Effects of D1 agonist on working memory and cognitive flexibility in a zebrafish model of aging

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Abstract

Part of the natural aging process is a decline in memory and executive function, even in the absence of disease. The dopaminergic system has been implicated in age associated alterations in cognitive flexibility and working memory. Here we examine the relationship between cognitive performance and dopamine function of young-adult and aging zebrafish (*Danio rerio*). We reveal an age-related decrease in working memory and cognitive flexibility when faced with a negative feedback loop for informing search strategies in the Free-Movement Pattern (FMP) Y-maze. We additionally found a selective role for dopamine D1-like receptor activation, by treatment with partial D1/D5 receptor agonist SKF-38393, for enhancing working memory performance in aged zebrafish, but not for restoring behavioural flexibility. We additionally noted that baseline performance levels were critical to the effect of SKF-38393 on cognitive flexibility. This reduction in behavioural plasticity was accompanied by a down-regulation of the dopamine transporter (*dat*) and a decrease in metabolic activity. Together, these findings suggest a selective role for cognitive enhancement via dopamine D1 receptors; however, beneficial effects are dependent on behavioural task and baseline performance, emphasising the need for caution when treating cognitive deficits with dopamine agonists to improve cognitive impairment of some tasks. This study further supports the use of zebrafish as a model of aging and cognitive decline.

*Keywords***:** FMP Y-maze; zebrafish; memory; cognitive flexibility; dopamine D1 receptor;

Introduction

During the process of 'natural' aging, the brain undergoes gradual structural and functional changes that cause deterioration in cognitive ability, even in the absence of neurodegenerative disease (Harada et al., 2013; Salthouse, 2009). The rate of decline is highly variable, with many able to maintain good health and mental ability into their late 80s, whilst others are greatly susceptible to debilitating cognitive impairment and disease (Deary et al., 2012). Individual differences in cognitive aging have driven the identification of underlying biological factors that can predict vulnerability to decline or development of age-related diseases (Berry et al., 2016). Many studies of healthy aging have implicated multiple components of the dopaminergic system in declining cognition, with individual differences in dopamine signalling playing a profound role in performance of cognitive tasks and response to dopamine-altering medications (Roshan Cools & D'Esposito, 2011; Kimberg et al., 1997; Volkow et al., 1998). As a result, dopamine has become a focus of research investigating age associated changes in cognition.

Dopamine plays an important role in many aspects of cerebral functions related to cognition such as attention, learning, working memory and mental flexibility (Girault & Greengard, 2004; Naderi et al., 2016). Three key modulators of dopamine function are dopamine synthesis, reuptake and activation of dopaminergic receptors (Klanker et al., 2013). Through pharmacological manipulations, a complex network of interactions involving these modulatory systems are used to maintain dopamine homeostasis (Roshan Cools & D'Esposito, 2011), dysregulation of which has been shown to have severe, and sometimes opposing, effects on cognition, particularly working memory and cognitive flexibility in health and disease (Cai & Arnsten, 1997; El-Ghundi et al., 2007; Rothmond et al., 2012; THOMAS J. BROZOSKI et al., 1979; Zahrt et al., 1997).

Zebrafish (*Danio rerio*) have recently emerged as a promising model of cognitive aging (Gerhard, 2007; Lili Yu et al., 2006). Unlike rodent and drosophila models that show rapid decline with age, zebrafish show similar gradual aging to humans. Zebrafish have a life span of approximately 3 years and changes in cognition are evident from around 2 years, increasing the accessibility of researching gradual senescence in a model of old age (Tsai et al., 2007). Similar to mammals, teleosts have homologues of neurotransmitters, associated systems and brain regions necessary to assess learning, memory and executive functions such as attention and cognitive flexibility (M. O. Parker et al., 2013). Ease of pharmacological manipulation and high throughput behavioural testing additionally add to the convenience and suitability of zebrafish to model the effects of aging and age-related diseases (Brock et al., 2017).

The FMP Y-maze has been developed to assess cognition in a range or organisms, including zebrafish, flies, rodents and a virtual task for assessing humans (submitted-Cleal, et al., 2020 [†])(Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Specific exploration patterns have been identified in a range of organisms using serial analysis of left and right turns. Strings of turns are subdivided into overlapping sequences of four consecutive turns, known as a tetragram, which combined give a total of 16 possible turning patterns ranging from all left turns (LLLL) to all right turns (RRRR). Previous work from our group discovered the use of working memory in pattern formation in the FMP Y-maze by using autocorrelation function and pharmacological manipulation of memory pathways. We identified that blockade of NMDA-receptors, muscarinic receptors and D1-receptors all played critical roles in establishing search patterns, and through autocorrelation function analysis it was noted that information was held and used to influence future decisions for decreasing amounts of time until there was no evident strategy for exploration, dependent on receptor antagonism (submitted-Cleal, et al. 2020). Working memory is based on the ability to recall previous arm entries, similar to the alternation task of the T- or Y-maze. Recording percentage-use of each tetragram sequence over the entire hour of exploration gives rise to a 'global' search strategy. Previous studies have identified that zebrafish have a very specific strategy of global exploration that is dominated by the use of an alternation strategy, which is characterised predominantly by alternating left and right turns (LRLR, RLRL) (Fontana, Cleal, & Parker, 2019). Similar strategies have also been reported in rodents, both in the FMP Y-maze and T-maze using the same analysis (submitted-Cleal, et al., 2020, Gross et al., 2011). The prolonged exploration time of the task, 1 h, not only permits examination of working memory, but also cognitive flexibility. Dividing the data into 6 equal 10 min time bins allows identification of changes in strategy over time. Fully functioning individuals should be able to use negative feedback (a lack of food reward or novelty) to update environmental information and adapt their strategy appropriately.

To understand the role of dopamine in healthy aging in zebrafish, we compared behavioural phenotypes of 6-month-old (mo) adults with aged, 24 mo adults, the latter of which are within the early stages of senescence. We hypothesised that 24 mo zebrafish may have begun experiencing mild cognitive decline. We examined changes in working memory and cognitive flexibility in a recently developed behavioural paradigm that can be used to assess both cognitive functions simultaneously with minimal handling and experimenter interference.

Materials and Methods

Ethical statement

The University of Portsmouth Animal Welfare and Ethical Review Board guidelines were followed for all experiments carried out as part of this study, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: P9D87106F].

Animals and housing

71 male and female wild type (AB) zebrafish (*Danio rerio*) aged 6 months old (middle age, n=33) and 24 months old (aged, n=38) were used to assess cognitive aging. Previous work in our lab has shown that there are no sex differences in the FMP Y-maze and therefore we did not examine the effect of sex as part of this study (Fontana, Cleal, & Parker, 2019). Zebrafish were bred in-house and raised in the University of Portsmouth Fish Facility. Fish were housed in groups of ~10-12 fish per 2.8L tank on a re-circulating system (Aquaneering Inc., San Diego, CA, USA), aquarium-water was maintained at pH 8.4 (±0.4). Previous work from our group and extensive pilot studies were used to calculate power analysis and inform sample sizes used in this study (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Room and tank temperatures were maintained at 25-27°C on a 14/10-hour light/dark cycle. From 5 days post fertilisation (dpf) fish were fed on ZM fry food until adulthood when they were fed on a daily diet of live brine shrimp (maintained at the fish facility) and dried flake food (ZM Systems, UK) 3 times/day (once/day at weekends). All fish used in this study were experimentally naïve. Once behavioural testing had been completed test fish were euthanized by rapid cooling (immersion in 2°C water), followed by decapitation and excision of the brain for downstream processing.
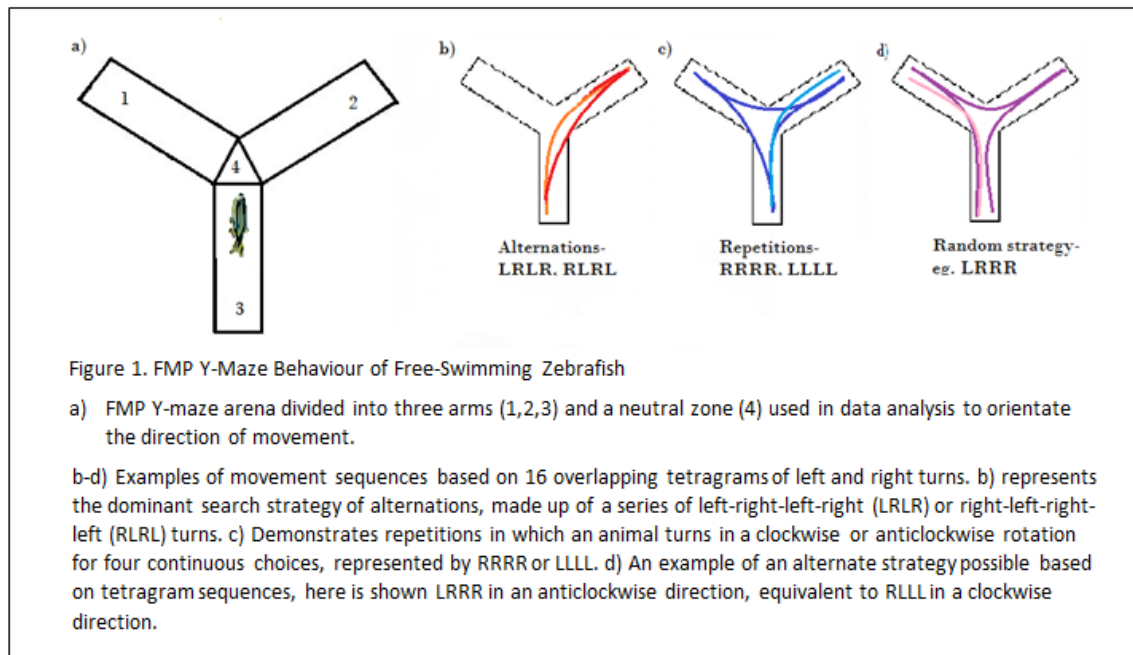
Drugs

To study the effect on working memory, cognitive flexibility and mRNA expression of dopamine receptors, adults were incubated for 30 min in 35 µM of the selective dopamine D1/D5 receptor partial agonist SKF-38393 hydrochloride (Medchemexpress), dissolved in aquarium-treated water, or aquarium-treated water (control). Drug was administered by placing fish in a 400 mL beaker filled with 300 mL of drug or water, and covered with a lid to prevent fish from escaping. Fish were netted into the beaker 30 mins prior to behavioural testing, immediately following treatment fish were transferred into the FMP Y-maze.

FMP Y-maze

The protocol was carried out as described in our previous papers (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Fish were recorded in a Y-maze for 1 hour. If the fish were adopting a random search strategy, it would be predicted that the distribution of tetragrams over a 1-hour period would be approximately stochastic (i.e., the relative frequency of each tetragram would be ~6.25%). *Figure 1* shows an example of a series of movement sequences performed in the FMP Y-maze, a combination of which provide a picture of the global search strategy used. To minimise stress, fish handling and experimenter visibility were both kept to a minimum. Behavioural testing was conducted using a commercially available, fully integrated testing environment, the Zantiks AD system for adult zebrafish (Zantiks Ltd., Cambridge, UK). Tanks were black, opaque acrylic with a transparent base. A white acrylic Y-maze insert was fitted into each tank. Two Y-maze inserts could be fitted per tank. The Y-maze dimensions were as follows: L500 mm x W200 mm x D1400 mm, with a 120° angle between arms. Tanks were filled with 3L of aquarium-water and placed into Zantiks behaviour units, one tank per unit. Each system was fully controlled via a web enabled device. Filming was carried out from above, which allowed live monitoring of fish within the behaviour system. Data output was automated, preventing any bias in the recording of arm entries.

Figure 1.

Figure 1. FMP Y-Maze Behaviour of Free-Swimming Zebrafish

a) FMP Y-maze arena divided into three arms (1,2,3) and a neutral zone (4) used in data analysis to orientate the direction of movement.

b-d) Examples of movement sequences based on 16 overlapping tetragrams of left and right turns. b) represents the dominant search strategy of alternations, made up of a series of left-right-left-right (LRLR) or right-left-right-left (RLRL) turns. c) Demonstrates repetitions in which an animal turns in a clockwise or anticlockwise rotation for four continuous choices, represented by RRRR or LLLL. d) An example of an alternate strategy possible based on tetragram sequences, here is shown LRRR in an anticlockwise direction, equivalent to RLLL in a clockwise direction.

RNA Extraction and cDNA Synthesis

Immediately following behavioural testing, fish were euthanised in ice water, brains were removed, snap-frozen in liquid nitrogen and stored at -80°C until further use. RNA was isolated using the RNeasy Micro kit (Qiagen) as described in the manufacture's protocol. Upon purification, the quality and concentration of all samples were assessed using the NanoDrop ND-1000 (Thermo Scientific). The purities of acceptable RNA samples (as measured by 260:280 and 230:260 absorbance ratios) were equal to or greater than 1.8. All samples were therefore of sufficient quality for expression-level analysis. Total cDNA was prepared using Applied Biosystems High Capacity RNA-to-cDNA Kit for RT-qPCR. Each 20 µL reaction was diluted 10-fold in nuclease-free water and used as the template for the real-time qPCR assays.

Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR (RT-qPCR) assays were used to validate relative gene expression based on SYBR green detection. Primers used in this study were predesigned primers from qPrimerDB (Lu et al., 2018) or based on previous studies (M. O. M. O. Parker et al., 2016; Tang et al., 2007). Primers were synthesised by Invitrogen and are listed in Table 1. The Roche LightCycler® 480 High Resolution Melting Master mix and the LightCycler® 96 (Roche Life Science) were used to amplify and detect the transcripts of interest. Thermal cycling conditions included an initial denaturation step at 95°C for 600s (recommended by manufacturer), 40 cycles of 95°C for 15s, 58°C for 20s and 72°C for 35s followed by melt curve analysis to confirm product specificity for all transcripts. Primers were tested with melt curve analysis and negative reverse transcription (RT) controls and negative template controls to optimise reaction conditions to generate a single melt peak in control samples, check for genomic contamination in negative RT controls and primer dimers in negative template controls. Elongation factor 1 alpha (*elfa*) was used as a housekeeping gene (Tang et al., 2007). Gene-expression levels were calculated using delta-delta CT method and normalised to *elfa*. Changes in expression were presented as means + SD of fold change to control group (n = 4-6 per group; assayed in duplicate).

Table 1. Primer sets used for qPCR

Gene	Primer sequence (Forward)	Primer sequence (Reverse)	Amplicon size
elfa	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGC ATTAC	87
dat	GTTGCTGACTTTAGGAATCGAC	GCGTAAACACATAGATTCCACC	179
drd1	TGGTTCCTTTCTGCAACCCA	AGTGATGAGTTCGCCCAACC	100
drd2a	ATACTTCCGCTCTTTGGATGAA	CGTGATGCATTTTCAAAGAAGC	119
drd2b	CAAAACCATGAGCAAGAGGAAA	GCAGCCAGCAAATAATGAAAAC	98
th	ACCGATATTGTCTGATCGAACA	AGTGAACCAGTACATTGTCGAT	112

Oxygen consumption measurements

Standard oxygen consumption rates of individual fish were measured based on wet weight (g) and amount of oxygen consumed during 1 h of free exploration of the FMP Y-maze. Method for measuring oxygen consumption were adapted from (Voutilainen et al., 2011). Briefly, each behaviour tank was filled with exactly 3 L of aquarium-water and an initial reading of temperature and oxygen saturation were recorded using a HQ30D portable dissolved oxygen meter. Zebrafish were netted directly into the maze which was covered with parafilm to create a closed system. Tanks were immediately placed into the behaviour unit and the trial started. At the end of 1 h of exploration oxygen and temperature readings were recorded for each fish. Zebrafish were then briefly anaesthetised using Aqua-Sed anaesthetic treatment (Aqua-Sed^{TM}, Vetark, Winchester, UK) in accordance to manufacturer guidelines. A wet weight was recorded for each fish in grams. Oxygen consumption was expressed as mg/L O_2 x g^{-1} x h^{-1}.

Statistical analysis

The primary endpoint for analysis was the number of choices for each of the 16 tetragrams as a proportion of total turns. Based on previous research we were interested particularly in the proportion of choices that represented alternations (LRLR, RLRL) and repetitions (RRRR, LLLL) as these tetragrams were the most commonly observed. One-way analysis of variance (ANOVA) in GraphPad Prism was used to determine the difference between tetragram frequency. For subsequent analyses, we were interested in putative changes in strategy during the search period, and we therefore included "time" as the within-subjects factor. We also included "total turns" as a covariate in all analyses, in order to control for general activity levels in statistical models. For age groups in which drug was added, we included drug (present vs absent) as a between-subjects factors. Comparison of two groups was

performed using Unpaired *t*-test. Two-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test in GraphPad Prism was used to determine the effects of age combined with drug treatment. Alpha values of $P \leq 0.05$ were considered statistically significant. Data were presented as either mean (±SEM).
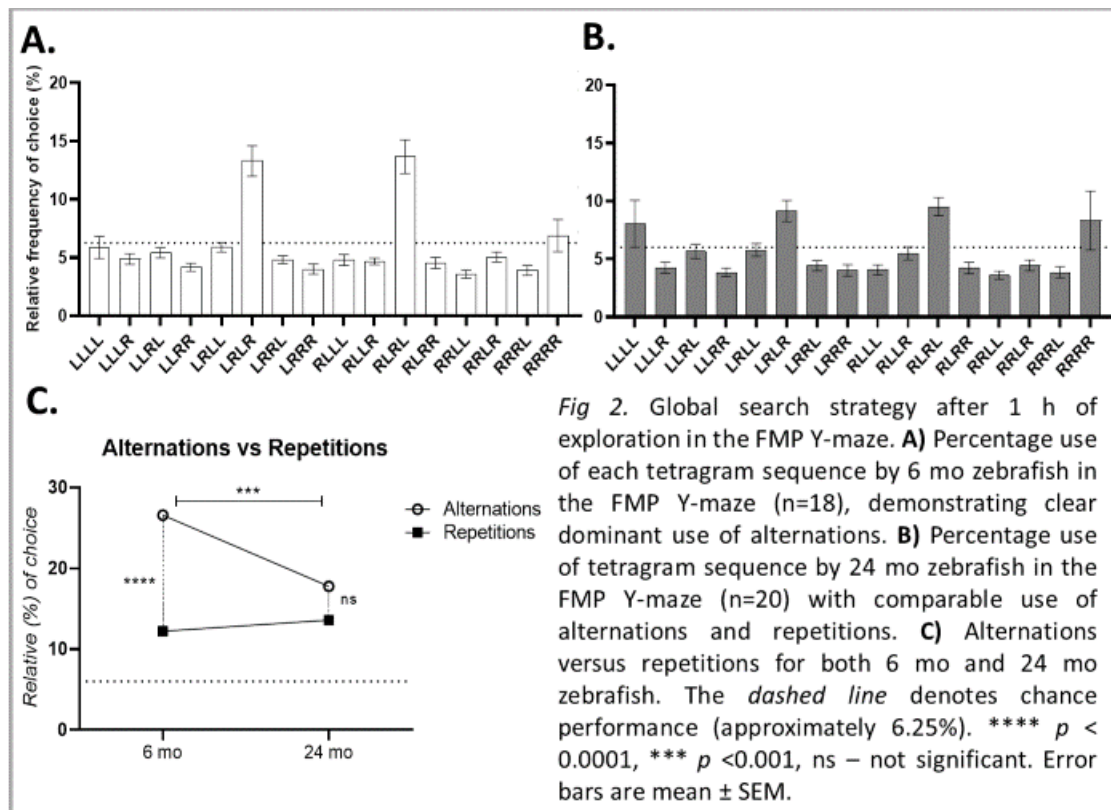
Results

Aging zebrafish show mild cognitive decline in the FMP Y-maze

Tetragram analysis revealed that global strategy (percentage use of each tetragram over the entire trial) relied on a similar pattern of turn choice, however, key differences were observed in the use of alternations (LRLR, RLRL) and repetitions (LLLL, RRRR). *Figure 2* shows tetragram frequency distribution for 1 h of free swimming in the FMP Y-maze for 6 mo v 24 mo zebrafish. Equal distribution of all possible tetragram configurations would be characteristic of a random search strategy (100% turns completed/16 tetragram configurations = 6.25%), whereas choices made above the 6.25% threshold are considered intentional and part of a global strategy. The majority of tetragrams were used randomly with bars falling below 6.25% (represented by the dashed line). 6 mo fish used alternations significantly more than all other search strategies (One-way ANOVA, F (7, 280 = 36.85, *p* <0.0001). However, there was a significant interaction between age and turn choice (Interaction, F (1, 68) = 11.94, *p* = 0.0009) with Tukey's *post hoc* test showing 6 mo zebrafish used alternations significantly more than 24 mo zebrafish (95% CI diff = 3.32-14.27, p = 0.0004***). This reduction in alternations in 24 mo zebrafish resulted in a change in the global strategy to split dominance between two tetragram sequences, alternations and repetitions, instead of just alternations as seen with the 6 mo zebrafish.
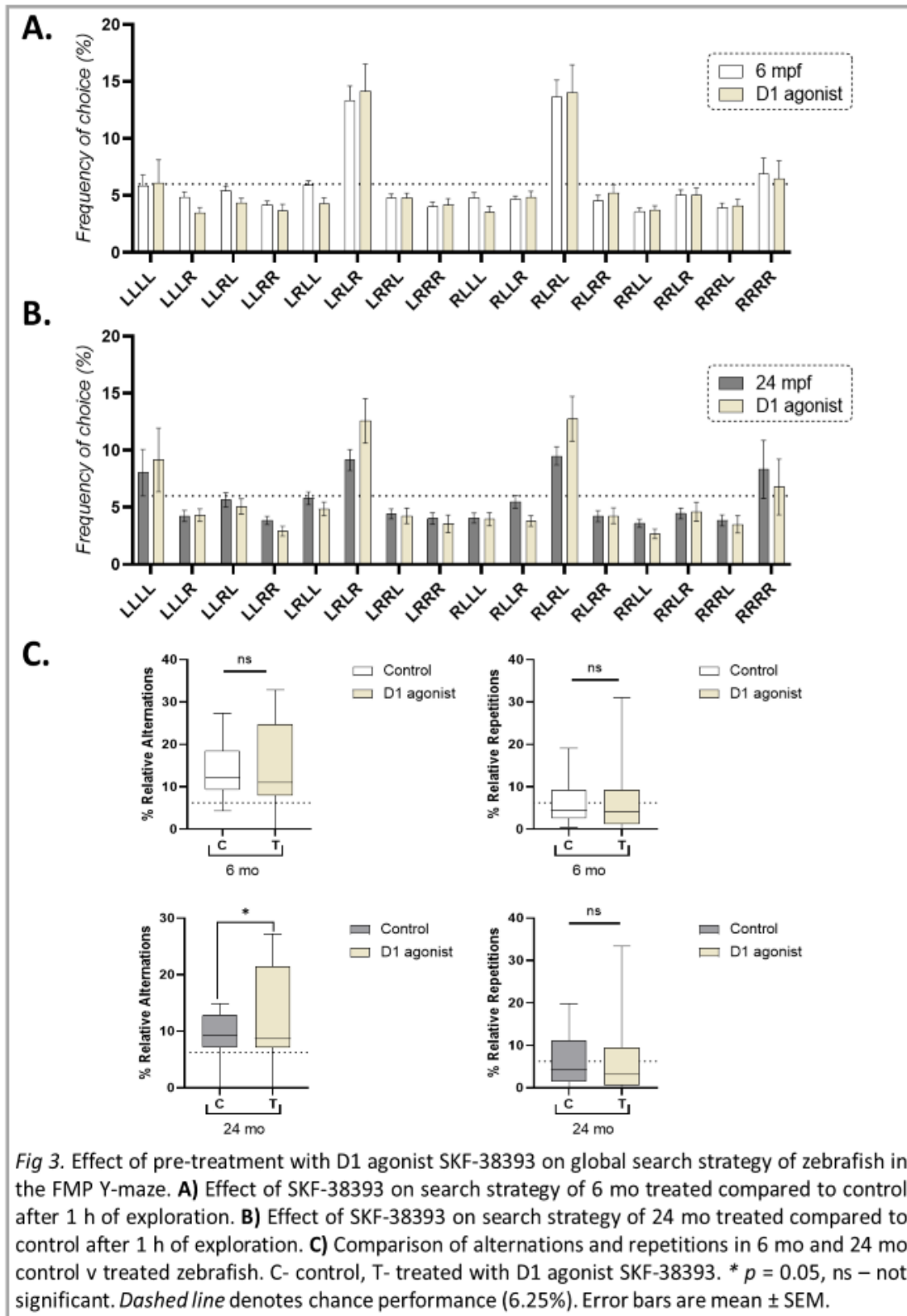
Figure 2.

Fig 2. Global search strategy after 1 h of exploration in the FMP Y-maze. **A)** Percentage use of each tetragram sequence by 6 mo zebrafish in the FMP Y-maze (n=18), demonstrating clear dominant use of alternations. **B)** Percentage use of tetragram sequence by 24 mo zebrafish in the FMP Y-maze (n=20) with comparable use of alternations and repetitions. **C)** Alternations versus repetitions for both 6 mo and 24 mo zebrafish. The *dashed line* denotes chance performance (approximately 6.25%). **** *p* < 0.0001, *** *p* <0.001, ns – not significant. Error bars are mean ± SEM.

Treatment with D1 agonist, SKF-38393, rescues working-memory deficit in aged zebrafish

Having shown a deficit in working memory of 24 mo zebrafish compared to 6 mo counterparts, shown by a change in global exploration strategy, we pre-treated zebrafish with D1 agonist SKF-38393 for 30 mins prior to testing in the FMP Y-maze. SKF-38393 treatment had no effect on global strategy in 6 mo zebrafish, with both alternations and repetitions showing no effect between treated (n=15) and untreated (n=18) (Two-way ANOVA, $F_{(1, 496)} = 0.44$, $p = 0.5078$). However, treatment of 24 mo zebrafish caused a significant increase in the use of alterations ($t = 2.33$, df = 74, $p = 0.0224$), without affecting use of repetitions ($t = 0.345$, df = 71, $p = 0.731$) (*Figure 3*). Working memory, as measured by global strategy showed a significant effect of age ($F_{(1,69)} = 9.037$, $p = 0.037$), but no significant interaction ($F_{(1,69)} = 2.546$, $p = 0.115$) and no main effect of treatment ($F_{(1,69)} = 1.643$, $p = 0.204$). However, Tukey's *post hoc* tests revealed that treatment with SKF-38393 rescued the deficit in alternations between aged 24 mo and middle aged 6 mo zebrafish (95% CI = -3.74-10.52, $p = 0.5969$) compared to controls (95% CI = 1.91-16.18**, $p =0.0073$).

Figure3.

Fig 3. Effect of pre-treatment with D1 agonist SKF-38393 on global search strategy of zebrafish in the FMP Y-maze. **A)** Effect of SKF-38393 on search strategy of 6 mo treated compared to control after 1 h of exploration. **B)** Effect of SKF-38393 on search strategy of 24 mo treated compared to control after 1 h of exploration. **C)** Comparison of alternations and repetitions in 6 mo and 24 mo control v treated zebrafish. C- control, T- treated with D1 agonist SKF-38393. * *p* = 0.05, ns – not significant. *Dashed line* denotes chance performance (6.25%). Error bars are mean ± SEM.
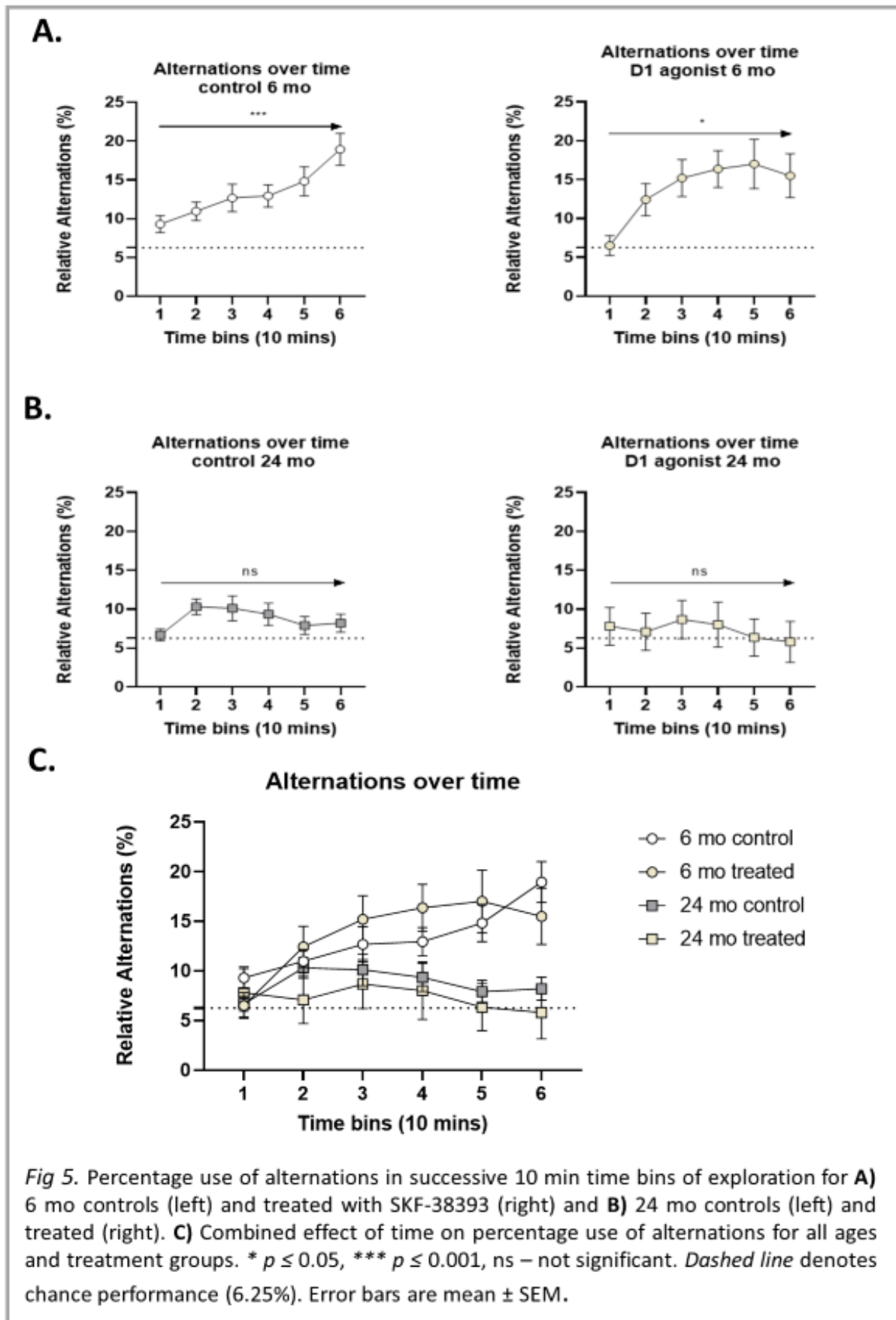
Healthy aging impacts cognitive flexibility which cannot be recovered by treatment with SKF-38393

The FMP Y-maze is a dual-action behavioural task which enables assessment of working memory based on global strategy, but also cognitive flexibility by analysing 'immediate' strategies consisting of exploration patterns for the total trial divided into equal length time bins (six 10 min time bins). This enables the identification of changes in strategy over time. *Figure 4* illustrates the percentage use of each tetragram sequence per 10 mins of exploration clearly denoting differences in the use of alternations over successive 10 min exploration intervals for 6 mo zebrafish, but a diminished effect of time on alternations in the 24 mo zebrafish. As alternations have been revealed as the dominant strategy and prone to change with age and treatment, we further explored the effect of time on alternation use. 6 mo control zebrafish showed the greatest effect of time on alternations as demonstrated in *Figure 5*. From the initial 10 mins of exploration, alternations were already used above random selection and continue to rise significantly with each successive time bin (One-way ANOVA, $F_{(5, 210)} = 4.33$, $p = 0.009$). The maximum mean difference between time bins was 9.7%, indicating that the alternation strategy was not static, but was altered in response to a constant environment. 24 mo aging zebrafish demonstrate a deficit in the ability to adapt their strategy over time, as shown by the stable use of alternations in consecutive time bins (One-way ANOVA, $F_{(5, 234)} = 1.35$, $p = 0.2449$), with a maximum mean difference of 3.6% between time bins. Treatment of 6 mo zebrafish with SKF-38393 did not affect global strategy as seen in *Figure 3*, however, it did appear to have a dampening effect of changes in alternation use over time (*Figure 5*). Though still significant, the effect was greatly reduced compared to controls (One-way ANOVA, $F_{(5, 164)} = 2.73$, $p = 0.0215$). In 24 mo zebrafish treated with SKF-38393 the global use of alternations was increased to a performance level equivalent to 6 mo controls; however, the drug treatment was unable to restore adaptability of search over time, resulting in a stable strategy (One-way ANOVA, $F_{(5, 208)} = 0.185$, $p = 0.9681$), which had a similar maximum mean difference of 2.9% between time bins.
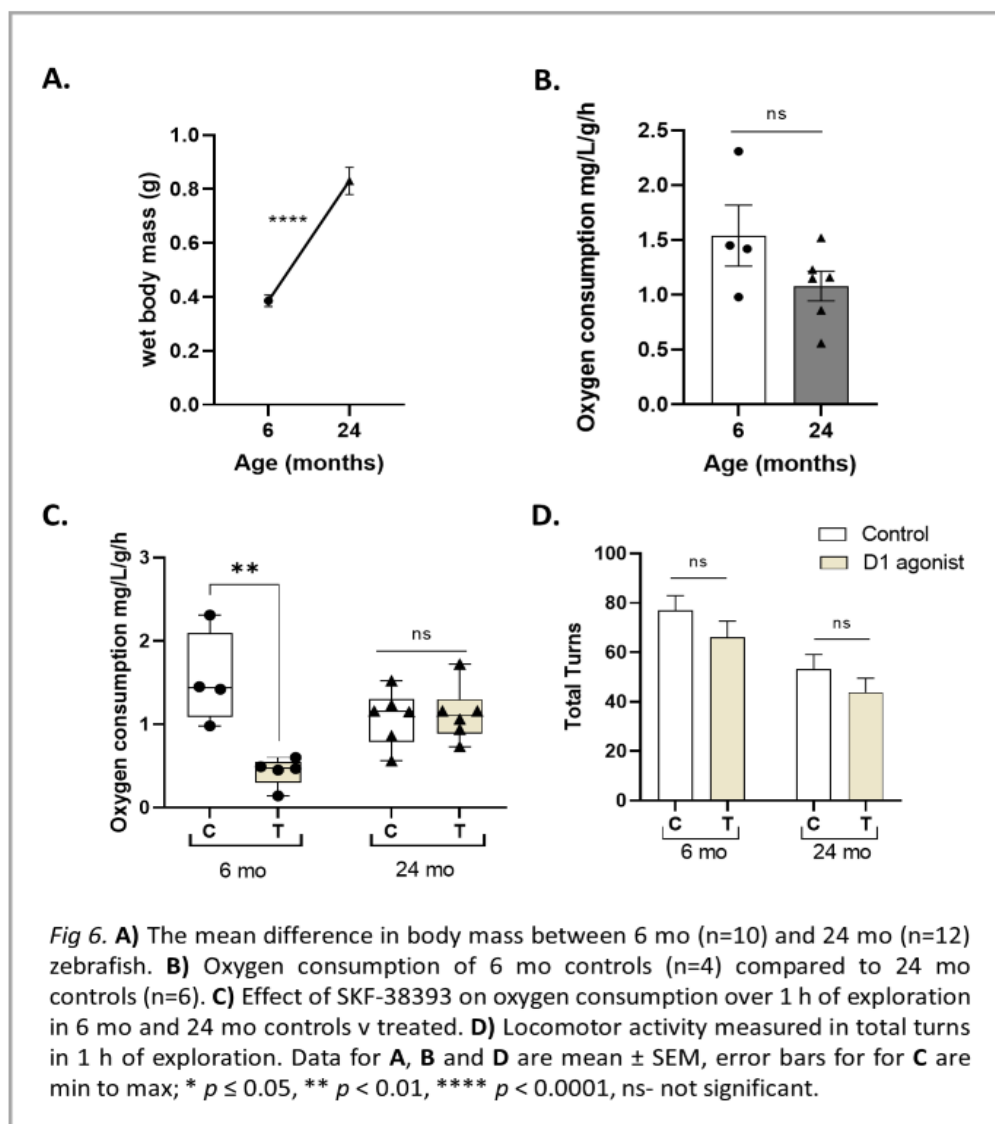
Figure 4.

Fig 4. shows the use of each tetragram per 10 min time bin for a 1 h trial of free FMP Y-maze exploration. **A)** depicts tetragram use for 6 mo adult controls compared to **B)** SKF-38393 treated. Both groups clearly illustrate an increasing use of alternations across successive time bins. **C)** shows the same frequency distribution for 24 mo aging adult controls versus **D)** SKF-38393 treated. Although the agonist treated group shows an increased percentage of alternation per time bin compared to controls, both groups have a heavily blunted effect of time, resulting in almost equal use of alternations in each 10 min time bin. Error bars are mean ± SEM.

Figure 5.

Fig 5. Percentage use of alternations in successive 10 min time bins of exploration for **A)** 6 mo controls (left) and treated with SKF-38393 (right) and **B)** 24 mo controls (left) and treated (right). **C)** Combined effect of time on percentage use of alternations for all ages and treatment groups. * $p \leq 0.05$, *** $p \leq 0.001$, ns – not significant. *Dashed line* denotes chance performance (6.25%). Error bars are mean ± SEM.

Metabolic rate is not a factor in changing search strategy with age

There appeared to be a substantial size difference between age groups, therefore to identify if metabolism played any role in the change in search strategy, wet weight and oxygen consumption over the course of the trial were recorded as an indirect measure of metabolism (Nelson, 2016). We identified a significant difference in wet body mass (t = 7.195, df = 19 , p <0.0001) with a mean wet weight of 0.39g for 6 mo and 0.83g for 24 mo zebrafish. We furthered this analysis by comparing oxygen consumption between age groups and found no significant difference (t = 1.660, df = 8 , p =0.1356). However, there was an effect of treatment on oxygen consumption (Two-way ANOVA, $F_{(1, 17)}$ = 11.41, p = 0.0036), Tukey's *post hoc* tests revealed that treatment with D1 agonist SKF-38393 caused a significant decrease in oxygen consumption in 6 mo (95% CI = 0.433-1.787, p = 0.0012), but had no effect on 24 mo fish (95% CI = -0.631-0.535, p = 0.9952) (*Figure 6*).

Figure 6.

Fig 6. **A)** The mean difference in body mass between 6 mo (n=10) and 24 mo (n=12) zebrafish. **B)** Oxygen consumption of 6 mo controls (n=4) compared to 24 mo controls (n=6). **C)** Effect of SKF-38393 on oxygen consumption over 1 h of exploration in 6 mo and 24 mo controls v treated. **D)** Locomotor activity measured in total turns in 1 h of exploration. Data for **A**, **B** and **D** are mean ± SEM, error bars for for **C** are min to max; * $p \le 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns- not significant.

Regulation of dopaminergic gene expression by aging

We investigated the effect of age on the dopaminergic system by analysing expression changes between 6 and 24 mo zebrafish. *Figure 7* shows the qPCR data of relative gene expression from whole brain tissue. We found no significant difference between 6 mo and 24 mo zebrafish for *dat* (t = 0.95, df = 7 , p =0.3722)*, drd1* (t = 1.25, df = 8 , p =0.2460)*, drd2a* (t = 1.20, df = 7 , p =0.2699)*, drd2b* (t = 0.21, df = 7 , p =0.8434) *or th* (t = 0.55, df = 7 , p =0.5985) mRNA expression levels.

Figure 7.

Fig 7. Quantitative real-time PCR analysis showing variations in the relative amounts of the *dat, drd1, drd2a, drd2b and th* mRNAs in whole brain tissue extracted from 6 mo controls (n=4) and 24 mo controls (n=6). Data were normalised to housekeeping gene *elfα* and defined as fold change relative to 6 mo controls. All data are mean ± SD; * $p ≤ 0.05$.
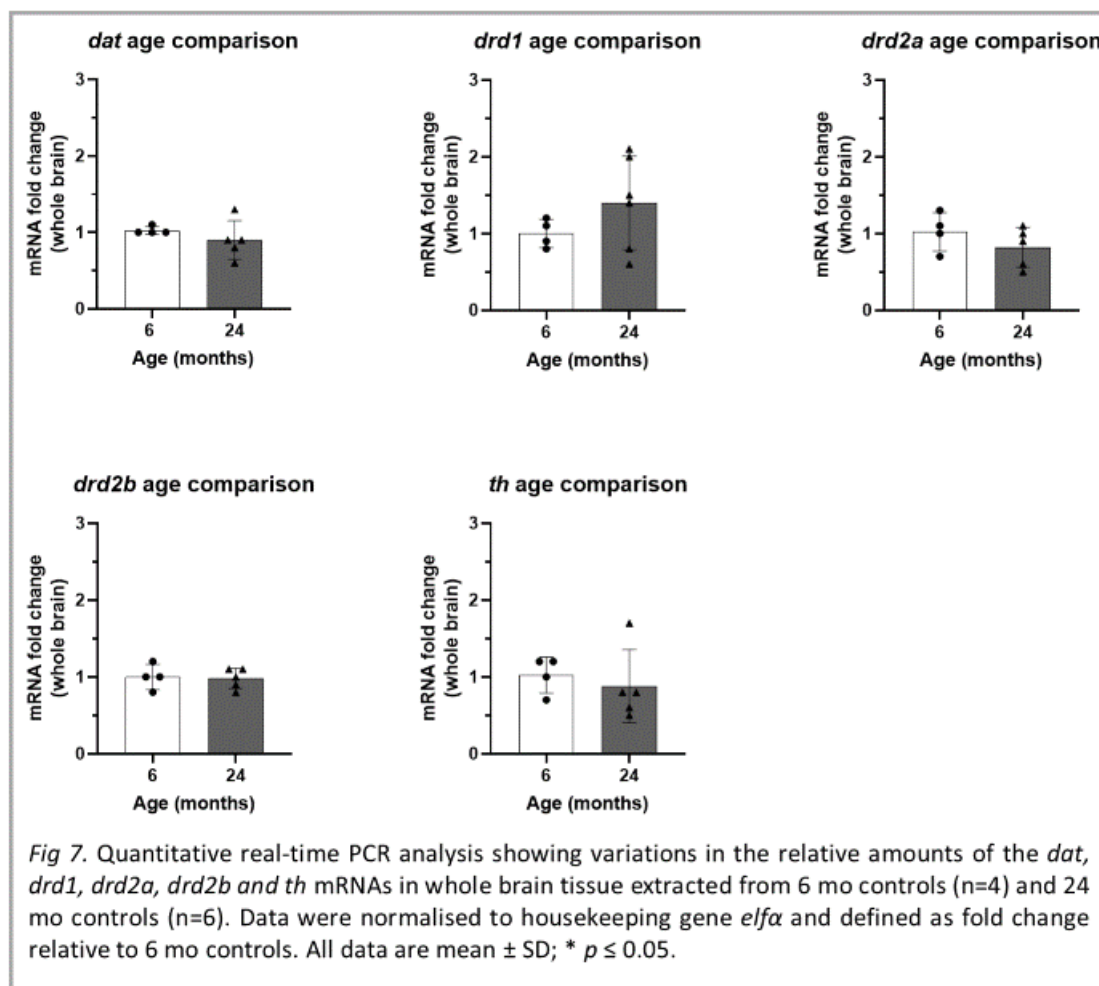
Effect of SKF-38393 on dopaminergic gene expression

The qPCR data in *Figure 8* shows that 35µM SKF-38393 was able to decrease the expression of *dat* in 6 mo zebrafish (t = 4.37, df = 8 , p =0.0024), but not 24 mo (t = 1.38, df = 10 , p =0.1972). However, 30 min treatment with SKF-38393 did not elicit expression changes in *drd1* (6 mo; t = 1.36, df = 7 , p =0.2170, 24 mo; t = 0.83, df = 10 , p =0.4266)*, drd2a* (6 mo; t = 1.33, df = 7 , p =0.2259, 24 mo; t = 1.31,

df = 8 , p =0.2265)*, drd2b* (6 mo; t = 1.17, df = 8 , p =0.2753, 24 mo; t = 0.18, df = 8 , p =0.8651) or *th*

(6 mo; t = 0.72, df = 8 , p =0.4950, 24 mo; t = 0.14, df = 8 , p =0.8894) in either age group.

Figure 8.

Fig 8. Quantitative real-time PCR analysis showing variations in the relative amounts of the *dat, drd1, drd2a, drd2b and th* mRNAs in whole brain tissue exposed to 35µM SKF-38393 in 6 mo (controls n=4, treated n=5) and 24 mo (controls n=6, treated n=6) zebrafish. Data were normalised to and defined as fold change relative to *elfα* controls. All data are mean ± SD; ** p < 0.01.
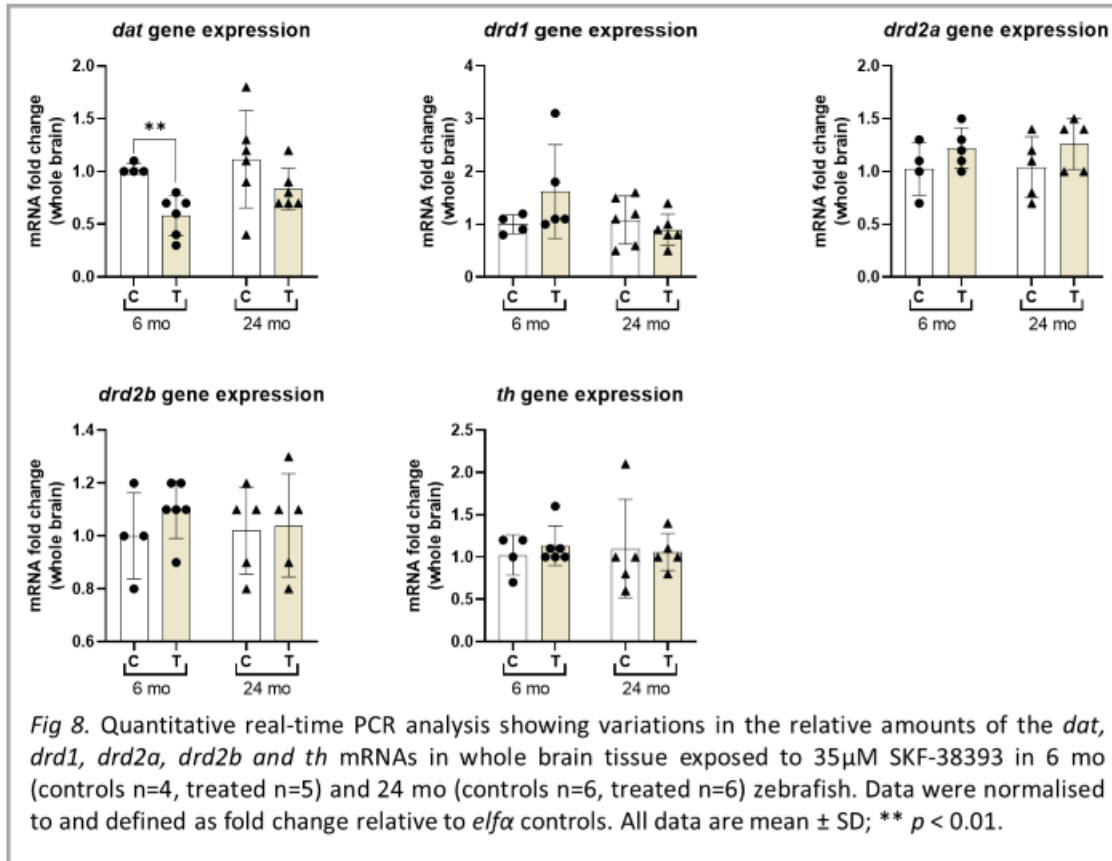
Discussion

Using a zebrafish model of aging we have investigated changes in working memory and cognitive flexibility between adulthood and old age, suggesting mild cognitive decline. The present findings indicate that working memory and strategy changes are both impaired in aged zebrafish; however, acute exposure to SKF-38393, a selective dopamine D1/D5 receptor partial agonist, enhanced working memory in aging, but had no detectable effect on behavioural flexibility. Real-time qPCR analysis identified a down regulation of *dat* mRNA expression in 6 mo adults treated with SKF-38393, but no effect on aged adults, supporting the role of *dat* in regulating dopamine availability and cognitive flexibility. These findings provide characterisation of cognitive changes in healthy aging which can be partially rescued by activating D1-like receptors, promoting the role of maintaining working memory, but not behavioural flexibility.

Changes in working memory in healthy aging zebrafish

Younger adult zebrafish have demonstrated a very specific global strategy used to explore the FMP Y-maze. Using tetragram sequence analysis, we identified patterns of choice selection of left and right turns in 6 mo adults that use alternation sequences (LRLR, RLRL) for more than 25% of the global search strategy, four times the use if selected randomly. All other sequences were used at a level equivalent to chance selection. Prior work from our group has demonstrated that changes in global spatial activity patterns, particularly those relating to alternations and repetitions, are representative of changes in working memory processing (submitted-Cleal, et al., 2020). Previous work has also demonstrated similar patterns of alternations in young adult zebrafish ranging from 3-6 mo (Cleal & Parker, 2018; Fontana, Cleal, & Parker, 2019; Fontana, Cleal, Clay, et al., 2019). Aging zebrafish at 24 mo have, however, demonstrated a marked change in global spatial activity patterns reducing the use of alternations by ~8%, bringing the use of alternations and repetitions almost in line at 18% and 16% respectively. This deficit in alternations may represents an inability to recall which arms of the maze have previously been entered and/or the order of entry, a process which has been shown to be dependent on working memory (Lalonde et al., 1986; Moran et al., 1995; Myhrer, 2003). These findings further support that zebrafish, like humans, have a natural decline in cognitive abilities as part of healthy aging, resulting in deficits in working memory (Berry et al., 2016; Dreher et al., 2008; Goldberg, 2017; Salthouse, 2009).

The role of aging in cognitive decline has been well documented in humans, with many animal models replicating similar deficits in cognitive performance (Gerhard, 2007; McQuail & Nicolle, 2012). Human and animal studies, have implicated disruption of the dopaminergic system in age related decline in executive functions such as working memory (Castner & Goldman-Rakic, 2004; Costa, 2014; Decker & McGaugh, 1991; Dreher et al., 2008; Godefroy et al., 1989). To this end we pre-treated adult and aging zebrafish with the partial dopamine D1/D5 receptor agonist to identify what role D1-like receptors play in the cognitive decline in healthy aging zebrafish. Similar to animal and human studies (Castner & Goldman-Rakic, 2004; Hemby et al., 2003; Molloy & Waddington, 1988; Wang et al., 2019), we found that treatment with a partial D1 agonist enhanced working memory in aging zebrafish, resulting in a rise in the use of alternations as part of the global strategy. 24 mo treated zebrafish increased alternations by nearly 7%, bringing alternation use to a level comparable with 6 mo zebrafish. The effect of SKF-38393 was age specific, as no such changes in working memory were observed in 6 mo zebrafish. Our findings support previous studies, implicating that boosting D1-like receptor activation is only beneficial if there is pre-existing dysregulation within the dopaminergic system (Roshan Cools & D'Esposito, 2011).

The role of metabolism and the effect of SKF-38393 on cognitive flexibility

Fine-scale analysis revealed that not only do fish use an overall, global strategy of alternations and repetitions to explore the FMP Y-maze, but this strategy is subject to change over time. We observed a pattern of increasing use of alternations throughout the trial in young, 6 mo adults. However, this natural tendency to modify search strategy with time ceases in aged adults. Throughout the life-span of an organism there is a continuous, dynamic equilibrium between goal stabilised and destabilised behaviours, in which an individual is required to balance focus on the current task against new information altering current goal perceptions (R. Cools, 2016). This cognitive flexibility has been strongly associated with the dopaminergic system and working memory (CAÑAS et al., 2003). In the FMP Y-maze the current goal could be perceived as foraging for potential food sources, or information seeking. This behavioural paradigm works on the basis of negative feedback (a continuous lack of food or reward) to update knowledge of the environment and inform decisions to selection appropriate behaviours, in this task reflected as search strategy. In young, healthy adult zebrafish the negative feedback loop dictates an increasing use of alternations over time. However, in line with previous studies in humans (Harada et al., 2013), aged zebrafish could not utilise negative feedback to update behaviour in response to the lack of environmental change and thus relied on the 'immediate' strategy, that was employed within the first 10 mins of novel exploration, as the only strategy used to explore the maze. This strategy was used regardless of how familiar the environment had become or in light of continuous lack of reward or novelty whilst exploring the FMP Y-maze. Changes in cognition is a normal aspect of healthy aging and similarly to humans, zebrafish exhibit age-related deficits in cognitive abilities (Adams & Kafaligonul, 2018). It is therefore unsurprising that in aging adults there is no longer an effect of time on alternations. The ability to adapt behaviour based on new information is fundamental to healthy cognitive processing, the disruption of which is common to psychiatric illness and neurodegenerative diseases (Pittenger, 2013; Waltz, 2017). The inability to adapt behaviour in response to the environment is of critical importance in aging conditions, and thus highlights the suitability of zebrafish to aid in informing human conditions of cognitive decline in aging.

An interesting finding during this study was that young adults that had shown a strong effect of time on search strategy, had this effect reduced following treatment with SKF-38393, and in aging adults, that had lost the ability to adapt strategy over time, treatment with the D1-like receptor agonist was unable to restore this behaviour. Dopamine acts as a neuromodulator, which is essential for achieving and maintaining cognitive control functions such as flexibly adjusting goal-directed behaviours (Ott & Nieder, 2019). Via D1-like receptor activation, dopamine can modulate working

memory performance and sustain goal-focused behaviour by stabilising neuronal activity and generating a high signal to noise ratio, reducing the influence of interfering (off target or distracting) stimuli (Durstewitz et al., 2000). However, in order to achieve goal-orientated behaviour with flexible adaptations a balance is required between a D1- and D2-dominant state, known as Dual state theory (R. Cools, 2016). Dual state theory implicates intermediate neural levels of dopamine, primarily acting via D1 receptors, resulting in low firing rate (lower energy use) and increased goal-directed behaviour as the D1-dominant state. The D2-dominant state, on the other hand, is characterised by fast firing rates (high energy use) and behavioural flexibility, e.g. set shifting or behavioral adaptations (R. Cools, 2016). A shift to the D1-dominant state by pharmacological intervention, for example following pre-treatment with a selective D1 receptor agonist, would result in system bias in favour of goal stabilisation, which is good for achieving the current goal (e.g. searching for food), but bad for adapting behaviour in response to new information (e.g. no food and a constant, unchanging environment, as presented in the FMP Y-maze) (R. Cools, 2016; Lianchun Yu & Yu, 2017).

We measured oxygen consumption in control and treated groups to identify if changes in metabolic activity played a role in the change in behaviour. Supporting the theory of dual state action between D1 - goal-directed behaviour and D2 - flexible behaviour. We found that 6 mo controls showed the greatest behavioural flexibility, but also had the highest mean oxygen consumption of 1.54 mg/L O_2 over 1 h of exploration. Treatment with SKF-38393 caused a significant decrease in oxygen consumption and a reduction in adaptive search behaviour over time. However, no such effect was evident in aged adults which had similar search strategies over time and similar oxygen consumption in control and treated groups. Our findings support over activation of the D1 receptor pathway, in 6 mo adults treatment with SKF-38393, potentially biasing the dopaminergic system in favour of a goal stabilised state, which decreased the amount of flexibility and reduced energy consumption by switching to a lower firing rate in dopaminergic neurons.

The lack of change in search patterns over time in aged adults and the accompanying lack of change in oxygen consumption between treated and control groups further supports this hypothesis. Examples of the Dual state theory have been evidenced in human, primate and computational studies (Durstewitz et al., 2000; Durstewitz & Seamans, 2008; Fallon & Cools, 2014; Ott & Nieder, 2019). Here we see that increasing D1 receptor signalling interferes with cognitive flexibility in younger adults and is unable to restore it in aged adults. Thus, biasing away from flexible behaviour appears to be a robust mechanism; however, as we have outlined above, in order to positively influence goal-directed behaviour a specific level of receptor activation is required. It is also noteworthy that these changes do not appear to be related to locomotion as there are no changes in total turns between controls and treated fish of either age group, despite changes in metabolism in 6 mo adults and changes in

global strategy in 24 mo fish in response to SKF-38393 treatment. This further supports the hypothesis that changes are mechanistic and dependent on age, opposed to physical, i.e. increased number of turns resulting in an increased number of alternations, or decreased activity decreasing oxygen consumption. Here we provide the first evidence that the Dual state theory may also apply to zebrafish. However, the modulatory effect of dopamine is complex, influenced by dopamine receptor activation and age. These findings provide further understanding of the role of the dopaminergic system in age related changes in cognition, but more work is necessary to fully elucidate the mechanisms and their potential manipulation to improve executive function in aging adults.

Molecular changes in dopaminergic gene expression

To further understand the role of dopamine in healthy aging we investigated the expression of genes critical to the dopaminergic system including tyrosine hydroxylase, dopamine transporter and the dopamine receptors (*drd1, drd2a* and *drd2b*). Contrary to previous studies, we did not observe any age-dependent alterations in expression levels of dopamine related genes. One possible explanation for this is that many studies assessing the role of aging on dopamine system regulation often use region-specific tissue for analysis, e.g. the hippocampus, prefrontal cortex, ventral or dorsal regions of the striatum (Araki et al., 2007; Godefroy et al., 1989; Hemby et al., 2003). The asymmetric distribution of dopamine receptors throughout the brain strongly correlates localization with functional specificity (El-Ghundi et al., 2007). It is therefore a possibility, that regional differences in expression may counteract one another when the system is subject to subtle changes in expression. It is also possible that differences in expression are not typical between 6 and 24 mo zebrafish, but as this has not been fully characterised in adult zebrafish more work is needed in this area.

However, larger or regionally uniform changes may still be detectable with whole brain gene expression analysis. To this end we examined if there were any detectable changes in expression related to fish treated with SKF-38393. We found a significant reduction in expression of *dat* in 6 mo treated adults compared to controls, an effect that was not replicated in 24 mo treated fish suggesting an age specific effect. *Dat,* a plasma membrane protein exclusively expressed in dopamine synthesising neurons, plays a crucial role in regulating the amplitude and duration of dopamine-mediated neurotransmission by clearing dopamine from the synaptic cleft (Bannon, 2005; Bannon et al., 2001; Mortensen & Amara, 2003). The constitutive process of transporter trafficking of dopamine allows for rapid up- and down- regulation of cell surface transporter expression and, thus, transport activity (Gulley & Zahniser, 2003). Downregulation of *dat,* thus resulting in an increase of synaptic dopamine availability by reducing reuptake, is a likely explanation for the reduction in cognitive flexibility over time in the treated 6 mo zebrafish. Studies investigating dopamine system function

modulating behaviour have identified a role for presynaptic dopamine transporters (*dats*). Studies examining cognitive flexibility in patients with Parkinson's Disease (PD) have found that patients taking dopamine-enhancing medication (e.g. dopamine receptor agonists or L-DOPA) perform poorly on reversal or reinforcement learning tasks compared to patients that are not receiving medication (Roshan Cools et al., 2001; Rutledge et al., 2009; Waltz, 2017). Additionally it was noted that learning rates that were enhanced by dopaminergic medications only impacted positive reward learning and had no effect on negative outcome learning (Rutledge et al., 2009). These findings are consistent with other studies using methylphenidate or cocaine- both substances blocking dopamine reuptake by inhibiting *dat* (Gatley et al., 1999). (Clatworthy et al., 2009) found that young healthy subjects orally administered methylphenidate had the least cognitive flexibility in reversal learning when associated with the greatest amount of striatal dopamine release; however, spatial working memory performance was improved with increasing amounts of striatal dopamine. Similarly studies of cocaine-use found that cognitive flexibility was selectively impaired, with some studies showing that working memory remained unaffected (Colzato et al., 2009; Stalnaker et al., 2009). Combined, these experiments support the findings from this study that increased synaptic dopamine, either by reducing dopamine transporters or treatment with dopamine-enhancing drugs, can impair behavioural flexibility in healthy subjects, or patients not treated with enhancing drugs, by causing an 'over-dose' of dopamine in regions with optimal dopamine levels, in relation to other dopamine depleted regions resulting in distinct functional changes, i.e. no effect on working memory, but a decrease in behavioural flexibility as seen here in 6 mo treated zebrafish compared to controls. This hypothesis would also explain the lack of effect of SKF-38393 on *dat* expression in 24 mo adults, which had already shown a deficit in cognitive flexibility in the control group, therefore pre-existing dysregulation of the dopamine system as a result of aging, prevented any 'over-dosing' effect causing changes in *dat* expression.

Conclusion

Our study is consistent with previous findings that the dopamine system plays a vital role in maintaining normal cognition and consequently appropriate behaviour selection in natural, healthy aging. We found that aged adult zebrafish have impaired working memory and cognitive flexibility compared to their younger counterparts, however, treatment with a D1 agonist could improve working memory performance in the FMP Y-maze. We also identified a role for 'over-dosing' of dopamine and regulation of *dat* expression in subjects without dopamine depletion, which consequently resulted in reduced cognitive flexibility in healthy treated adults compared to controls.

Further study is required to fully elucidate the mechanisms underlying responses to dopamine-enhancing drugs, with particular focus on regional brain changes and specific behavioural impairment and enhancement. Our work further supports the use of zebrafish as a model organism for studying behavioural changes and cognitive decline in aging.

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