, followed by 80 min of visual stimuli, a
10-minute period without visual or acoustic stimuli, and 30 min with acoustic stimuli. Larvae were not exposed to visual stimuli and acoustic stimuli at the same time.

The visual stimuli consisted of a series of moving lines that were red, green or blue. Prior studies have shown that zebrafish larvae will swim in the same direction as moving lines, a behavior that is called an optomotor response or OMR [16,19]. Our previously-developed assays for visually-guided behaviors indicate 5 dpf larvae consistently respond for visually-guided behaviors indicate 5 dpf larvae consistently respond to visual or acoustic stimuli, and 30 min with acoustic stimuli. Values of activity (% move) and position (% up) were averaged in individual larvae during 10-minute periods, for a total of 18 periods. These values were subsequently averaged between larvae in the same treatment group. We examined a total of 10 treatment groups. For clarity, only 3 treatment groups were graphed (Fig. 3), but all 10 treatment groups were analyzed in detail as shown in subsequent figures. The 18-period graphs show that multiple calcineurin-sensitive behaviors can be examined in a single assay. The assay provides quantitative information on activity, visually-guided behaviors and acoustic startle responses.

2.7. Cluster analysis of behavioral profiles

Changes in larval activity, vision, startle response, habituation and excitability as compared to the DMSO vehicle controls were summarized in a ‘behavioral profile’. These behavioral profiles were generated for each compound used in this study. Similar profiles were grouped by hierarchical cluster analysis. The cluster analysis was carried out in Cluster 3.0 without filtering or adjusting data and using the Euclidian distance similarity metric with complete linkage. The clusters were shown in TreeView (version 1.1.6r4) using a spectrum from green (25% decrease) to red (25% increase).

3. Results

3.1. Measurements of behavior in zebrafish larvae

Zebrafish larvae were examined at 5 days post-fertilization (dpf) using an imaging system with four 96-well plates for automated analysis of behavior in a 384-well format (Fig. 2). The high-resolution imaging system is capable of measuring movement and location of individual larvae in each well. At 5 dpf, zebrafish larvae are approximately 4 mm long, swim freely and respond to visual and acoustic stimuli. The visual stimuli in this study consisted of moving lines (red, green or blue), projected through the bottom of opaque 96-well plates. Zebrafish larvae swim in the same direction as moving lines through their innate optomotor response or OMR [16,19]. The acoustic stimuli consisted of sound pulses at 20-second intervals or 1-second intervals. Larvae are known to display repeated startle responses to infrequent acoustic pulses at 20-second intervals, but habituate to frequent pulses at 1-second intervals [20,21].

Zebrafish larvae were treated at 5 dpf with various modulators of calcineurin signaling, starting 3 h before imaging. The larvae were then imaged for a total of 3 h using a behavioral assay with various visual and acoustic stimuli (Fig. 3). The behavioral assay started with a 1-h period without stimuli, followed by 80 min with visual stimuli, 10 min without visual or acoustic stimuli, and 30 min with acoustic stimuli. Values of activity (% move) and position (% up) were averaged in individual larvae during 10-minute periods, for a total of 18 periods. These values were subsequently averaged between larvae in the same treatment group. We examined a total of 10 treatment groups. For clarity, only 3 treatment groups were graphed (Fig. 3), but all 10 treatment groups were analyzed in detail as shown in subsequent figures. The 18-period graphs show that multiple calcineurin-sensitive behaviors can be examined in a single assay. The assay provides quantitative information on activity, visually-guided behaviors and acoustic startle responses.

3.2. Pharmacological treatments

To determine if larval zebrafish behavior is affected by modulation of calcineurin signaling, we imaged 5 day-old larvae in the following 10 treatment groups: 10 μM cyclosporine A (CsA), 1 μM tacrolimus (FK506), 5 or 10 μM proINDY, a combination of CsA + 5 or 10 μM proINDY, or a combination of FK506 + 5 or 10 μM proINDY, DMSO as a control, and a combination of FK506 + 5 or 10 μM proINDY, DMSO as a control.

The ImageJ macro and MS Excel template are available in the Supplementary Information.
vehicle control, and 1 μM rapamycin as a control for target specificity. Rapamycin and FK506 are both macrolide immunosuppressants with similar structures, however, rapamycin affects Target of Rapamycin (TOR) signaling instead of calcineurin signaling [22]. The concentrations of CsA, FK506 and rapamycin were selected based on prior studies in zebrafish embryos and larvae [23,24]. The two concentrations of proINDY, a membrane-permeable form of INDY (inhibitor of DYRK), were selected based on studies in cell lines and Xenopus embryos [25]. We found that none of the treatments interfered with larval survival 1 or 2 days after treatment.

3.3. Activity

Activity was examined both early in the behavioral assay, as an average of activity during the first hour of imaging, and late in the assay, during period 15 (Fig. 4). Early and late activity values were determined when larvae were imaged without visual or acoustic stimuli. We found that the calcineurin inhibitors CsA and FK506 increased early larval activity in comparison to the DMSO control (Fig. 4a). Rapamycin also increased early activity, with activity values that exceeded activity in the DMSO control and FK506 treatment group. In contrast, proINDY treatments induced a decrease in early activity, in comparison to DMSO controls. We examined if the effects of CsA and FK506 could be rescued by the addition of proINDY and, conversely, if the effects of proINDY could be rescued by the addition of CsA and FK506. Our results show that this was indeed possible. For example, co-treatment of 10 μM CsA + 5 μM proINDY induced a decrease in early activity as compared to the CsA treatment alone, and induced an increase in early activity as compared to the proINDY treatment alone.

Similar results were obtained in the analysis of late activity (Fig. 4b). CsA, FK506 and rapamycin increased activity and proINDY decreased activity in comparison to the DMSO control. In addition, we again found a rescue of activity in co-treatments of CsA + proINDY and FK506 + proINDY. While the overall patterns of early and late activity were very similar in the rapamycin group and the FK506 group, we did observe a noticeable difference between the two treatments. Early activity is
higher in the rapamycin group than the FK506 group (Fig. 4a), while late activity is lower in the rapamycin group than the FK506 group (Fig. 4b). Thus, the effects of FK506 and rapamycin on early and late activity were similar, but not the same. Based on the results described above, we conclude that the calcineurin-NFAT signaling pathway regulates activity in zebrafish larvae.

### 3.4. Visually-guided behaviors

The optomotor response or OMR was examined in 5 dpf larvae using red, green and blue lines as well as red lines that move 16 times faster than all other lines. The optomotor response was calculated by subtracting the average larval position in two subsequent 10-minute periods, when lines moved down and then up (see Fig. 3). Optomotor responses were examined in all 10 treatment groups to determine if visually-guided behaviors are affected by modulation of calcineurin.
signaling (Fig. 5). We first analyzed the visually-guided response to red lines (Fig. 5a). Larvae treated with the calcineurin inhibitors CsA and FK506 displayed decreased optomotor responses, compared to the DMSO vehicle control. The optomotor response of the rapamycin treatment group was higher than the optomotor response of the FK506 treatment group. Treatment with 5 μM ProINDY led to an increased optomotor response in comparison to the DMSO control. This excessive optomotor response can be rescued by co-treatment with CsA or FK506. Similar results were obtained when analyzing responses to green lines (Fig. 5b) and blue lines (Fig. 5c). The analysis of larval responses to fast red lines (Fig. 5d) revealed a similar CsA-induced decrease in the optomotor response and proINDY-induced increase in the optomotor response. Based on the differential OMR observed across treatment groups, we conclude that the calcineurin-NFAT signaling pathway affects optomotor responses in zebrafish larvae.

3.5. Acoustic startle responses

Previous studies have shown that zebrafish larvae will repeatedly startle when exposed to infrequent acoustic stimuli at 20-second intervals, but habituate to frequent acoustic stimuli at 1-second intervals [20,21]. We examined these startle responses in four 10-minute periods: period 15 without acoustic stimuli, period 16 with infrequent acoustic stimuli, period 17 with frequent stimuli and period 18 with infrequent stimuli (Fig. 6). As anticipated, DMSO-treated control larvae displayed stable activity during period 15, increased activity during period 16, gradually decreasing activity during period 17, and increased activity during period 18 (Fig. 6a). In contrast, the CsA-treated larvae displayed increased activity throughout period 17, which indicates that larvae lost the ability to habituate, and continuously startled in response to frequent acoustic pulses at 1-second intervals. To examine these effects in more detail, we measured habituation, acoustic startle, and 1-second excitability in all 10 treatment groups. CsA and FK506-treated larvae displayed decreased habituation, as compared to the DMSO-treated controls (Fig. 6b). In contrast, rapamycin-treated larvae displayed normal habituation, which did not differ significantly from the DMSO controls and was elevated compared to habituation in the FK506-treated larvae. Startle responses were slightly elevated after treatment with proINDY (Fig. 6c). CsA and FK506 treatment led to an increase in excitability, compared to excitability in the DMSO controls (Fig. 6d). Rapamycin treatment also increased excitability in comparison to the DMSO controls, although the level of excitability was lower than observed in the FK506-treated larvae. The effects of CsA and FK506 on larval excitability could not be rescued by co-treatment with proINDY. In fact, co-treatment of FK506 and proINDY led to a large increase in excitability, which was higher than the excitability observed with either compound alone.

Based on the effects of CsA and FK506, we conclude that habituation, startle responses and excitability are regulated by calcineurin signaling. However, the adverse effect of proINDY on FK506-induced hyperexcitability is not easily explained by calcineurin-NFAT signaling and may suggest the involvement of other calcineurin signaling pathways.

3.6. Recovery of behavior at 6 and 7 dpf

Zebrafish larvae were treated and imaged at 5 dpf, rinsed, grown in egg water, and imaged again at 6 and 7 dpf to assess the recovery of behavior. To evaluate the effects of five single treatments on multiple behaviors at 5, 6 and 7 dpf, we calculated treatment-induced changes as compared to the DMSO control and color coded the differences in Fig. 6. Responses to acoustic stimuli. A) Example of one imaging experiment analyzed during the final 40 min in 6-second intervals. B) Habituation to acoustic stimuli at 1-second intervals (first 5 min minus last 5 min of period 17). C) Startle responses (average activity in period 16 minus period 17). D) Excitability by acoustic stimuli at 1 s intervals (average activity in period 17 minus period 16). Larvae were treated at 5 dpf with DMSO (2 μl/mL), cyclosporine (CsA, 10 μM), tacrolimus (FK506, 1 μM), rapamycin (RM, 1 μM), proINDY (5PI = 5 μM proINDY, 10PI = 10 μM proINDY), or a combination of two treatments. Differences between corresponding groups were examined for significance using a Chi-square test with a Bonferroni correction for multiple comparisons. N in panel A = 96 larvae per treatment group. N in panel B, C and D = 912, 372, 188, 263, 383, 275, 190, 178, 93 and 86 larvae in the 10 subsequent treatment groups. * p < 0.05/14), ** p < 0.01/14), *** p < 0.001/14).
behavior (Fig. 7). The color-coded figure provides a summary of all experiments at 5 dpf, highlighting the observed changes in behavior at the time of initial imaging. CsA and FK506 treatments increased activity, decreased optomotor responses and increased excitability. In contrast, proINDY treatments decreased activity, increased optomotor responses and increased excitability. A few treatments showed a 6 dpf withdrawal effect, where changes in behavior were opposite to the changes in behavior at 5 dpf. For example, in the CsA treatment group activity was elevated at 5 dpf and decreased at 6 dpf. In the same treatment group, the optomotor response to fast red lines was suppressed at 5 dpf and elevated at 6 dpf. The summary figure shows that most, but not all, behaviors recovered in two days after treatment.

3.7. Cluster analysis of behavioral profiles

Values of multiple behaviors, as shown in Fig. 7, are often referred to as ‘behavioral profiles’ and are well suited for hierarchical cluster analysis. The cluster analysis evaluates if various treatments induce similar behavioral profiles. Cluster analysis of the 5 dpf data revealed that CsA and FK506 treatments cluster together, indicating a profile specific to calcineurin inhibition (Fig. 8). The cluster analysis has sufficient phenotypic resolution to separate the behavioral profile of FK506 from rapamycin, two macrolide immunosuppressants with similar structures but different molecular targets [22]. Both concentrations of proINDY induce similar changes in behavior and cluster together. The proINDY behavioral profile is nearly opposite to the profile induced by inhibition of calcineurin. Based on these results, we conclude that modulators of calcineurin signaling induce specific behavioral profiles that can be grouped by cluster analyses.

4. Discussion

4.1. Modulation of calcineurin signaling

The current study shows that zebrafish larvae serve as a valuable model to study the risks and benefits of treatments that modulate calcineurin signaling. Using automated analyses of behavior, we found that the calcineurin inhibitors CsA and FK506 increase activity and decrease optomotor responses. Conversely, the DYRK inhibitor proINDY induces opposite effects, i.e. a decrease in activity and an increase in optomotor responses. These results are consistent with models of calcineurin-NFAT signaling (Fig. 1) and suggest that small-molecule treatments aimed to restore calcineurin signaling have beneficial effects on activity and visually-guided behaviors. This idea is supported by the observed rescues of behavior in co-treatments of calcineurin inhibitors and proINDY.

4.2. Other signaling pathways

Some changes in behavior cannot be easily explained by the calcineurin-NFAT model. Specifically, CsA and FK506 treatments lead to an increase in acoustic excitability. These larvae continuously startle, without habituation, in response to acoustic stimuli at 1-second intervals. This behavior is not rescued by co-treatment with proINDY. Instead, such co-treatments induce an exacerbated phenotype. These results indicate that excitability may not be regulated by calcineurin-NFAT signaling and suggest the involvement of other calcineurin signaling pathways. For example, calcineurin may act by dephosphorylation of other signaling proteins such as CREB, GSK-3 and BAD [5]. Overall, the observed hyperexcitability phenotype indicates that small-molecule treatments aimed to restore calcineurin signaling can

| Treatment | dpf | 1hr | R | G | B | FR | RGB | S | Hab | E |
|-----------|-----|-----|---|---|---|----|-----|---|-----|---|
| Δ 10 μM CsA | 5   | 17  | 21 | -15| -5 | -20| -18 | -15| -4  | -8 |
|           | 6   | -27 | -9 | -2 | -6 | 5  | 20  | 6  | 3   | 4  |
|           | 7   | 3   | -1 | 7  | -2 | 5  | 2   | 3  | 6   | 3  |
| Δ 1 μM FK506 | 5   | 5   | 25 | -17| -9 | -14| -6  | -13| -1  | -5  |
|           | 6   | -15 | 15 | -4 | -6 | 8  | 31  | 8  | -4  | -4  |
|           | 7   | 15  | 12 | 0  | 1  | 5  | 4   | 2  | -6  | -2  |
| Δ 1 μM RM   | 5   | 13  | 9  | -5 | 0  | -4| -10 | -5 | 0   | 2   |
|           | 6   | -5  | 0  | 3  | 6  | -1 | -2  | -1 | 2   | -4  |
|           | 7   | -24 | 4  | 6  | 1  | -4| 1   | -4 | 0   | 0   |
| Δ 5 μM PI   | 5   | -13 | -20| 13 | 11 | 19 | 9   | 14 | 2   | -2  |
|           | 6   | -7  | -1 | 2  | 0  | 5  | 2   | 3  | 4   | -6  |
|           | 7   | 7   | -1 | 2  | 2  | 4  | 2   | 2  | 1   | -2  |
| Δ 10 μM PI  | 5   | -3  | -10| 13 | 16 | 8  | 12  | 2  | -1  | 14  |
|           | 6   | 1   | -2 | 5  | -10| 0  | 4   | 6  | 1   | -5  |
|           | 7   | 1   | -3 | -1| 2  | 5  | 3   | 3  | 0   | -1  |

Fig. 7. Recovery of behavior in 6 and 7 day-old larvae. After treatment and initial imaging at 5 days post fertilization (dpf), zebrafish larvae were grown in egg water and imaged again at 6 and 7 dpf. Changes in behavior were calculated (Treatment - DMSO) and color coded for a visual evaluation of altered behaviors. Note that most, but not all behaviors, recovered two days after the treatment at 7 dpf. Measurements of behavior included activity in the first hour (1 h) and period 15 (P15), optomotor responses to moving lines in red (R), green (G), blue (B), fast red (FR) and all colors and speeds combined (RGB), startle response (S), habituation (Hab) and excitability (E).

Fig. 8. Hierarchical cluster analysis of behavioral profiles. Behaviors at 5 dpf were analyzed in Cluster 3.0, using the Euclidian distance metric with complete linkage. The clusters were then color coded in TreeView using a spectrum from green (25 % decrease) to red (25 % increase). Three main clusters were identified, each with a distinct behavioral profile: 1) the calcineurin inhibitors FK506 and CsA, 2) DMSO and rapamycin (RM), and 3) the DYRK1A inhibitor proINDY (PI) at two concentrations. Measurements behavior include activity in the first hour (1 h) and period 15 (P15), optomotor responses to moving lines in red (R), green (G), blue (B), fast red (FR) and all colors and speeds combined (RGB), startle response (S), habituation (Hab) and excitability (E).
induce adverse side effects.

4.3. Cluster analysis

Multiple measures of behavior were organized in behavioral profiles, which are suitable for hierarchical cluster analysis. Cluster analyses are typically used to examine gene expression patterns, but have also been successfully used in the analysis of behavior [11–13]. The cluster analysis performed in this study revealed a specific behavioral profile for calcineurin inhibition. In addition, the analysis had sufficient phenotypic resolution to distinguish FK506 and rapamycin, which are both macrolide immunosuppressants with similar structures, but that affect different signaling pathways [22].

4.4. Clinical significance

Modulated calcineurin signaling is associated with neural dysfunction in Down syndrome and Alzheimer’s disease. In Down syndrome (trisomy 21), the extra copy of chromosome 21 leads to overexpression of both the Down Syndrome Critical Region gene 1 (DSCR1), also called the Regulator of Calcineurin (RCAN1), and a dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A), which both suppress calcineurin signaling pathways [4]. The suppressed calcineurin signaling pathway may affect fetal development as well as neural function later in life. INDY and other DYRK inhibitors have been proposed as therapeutics to restore calcineurin-NFAT signaling in Down syndrome [25–27]. People with Down syndrome frequently develop Alzheimer’s disease in their fifties or sixties, although it is unclear if this is caused by a suppression of calcineurin signaling or by the gene for the Amyloid Precursor Protein (APP), which is also located on chromosome 21 [5, 26, 28]. In fact, various studies have shown that calcineurin signaling is elevated, rather than suppressed, in Alzheimer’s disease [5–8]. The activation of calcineurin leads to dephosphorylation of various proteins, including the nuclear factor of activated T-cells (NFAT), BCL2-associated death protein (BAD) and glycogen synthase kinase-3 (GSK-3), which in turn induce various hallmarks of Alzheimer’s disease, such as inflammation, cell death, and hyperphosphorylation of tau [5]. Based on this model, the inhibition of calcineurin with cyclosporine or tacrolimus may serve as a viable therapeutic strategy for treating early stage Alzheimer’s disease [5,9].

The automated analysis of zebrafish behavior may be used to examine other previously identified DYRK and calcineurin inhibitors that may restore neural function without adverse side effects. Such inhibitors are likely to have clinical significance in various calcineurin-related disorders, including Down syndrome and Alzheimer’s disease [3–8, 26, 28]. Calcineurin and DYRK inhibitors that are not used in medicine would need to be further examined for efficacy and safety in a mammalian model system, such as the mouse, before initiating clinical trials. This route makes use of the strengths of various model systems, i. e. zebrafish are well suited for large-scale screens and mice are well suited for more detailed pre-clinical studies. In addition, a comparative approach using both zebrafish and mice can reveal fundamental mechanisms that are critical to neural function, since these core mechanisms have been conserved in the past 400 million years [29]. A more direct bench-to-bedside approach may be possible if specific calcineurin inhibitors or DYRK inhibitors are already used in medicine or are natural products that are part of our diet. In these cases, human population studies could reveal beneficial effects in neural function, similar to the beneficial effects of CsA and FK506 in the prevention of Alzheimer’s disease [9].

Author contributions

S.T.E. performed and analyzed most of the experiments, participated in project and experimental design, and co-wrote the manuscript. R.I. carried out experiments showing that CsA affects larval vision and habituation and evaluated various approaches for data analysis. R.K. and J.K. contributed to the hierarchical cluster analysis of behavioral profiles and J.K. provided critical feedback on the prevention and treatment of Alzheimer’s disease. R.C. conceived the initial idea of the project, participated in experimental and project design and co-wrote the manuscript. All authors contributed to the final version of the manuscript.

Funding

This work was supported by the following grants from the National Institutes of Health and the National Science Foundation: NIH R01GM136906 (R.C.), NIH R01EY024562 (R.C.), NSF EPSCoR 1655221 (R.C.), NIH P20GM119943 (J.A.K.), and NIH P01AG051449 (J.A.K.).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgment

We would like to thank Bethany Arabic for prior work on the optimization of visual and acoustic stimuli.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2021.113544.

References

[1] J.R. Azzi, M.H. Sayegh, S.G. Mallat, Calcineurin inhibitors: 40 years later, can’t live without, J. Immunol. 191 (12) (2013) 5785–5791.
[2] J.L. Furner, C.M. Norris, Calcineurin and glial signaling: neuroinflammation and beyond, J. Neuroinflammation 11 (2014) 158.
[3] J. Saraf, P. Bhattacharya, K. Kalia, A. Borah, D. Sarmah, H. Kaur, K.R. Dave, D. R. Yavagal, A friend or foe: calcineurin across the gamut of neurological disorders, ACS Cent. Sci. 4 (7) (2018) 805–819.
[4] J.R. Arron, M.M. Winslow, A. Polleri, C.P. Chang, H. Wu, X. Gao, J.R. Neilton, L. Chen, J.J. Heit, S.K. Kim, N. Yamashita, T. Miyakawa, U. Francke, L.A. Graef, G. R. Crabtree, NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21, Nature 441 (7093) (2006) 595–600.
[5] L.C. Reese, G. Tagialatela, A role for calcineurin in Alzheimer’s disease, Curr. Neuropharmacol. 9 (4) (2011) 685–692.
[6] S. Kocahan, Z. Dogan, Mechanisms of Alzheimer’s disease pathogenesis and prevention: the brain, neural pathology, N-methyl-D-aspartate receptors, tau protein and other risk factors, Clin. Psychopharmacol. Neurosci. 15 (1) (2017) 1–8.
[7] E. Popugaeva, E. Pichikaya, I. Bezpevcuny, Dysregulation of neuronal calcium homeostasis in Alzheimer’s disease - a therapeutic opportunity? Biochem. Biophys. Res. Commun. 483 (4) (2019) 978–1004.
[8] P. Sompol, C.M. Norris, Cs(2+), astrocyte activation and calcineurin/NFAT signaling in age-related neurodegenerative diseases, Front. Aging Neurosci. 10 (2018) 199.
[9] G. Tagialatela, C. Rastellini, L. Cicalese, Reduced incidence of dementia in solid organ transplant patients treated with calcineurin inhibitors, J. Alzheimers Dis. 47 (2) (2015) 259–333.
[10] R.J. Thorn, A. Dombroski, K. Eller, T.M. Domínguez-Gonzalez, D.E. Clift, P. Baek, R.J. Seto, E.S. Kahn, S.K. Tucker, R.M. Colwill, J.K. Sello, R. Creton, Analysis of vertebrate vision in a 384-well imaging system, Sci. Rep. 9 (1) (2019) 13989.
[11] G. Bruni, A.J. Remzekamp, A. Veliench, M. McCarroll, L. Gendlev, E. Fertsch, J. Taylor, P. Lakhani, D. Lensen, T. Evron, P.J. Lello, X.P. Huang, S. Kolczewski, G. Carey, B.J. Caldarone, E. Prinssen, B.L. Roth, M.J. Keiser, R.T. Peterson, D. Kokel, Zebrafish behavioral profiling identifies multtarget antipsychotic-like compounds, Nat. Chem. Biol. 6 (3) (2010) 231–237.
[12] D. Kokel, J. Bryan, C. Laggner, R. White, C.Y. Cheung, R. Mateus, D. Healey, S. Kim, A.A. Werlich, S.J. Haggarty, C.A. Macrae, B. Shoichet, R.T. Peterson, Rapid behavior-based identification of neuroactive small molecules in the zebrafish, Nat. Chem. Biol. 6 (3) (2010) 231–237.
[13] R.J. Seto, E.S. Kahn, S.K. Tucker, R.M. Colwill, J.K. Sello, R. Creton, Analysis of vertebrate vision in a 384-well imaging system, Sci. Rep. 9 (1) (2019) 13989.
[14] L.R. Nery, N.S. Eltz, C. Hackman, R. Fonseca, A. Altenhofen, H.N. Guerra, V. Freitas, C.D. Bonan, M.R. Viana, Brain intraventricular injection of amyloid-beta in zebrafish embryo impairs cognition and increases tau phosphorylation, effects reversed by lithium, PLoS One 9 (9) (2014), e105862.
[15] R.M. Colwill, R. Creton, Imaging escape and avoidance behavior in zebrafish larvae, Rev. Neurosci. 22 (1) (2011) 65–73.
[16] R.J. Thorn, D.E. Clift, G. Ojo, R.M. Colwill, R. Creton, The loss and recovery of vertebrate vision examined in microplates, PLoS One 12 (8) (2017), e0183414.
[17] W.C. Liew, L. Orban, Zebrafish sex: a complicated affair, Brief. Funct. Genomics 13 (2) (2014) 172–187.
[18] D. Clift, H. Richendrfer, R.J. Thorn, R.M. Colwill, R. Creton, High-throughput analysis of behavior in zebrafish larvae: effects of feeding, Zebrafish 11 (5) (2014) 455–461.
[19] E.A. Naumann, J.E. Fitzgerald, T.W. Dunn, J. Rihel, H. Sompolinsky, F. Engert, From whole-brain data to functional circuit models: the zebrafish optomotor response, Cell 167 (4) (2016) 947–960, e20.
[20] M.A. Wolman, R.A. Jain, L. Liss, M. Granato, Chemical modulation of memory formation in larval zebrafish, Proc. Natl. Acad. Sci. U. S. A. 108 (37) (2011) 15468–15473.
[21] J.D. Best, S. Berghmann, J.J. Hunt, S.C. Clarke, A. Fleming, P. Goldsmith, A. G. Roach, Non-associative learning in larval zebrafish, Neuropsychopharmacology 33 (5) (2008) 1206–1215.
[22] S. Vellanki, A.E. Garcia, S.C. Lee, Interactions of FK506 and rapamycin with FK506 binding protein 12 in opportunistic human fungal pathogens, Front. Mol. Biosci. 7 (2020), 588913.
[23] D.E. Clift, R.J. Thorn, E.A. Passarelli, M. Kapoor, M.K. LoPiccolo, H.A. Richendrfer, R.M. Colwill, R. Creton, Effects of embryonic cyclosporine exposures on brain development and behavior, Behav. Brain Res. 282 (2015) 117–124.
[24] A.M. Siebel, F.P. Menezes, I. da Costa Schaefer, B.D. Petersen, C.D. Bonan, Rapamycin suppresses PTZ-induced seizures at different developmental stages of zebrafish, Pharmacol. Biochem. Behav. 139 (Pt B) (2015) 163–166.
[25] Y. Ogawa, Y. Nonaka, T. Goto, E. Ohnishi, T. Hiramatsu, I. Kii, M. Yoshida, T. Ikura, H. Onogi, H. Shibuya, T. Hosoya, N. Ito, M. Hagiwara, Development of a novel selective inhibitor of the Down syndrome-related kinase DyRK1A, Nat. Commun. 1 (2010) 86.
[26] H. Kim, K.S. Lee, A.K. Kim, M. Choi, K. Choi, M. Kang, S.W. Chi, M.S. Lee, J.S. Lee, S.Y. Lee, W.J. Song, K. Yu, S. Cho, A chemical with proven clinical safety rescues Down-syndrome-related phenotypes in through DYRK1A inhibition, Dis. Model. Mech. 9 (8) (2016) 839–848.
[27] D.B. Jarhad, K.K. Manhelkar, H.R. Kim, M. Noh, L.S. Jeong, Dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) inhibitors as potential therapeutics, J. Med. Chem. 61 (22) (2018) 9791–9810.
[28] P. Castro, S. Zaman, A. Holland, Alzheimer’s disease in people with Down’s syndrome: the prospects for and the challenges of developing preventative treatments, J. Neurol. 264 (4) (2017) 804–813.
[29] R. Gerlai, Evolutionary conservation, translational relevance and cognitive function: the future of zebrafish in behavioral neuroscience, Neurosci. Biobehav. Rev. 116 (2020) 426–435.