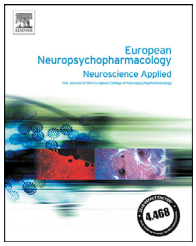
European Neuropsychopharmacology (2019) 000, 1–13



www.elsevier.com/locate/euroneuro

Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil

Héctor Carreño Gutiérrez[a], Irene Vacca[a], Gido Schoenmacker[b], Madeleine Cleal[c], Anna Tochwin[d], Bethan O'Connor[a], Andrew M.J. Young[a], Alejandro Arias Vasquez[b], Matthew J. Winter[d], Matthew O. Parker[c], William H.J. Norton[a,*]

[a] *Department of Neuroscience, Psychology and Behaviour, College of Life Sciences, University of Leicester, Leicester LE1 7RH, UK*
[b] *Radboudumc Human Genetics/Radboud University Institute for Computing and Information Sciences (iCIS)/Donders Centre for Neuroscience, Nijmegen, the Netherlands*
[c] *School of Health Sciences and Social Work, University of Portsmouth, Portsmouth PO1 2FR, UK*
[d] *Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK*

Received 1 May 2019; received in revised form 4 October 2019; accepted 10 October 2019
Available online xxx

KEYWORDS
Zebrafish;
Aggression;
Drug screen;
Behaviour;
Caffeine;
Sildenafil

Abstract
Although aggression is a common symptom of psychiatric disorders the drugs available to treat it are non-specific and can have unwanted side effects. In this study we have used a behavioural platform in a phenotypic screen to identify drugs that can reduce zebrafish aggression without affecting locomotion. In a three tier screen of ninety-four drugs we discovered that caffeine and sildenafil can selectively reduce aggression. Caffeine also decreased attention and increased impulsivity in the 5-choice serial reaction time task whereas sildenafil showed the opposite effect. Imaging studies revealed that both caffeine and sildenafil are active in the zebrafish brain, with prominent activation of the thalamus and cerebellum evident. They also interact with 5-HT neurotransmitter signalling. In summary, we have demonstrated that juvenile

* Corresponding author.
E-mail address: whjn1@le.ac.uk (W.H.J. Norton).

https://doi.org/10.1016/j.euroneuro.2019.10.005

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

zebrafish are a suitable model to screen for novel drugs to reduce aggression, with the potential to uncover the neural circuits and signalling pathways that mediate such behavioural effects.

1. Introduction

Aggression is an adaptive behaviour that animals use to protect offspring, defend or obtain resources and establish social hierarchies (Nelson and Trainor, 2007). However, heightened aggression can cause injury or death meaning that expression of this behaviour must be tightly controlled. In humans, aggression can also be a non-specific symptom of several psychiatric disorders including attention-deficit/hyperactivity disorder (ADHD), bipolar disorder, conduct disorder, schizophrenia and substance abuse disorder (Nevels et al., 2010).

Human aggression can be managed by a combination of behavioural, cognitive and pharmacological therapies. Current drugs used to treat aggression include psychostimulants, mood stabilisers, anti-epilepsy drugs, selective 5-HT reuptake inhibitors and agonists of alpha-2 adrenergic receptors (Connors et al., 2006; Loy et al., 2012; Nevels et al., 2010; Tcheremissine and Lieving, 2006). However, these drugs are not specific and often target a small number of signalling pathways. Furthermore, these drugs do not work in all patients and can have unwanted side-effects making them unsuitable for long-term use (Nevels et al., 2010; Polzer et al., 2007; Scotto Rosato et al., 2012; Wernicke et al., 2003). The existence of two broad subtypes of aggression (impulsive/reactive and instrumental/proactive) with distinct genetic and neurological bases complicates treatment of this behaviour (Blair, 2010; Kempes et al., 2005; Montoya et al., 2012; Nevels et al., 2010; Scotto Rosato et al., 2012). Each subtype is associated with particular psychiatric disorders and may respond to different drugs (Pappadopulos et al., 2006). It is clear that there is an unmet clinical need for safer, more potent drugs to treat aggression (Nevels et al., 2010).

Traditionally, drug screens have focussed on finding compounds that interact with defined molecular targets. However, lack of knowledge of the molecular basis of complex behaviours such as aggression limits this approach. Recent research has shown that behaviour-based screens can be used to identify novel psychoactive compounds (MacRae and Peterson, 2015; Rihel and Schier, 2012). Compared to in vitro screens, whole organism paradigms maintain the complex architecture of the brain making them ideal to examine drugs that act at multiple receptors. The zebrafish is an excellent vertebrate model for in vivo drug discovery (Cunliffe, 2016) due to their short generation time, small size and the possibility to apply compounds by immersion. Numerous robust tests to measure both larval and adult zebrafish behaviour have already been established (Norton, 2013). Small molecules that can alter sleep, photomotor response, light/dark behaviour, locomotion, feeding, freezing and susceptibility to seizures have been identified demonstrating the feasibility of this approach (Baxendale et al., 2012; Copmans et al., 2016; Fraser et al., 2017; Jordi et al., 2018; Kokel et al., 2010; Rennekamp et al., 2016; Rihel et al., 2010; Vliet et al., 2017).

Previous research in our laboratory and others has used zebrafish to study the genetic and pharmacological basis of aggression (Carreno Gutierrez et al., 2018; Jones and Norton, 2015; Norton, 2019). Zebrafish display characteristic agonistic postures that include erection of the fins, thrashing the tail, biting, splashing and directing short bouts of swimming against an opponent (Gerlai et al., 2000; Laan et al., 2018). We have identified *fibroblast growth factor receptor 1a* as novel aggression-linked gene (Norton et al., 2011) and demonstrated that nitric oxide and 5-HT interact to control this behaviour (Carreno Gutierrez et al., 2017). We have also built a platform to measure mirror-induced aggression in juvenile zebrafish and validated software to automatically quantify our data (Carreno Gutierrez et al., 2018).

In this study we set out to identify novel drugs that can reduce zebrafish aggression. We selected ninety drugs from published research and recorded both aggression and locomotion. We used a three-tiered strategy that increased in stringency at each step to identify the most promising candidates. We identified caffeine and sildenafil as drugs that consistently decrease aggression with a lesser effect upon locomotion. Both drugs reduce aggression at different life stages but had an opposite effect upon cognitive impulsivity. By combining whole brain imaging and neurochemistry we determined the brain areas and neurotransmitter signalling pathways that mediate activity of these drugs. As well as determining novel compounds to treat aggression, this research further demonstrates the utility of zebrafish for phenotype-based drug screens.

2. Experimental procedures

2.1. Zebrafish strains, care and maintenance

Adult and juvenile zebrafish were maintained at the Universities of Leicester, Portsmouth and Exeter using standard protocols and in accordance with institutional guidelines for animal welfare. All experiments were carried out using AB wild-type zebrafish apart from the neural imaging (see below).

2.2. Screen design

The drug screen was performed as described in Carreno Gutierrez et al. (2018) and Norton (2019). Behaviour was measured in small plastic tanks (9 × 4.2 × 4 cm) with three white opaque walls and one clear wall. Each tank was filled with 100 ml water from the main aquarium facility. Groups of 20 juvenile ze-

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

brafish were habituated to these arenas overnight. Experiments were carried out between 11:00 and 15:00. On the experimental day fish were placed into individual tanks using a wide-tipped Pasteur pipette. Aggression and locomotion was measured in twelve juvenile (one month-old) zebrafish in parallel using a custom-built cubicle (Carreno Gutierrez et al., 2018). The ambient light (150 Lux) and temperature was controlled. Infrared lighting was provided using a light source that formed the floor of the apparatus.

In the first round of the screen we tested ninety drugs on up to twelve juvenile zebrafish. A description of the drugs used in this study is provided in Supplemental Tables 1 and 3. Drugs were selected by either searching PubMed with the keywords "aggression, drug, pharmacology (round 1 of the screen), or by using the Chemmine website (http://chemmine.ucr.edu) as described below. The chemical and physical properties of the drugs were identified in the PubChem database (Supplemental Tables 1 and 3). Drug concentrations were selected according to previous studies and taking into account the small size of juvenile zebrafish (for references refer to Supplemental Table 1). Each drug was applied at three different concentrations. Fish were immersed in 100 ml of drug solution for 30 min before being placed into the cubicle and recording behaviour. Control fish received a mock treatment (immersion in aquarium water containing 0.01% DMSO as a vehicle control) and underwent the same handling procedure. The second round of screening used up to twenty-four juvenile zebrafish. We then chose to further analyse caffeine and sildenafil in a third round of testing using around fifty juvenile zebrafish.

Juvenile aggression was compared with- and without drug treatment. Behaviour was recoded using video cameras and ViewPoint aggression software automatically quantified aggression and locomotion (Carreno Gutierrez et al., 2018). Each experiment lasted 12 min. In the first 2 min juveniles were habituated to the tank following transfer. During the next five min locomotion was recorded without visual access to the mirror. Locomotion was quantified by videotracking. The divider was then removed and aggressive interaction with the mirror was recorded for another 5 min. Aggression was defined as the amount of time a fish spent with its body at an angle of 22.5° to the mirror and its nose touching the mirror. Aggression was expressed as "aggression units", an arbitrary unit that corresponds to the total amount of time spent fighting the mirror image (Carreno Gutierrez et al., 2018). Locomotion was expressed as "locomotion units" based upon the total number of pixels moved during an experiment (Carreno Gutierrez et al., 2018).

2.3. Adult behaviour

Adult behavioural experiments were performed using AB wild-type zebrafish that were between three and five months old at the start of testing. Fish were recorded using FlyCapture2 2.5.2.3 software and a digital camera from Point Grey Research. The age, sex, and size of fish were carefully matched. Caffeine (25, 50, 100 and 200 μM) and sildenafil (1, 5, 10 and 50 μM) were applied by immersion for 30 min. A full description of adult behaviour methods is included in the Supplemental material.

2.4. High pressure liquid chromatography

The amine and metabolite content of homogenates of telencephalon, diencephalon, optic tectum and hindbrain was assessed by high performance liquid chromatography (HPLC) with electrochemical detection. Separation was achieved on a 150 × 1.0 mm C18 column (LUNA(2), Phenomenex, UK) and dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were detected electrochemically (750 mV versus an Ag/AgCl reference electrode: ANTEC Intro, ANTEC Inc, Netherlands). Peak area for each compound was determined using a PC-based integrator (ChromPerfect, Justice Laboratories, USA), and results were expressed as fmol/mg of brain. A full description of HPLC methods is included in the Supplemental material.

2.5. Data analysis

Statistical analysis of aggression, locomotion and anxiety like behaviour was performed in GraphPad Prism 6 and MATLAB R2018a. The data set was assessed for normality using a Shapiro-Wilks test. A student's *t*-test or Mann-Whitney *U test* was then used to compare control and drug-treated animals. When comparing more than two groups, a one-way ANOVA or Kruskal-Wallis test was used. If significant this was followed up with post hoc Mann-Whitney *U*-tests to determine which dosage groups were different from controls. Multiple testing correction for compounds was performed using false discovery rate (FRD) controlling correction (Benjamini and Hochberg, 1995). Post-hoc tests were not corrected. Data analysis for the 5-Choice serial reaction time task (5-CSRTT) and fear conditioning was carried out in SPSS. Data pertaining to correct responses on the 5-CSRTT and premature (impulsive) responses were assessed using general linear mixed effects models. In both models, fixed effects were 'dose', and random effects were 'subject ID'. This allowed us to examine differences for each dose from control, taking each fishes' repeated measures into account while controlling for non-independence of replicates. Data pertaining to fear conditioning were analysed using a 2-way mixed design analysis of variance (ANOVA), with drug treatment (5 levels [0, 50 μM caffeine, 100 μM caffeine, 5 μM sildenafil, 50 μM sildenafil]) as the between subjects factor and conditioning (2 levels [baseline, post-conditioning]) as the within-subjects factor. The dependent variable was preference for the conditioned stimulus. High pressure liquid chromatography data were subjected to 2-way ANOVA with significant main effects of drugs interrogated post hoc with Dunnett's multiple comparison test. All data are presented as mean ±SEM. Type-1 error rate was set at alpha = 0.05 for all analyses. Asterisks in figures indicate significant differences: (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

3. Results

3.1. First round screen of ninety drugs

Our laboratory has developed a protocol to automatically record and quantify juvenile zebrafish aggression and locomotion (Carreno Gutierrez et al., 2018). Using this setup we conducted a biased screen of ninety drugs that affect aggression or aggression-related signalling pathways in other species (Supplemental Table 3). We immersed one month-old wild-type juvenile zebrafish in three concentrations of each drug and recorded their behaviour thirty minutes later. We first measured locomotion followed by aggressive interaction with a mirror (Carreno Gutierrez et al., 2018; Gerlai et al., 2000). A positive hit was defined as a substance that would decrease aggression with little to no effect upon locomotion. To maximise the number of compounds tested we used between nine and twelve juvenile fish per drug treatment. Depending on the compounds and doses used we found both increases and decreases in aggression compared to the control fish (Supplemental Table 1) even though the p value did not reach significance due to the small number of animals analysed. The thirty compounds that were

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

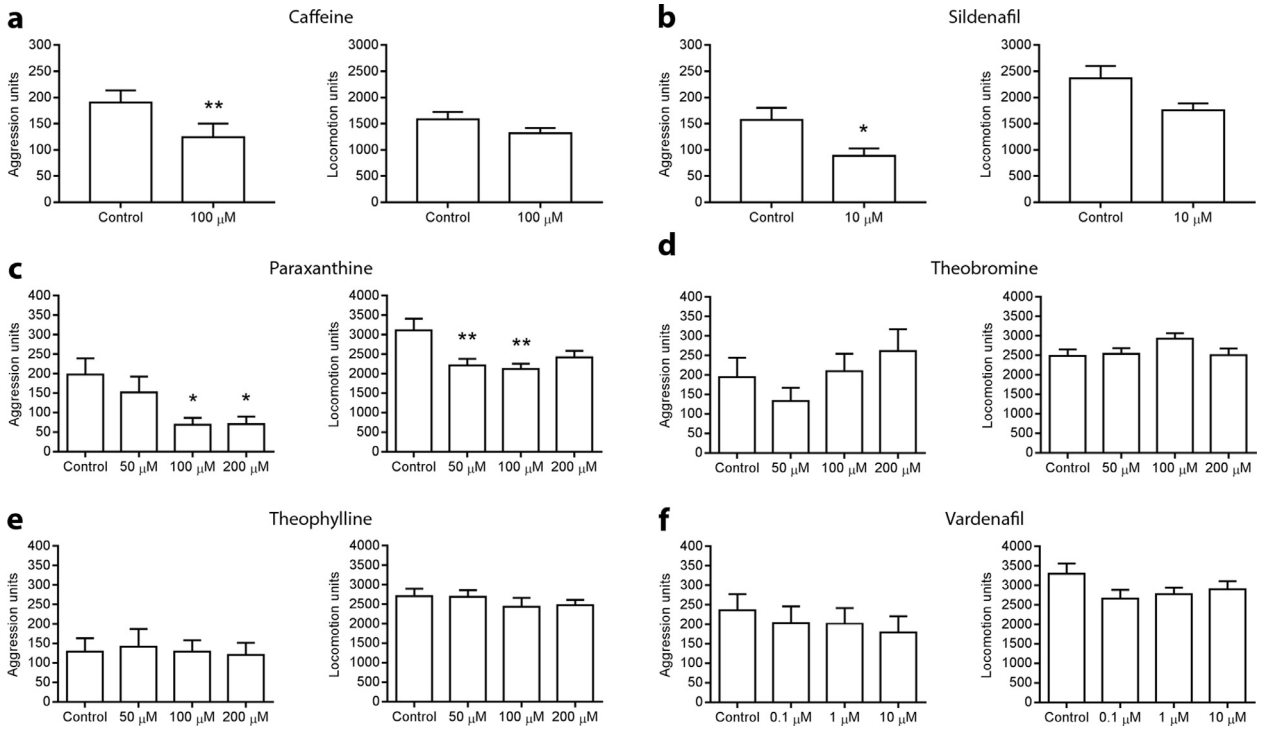


Fig. 1 Caffeine, sildenafil and paraxanthine decrease juvenile zebrafish aggression. Graphs showing aggression and locomotion following application of (a) caffeine (Aggression: Mann-Whitney $U = 928.5$, $p = 0.0025$; $n = 56$ control, $n = 50$ caffeine. Locomotion: Mann-Whitney $U = 1205$, $p = 0.2193$), (b) sildenafil (Aggression: Mann-Whitney $U = 1081$, $p = 0.0204$; $n = 56$ control, $n = 52$ sildenafil. Locomotion: Mann-Whitney $U = 686$, $p = 0.0578$), (c) paraxanthine (Aggression: ANOVA F (3, 122) = 3.871, $p = 0.0110$; control vs. 50 μM: $p = 0.6213$, control vs. 100 μM: $p = 0.0154$, control vs. 200 μM: $p = 0.0169$; $n = 36$ control, $n = 30$ paraxanthine. Locomotion: ANOVA F (3, 122) = 4.653; control vs. 50 μM: $p = 0.0094$, control vs. 100 μM: $p = 0.0034$); $n = 36$ control, $n = 30$ paraxanthine. (d) Theobromine (Aggression: ANOVA F (3, 116) = 1.284, $p = 0.2831$; $n = 30$ control, $n = 30$ theobromine. Locomotion: ANOVA F (3, 116), $p = 0.1512$); $n = 30$ control, $n = 30$ theobromine. (e) Theophylline (Aggression: ANOVA F (3, 118) = 0.0568, $p = 0.9821$; $n = 35$ control, $n = 30$ 50 μM, n - 28 100 μM, $n = 29$ 200 μM theophylline. Locomotion: ANOVA F (3, 118) = 0.5718, $p = 0.6347$); $n = 35$ control, $n = 30$ 50 μM, n - 28 100 μM, $n = 29$ 200 μM theophylline. (f) Vardenafil (Aggression: ANOVA F (3, 119) = 0.3432, $p = 0.7941$; $n = 35$ control, $n = 30$ 0.1 μM, n - 28 1 μM, $n = 30$ 10 μM vardenafil. Locomotion: ANOVA F (3, 120) = 1.619, $p = 0.1885$); $n = 35$ control, $n = 30$ 0.1 μM, n - 28 1 μM, $n = 30$ 10 μM vardenafil. One-way ANOVA followed by Dunnett's post hoc; $n = 28$–36 each experimental group. * $p < 0.05$, ** $p < 0.01$.

most effective at reducing aggression were selected to be retested.

3.2. Second round screen of positive hits and validation of caffeine and sildenafil activity

We re-tested these thirty compounds in a second batch of experiments using up to thirty juvenile zebrafish. Despite using a larger number of animals we still generated relatively few significant results after correcting for the false discovery rate due to multiple comparisons (Supplemental Table 1). In some cases (e.g. tacrine) a reduction of aggression was also accompanied by a significant decrease in locomotion, making them unsuitable for further analysis. We therefore chose caffeine and sildenafil for further examination based upon their ability to reduce aggression, limited effect upon locomotion and known safety profile in humans. We tested caffeine and sildenafil rigorously using a large number of juvenile zebrafish (Fig. 1 and Supplemental Table 1). Caffeine deceased aggression at 100 μM without affecting locomotion (Fig. 1(a)) and sildenafil decreased aggression at 10 μM with only a trend effect upon locomotion (Fig. 1(b)).

3.3. Identification and characterisation of drugs structurally related to caffeine and sildenafil

We next identified chemical compounds that are structurally related to caffeine and sildenafil. We used the ChemMine website to search the Pubchem database for compounds with a similar molecular structure to caffeine and sildenafil (Supplemental Table 4). We clustered these drugs using multidimensional scaling and identified the caffeine derivatives paraxanthine, theobromine and theophylline and the sildenafil derivative vardenafil (Supplemental Fig. 2 and Supplemental Table 4). Paraxanthine significantly reduced juvenile zebrafish aggression at the two highest concentrations used (100 μM and 200 μM; Fig. 1(c)) whereas the other drugs did not (Fig. 1(d)-(f)). This demonstrates

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

that paraxanthine may also represent a suitable compound to decrease zebrafish aggression.

3.4. Caffeine and sildenafil alter neural activity in the larval zebrafish brain

Caffeine and sildenafil can both cross the blood brain barrier and act centrally to modify behaviour (Kouvelas et al., 2009; Watson et al., 2002). We used transgenic elavl3:GCaMP6s-expressing zebrafish to image activity in response to caffeine or sildenafil in the brain of four day-old larvae (Winter et al., 2017) (Supplemental Fig. 3). Although four day-old zebrafish cannot perform complex behaviours such as aggression, this approach allowed us to identify a signature of neural activity triggered by these drugs. Application of 100 μM caffeine caused a trend to a non-significant increase in sharp pulses of neural activity (peak intensity; Fig. 2(a) and Supplemental Table 2) in nearly all brain areas examined. The increase in peak intensity reached significance in the diencephalon and anterior commissure as well as the eminentia granularis and the caudal lobe of the cerebellum (Supplemental Table 2). Area under the curve (AUC) analysis also revealed a trend decrease of activity in the inferior raphe, interpeduncular nucleus and tectum stratum periventriculare (Fig. 2(b) and film 1,2 (film 1: water treated; film 2: caffeine treated). Immersion in sildenafil led to trend decreases in peak intensity in the olfactory bulb and eyes, habenulae, eminentia granularis, tangential vestibular neurons and vagal ganglia (Fig. 2(c) and Supplemental Table 2). Trend increases in peak intensity were seen in the anterior commissure, diencephalon, tegmentum, rhombencephalon and noradrenergic neurons of the interfascicular and vagal areas. Application of 10 μM sildenafil significantly increased AUC fluorescence in the eminentia thalami and caused a trend increase in many other diencephalic areas (Fig. 2(d), Supplemental Table 2 and film 1,3 (film 1: water treated; film 3: sildenafil treated). Together, these results show that different regions of the brain respond to caffeine or sildenafil application.

3.5. Caffeine and sildenafil reduce adult zebrafish aggression

We have previously shown that juvenile and adult zebrafish express similar aggression levels across the lifespan (Carreno Gutierrez et al., 2018). We assessed the ability of caffeine and sildenafil to reduce adult zebrafish mirror-induced aggression. Both caffeine (Fig. 3(a)) and sildenafil reduced (Fig. 3(d)) adult aggression and had a less pronounced effect on locomotion (Fig. 3(c) and (f)) in agreement with our juvenile zebrafish data. The time spent in the mirror zone was only altered by the highest concentration of caffeine (Fig. 3(b)) suggesting that the reduction of aggression was selective. We also measured anxiety-like behaviour in the novel tank test (NTT). Immersion in caffeine or sildenafil did not alter the time spent in the top (Fig. 3(g) and (k)) of the novel tank, the time spent freezing, angular velocity (Fig. 3(i) and (m)) or total distance swum (apart from 100 μM caffeine; Fig. 3(j) and (n)). This suggests that neither caffeine nor sildenafil are anxiogenic at the concentrations that we tested.

3.6. Caffeine and sildenafil alter attention and impulsivity but not learning

In mammals, aggression can be divided into impulsive/reactive and instrumental/proactive subtypes (Blair, 2010). We investigated whether caffeine and sildenafil might modify zebrafish aggression by altering their levels of attention, impulsivity or learning (Supplemental Fig. 1). We trained ten adult wild-type zebrafish to associate a light with a food reward in the 5-choice serial reaction time task (5-CSRTT). We then introduced a 10 s variable pre-stimulus interval and tested fish for seven days. Fish were treated with either sildenafil (5 μM/50 μM), caffeine (50 μM/100 μM) or no drug (alternating between drug and no-drug) each day. Caffeine decreased the number of correct responses in the 5-CSRTT and increased premature (impulsive) responses (Fig. 4(a) and (b)). Conversely, sildenafil showed an opposite phenotype, with an increase in the number of correct responses and a decrease in premature (impulsive) responses that did not reach significance (Fig. 4(c) and (d)). We examined whether acute exposure to caffeine or sildenafil affected learning by measuring change in preference for a conditioned stimulus following pairing with a brief, mild electric shock. Neither caffeine nor sildenafil altered behaviour in this test compared to control zebrafish, suggesting that aversive learning is not affected by these drugs (Fig. 4(e)).

3.7. Acute caffeine and sildenafil application heightens 5-HT levels in the brain

The behavioural profile of caffeine and sildenafil prompted us to investigate the neurotransmitter signalling pathways that they modulate. We immersed adult wild-type zebrafish in 100 μM caffeine or 10 μM sildenafil and used HPLC to measure the basal levels of 5-HT, DA and their metabolites. Application of either drug increased the concentration of 5-HT in the telencephalon, diencephalon, optic tectum and rhombencephalon, and 5-HIAA in the telencephalon, diencephalon and optic tectum compared to control treated zebrafish (Fig. 5(a)-(d)). The levels of dopamine, DOPAC and HVA were unaffected by drug application. Caffeine application decreased the utilisation ratio of 5-HIAA/5-HT in the diencephalon and rhombencephalon (Fig. 5(f) and (h)), whereas sildenafil decreased the utilisation ratio of both 5-HIAA/5-HT and HVA/DA in the rhombencephalon (Fig. 5(h)). This demonstrates that both caffeine and sildenafil act centrally to modify monoaminergic neurotransmission.

4. Discussion

In this study we have used a biased screen to identify drugs that reduce zebrafish aggression but not locomotion. We applied three concentrations of each drug to maximise the number of positive hits identified. Our screen had a hit rate of 3.1% (two out of 94 drugs) that is similar to other screens

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

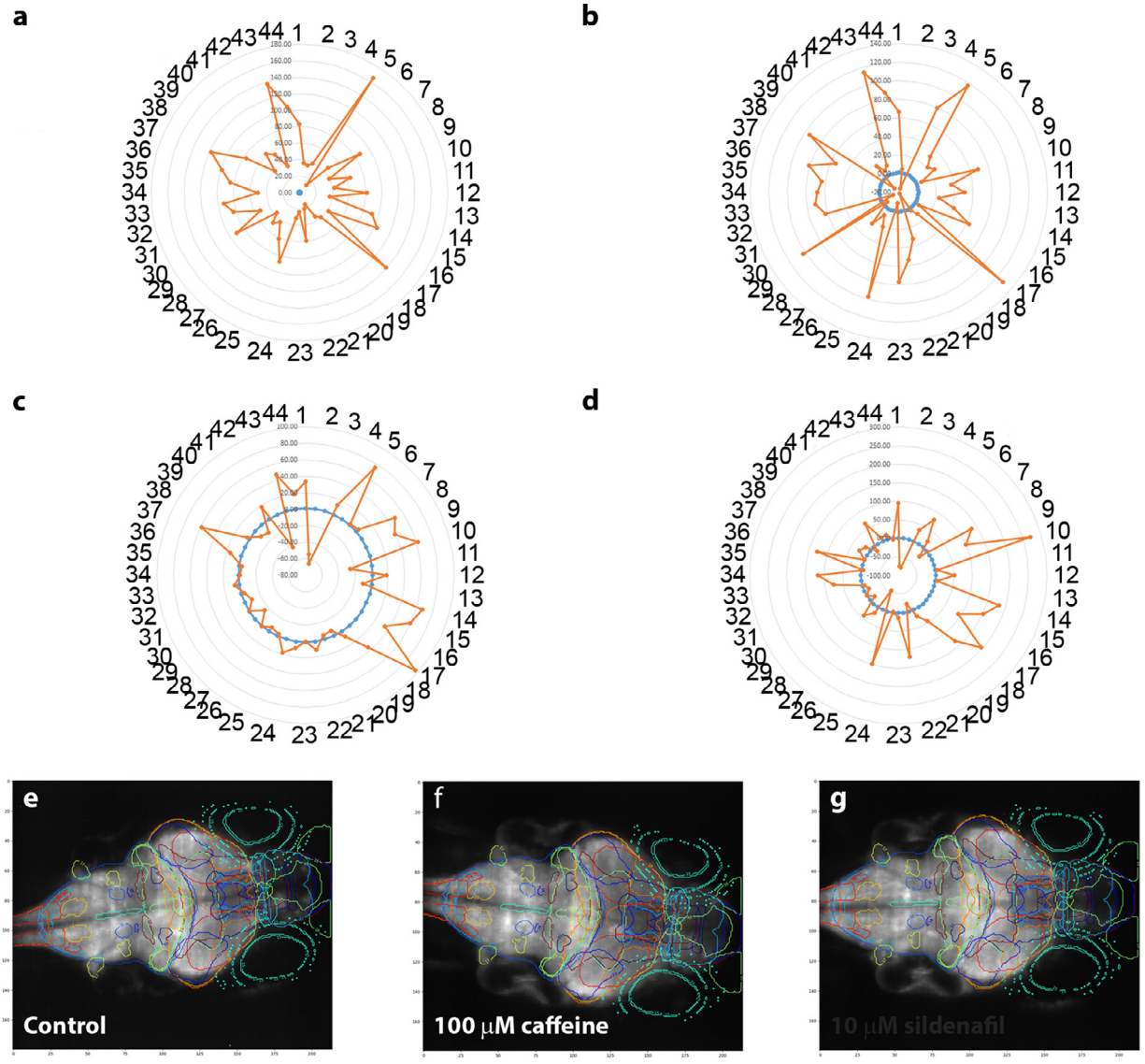


Fig. 2 Neural activity following drug application. (a) Radar plot showing 100 μM caffeine peak intensity. (b) Radar plot showing 100 μM caffeine area under curve. (c) Radar plot showing 10 μM sildenafil peak intensity. (d) Radar plot showing 10 μM sildenafil area under curve. Orange lines in a-d show response to drug and blue lines show control response to water. Legend: 1. Eyes; 2. Olfactory bulb; 3. Olfactory epithelium; 4. Telencephalon; 5. Anterior commissure; 6. Pallium; 7. Subpallium; 8. Diencephalon; 9. Dorsal thalamus; 10. Eminentia thalami; 11. Habenulae; 12. Intermediate hypothalamus; 13. Pineal; 14. Posterior tuberculum; 15. Preoptic area; 16. Pretectum; 17. Rostral hypothalamus; 18. Ventral thalamus; 19. Mesencephalon; 20. Tectum stratum periventriculare; 21. Tectum neuropil; 22. Tegmentum; 23. Torus longitudinalis; 24. Torus semicircularis; 25. Rhombencephalon; 26. Area postrema; 27. Cerebellum; 28. Corpus cerebelli; 29. Eminentia granularis; 30. Inferior olive; 31. Interpeduncular nucleus; 32. Lateral reticular nucleus; 33. Lobus caudalis cerebelli; 34. Locus coeruleus; 35. Mauthner neuron; 36. Medial vestibular nucleus; 37. Noradrenergic neurons; 38. Inferior raphe; 39. Superior raphe; 40. Tangential vestibular nucleus; 41. Valvula cerebelli; 42. Vagal ganglia; 43. Spinal cord; 44. Neuropil region. (e) Control maximum projection of neural activity. (f) 100 μM caffeine maximum projection of neural activity. g) 10 μM sildenafil maximum projection of neural activity. $n = 8$ control, $n = 8$ 100 μM caffeine and $n = 8$ 10 μM sildenafil. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for drugs that alter sleep (8.2%) (Rihel and Schier, 2012) and seizure liability (2.3%) (Baxendale et al., 2012) in zebrafish. Caffeine and sildenafil significantly reduced both juvenile and adult aggression with a smaller effect on locomotion (Figs. 1 and 3). These drugs also had opposite effects upon attention and impulsivity (Fig. 4(a)-(d)) but did not alter anxiety-like behaviour (Fig. 3(g)-(n)) or learning (Fig. 4(e)).

A small-scale screen of drugs with a similar molecular structure as caffeine and sildenafil demonstrated that paraxanthine can also reduce aggression (Fig. 1(c)). Although we have not characterised paraxanthine in detail, this approach shows the feasibility of using zebrafish to screen for derivatives of drugs with potentially more favourable toxicological and behavioural profiles.

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

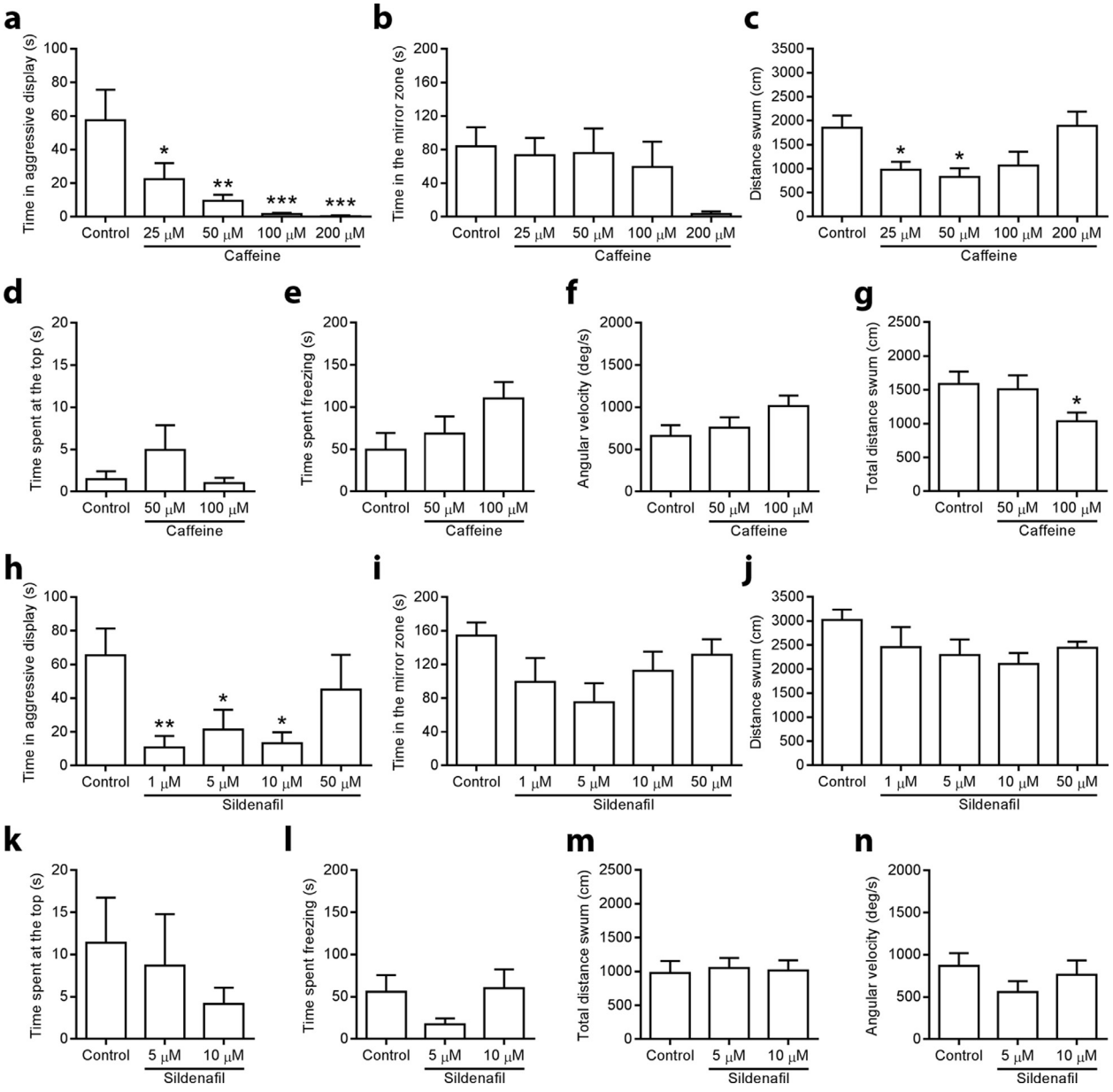


Fig. 3 Effect of caffeine and sildenafil on adult zebrafish behaviour. (a-c) Mirror-induced aggression upon caffeine treatment. (a) Time spent fighting the mirror in the aggression test (ANOVA: $F = 5.809$, $p = 0.0007$; control vs. 25 μM: $p = 0.0445$, control vs. 50 μM: $p = 0.0034$, control vs. 100 μM: $p = 0.0008$, control vs. 200 μM: $p = 0.0009$; $n = 9$-11 each). (b) Time spent near the mirror (ANOVA: F (4, 47) $= 1.68$, $p = 0.1708$); $n = 9$-11 each. (c) Total distance swum (ANOVA: F (4, 47) $= 4.568$, $p = 0.0034$; control vs. 25 μM: $p = 0.0383$, control vs. 50 μM: $p = 0.0101$, control vs. 100 μM: $p = 0.0726$, control vs. 200 μM: $p = 0.9998$); $n = 9$-11 each. (d-g) Novel tank diving upon caffeine treatment. (d) Time spent at the top (Kruskal-Wallis $H(2) = 1.315$, $p = 0.5182$; $n = 13$-14 each). (e) Time spent freezing (ANOVA: F (2, 38) $= 2.437$, $p = 0.1010$); $n = 13$-14 each. (f) Angular velocity (ANOVA: F (2, 38) $= 2.175$, $p = 0.1275$); $n = 13$-14 each. (g) Total distance swum (Kruskal-Wallis $H(2) = 6.038$, $p = 0.0488$; control vs. 50 μM: $p > 0.999$, control vs. 100 μM: $p = 0.0408$); $n = 13$-14 each. (h-j) Mirror-induced aggression upon sildenafil treatment. (h) Time spent fighting the mirror in the aggression test (Kruskal-Wallis: $H(4) = 13.27$, $p = 0.0100$; control vs. 1 μM: $p = 0.0046$, control vs. 5 μM: $p = 0.0366$, control vs. 10 μM: $p = 0.0274$, control vs. 50 μM: $p = 0.5010$; $n = 10$-12 each). (i) Time spent near the mirror (ANOVA: F (4, 48) $= 1.865$, $p = 0.1319$); $n = 10$-12 each. (j) Total distance swum (ANOVA: F (4, 48) $= 1.664$, $p = 0.1739$); $n = 10$-12 each. (K-N) Novel tank diving upon sildenafil treatment. (k) Time spent at the top (Kruskal-Wallis: $H(2) = 0.0179$, $p = 0.9910$); $n = 10$-12 each. (l) Time spent freezing (Kruskal-Wallis: $H(2) = 1.381$, $p = 0.5014$); $n = 10$-12 each. (m) Angular velocity (Kruskal-Wallis: $H(2) = 1.776$, $p = 0.4114$); $n = 10$-12 each. (n) Total distance swum (ANOVA: F (2, 33) $= 0.0565$, $p = 0.9451$); $n = 10$-12 each. One-way ANOVA followed by Dunnett's post hoc or Kruskal Wallis test followed by Dunn's post hoc. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

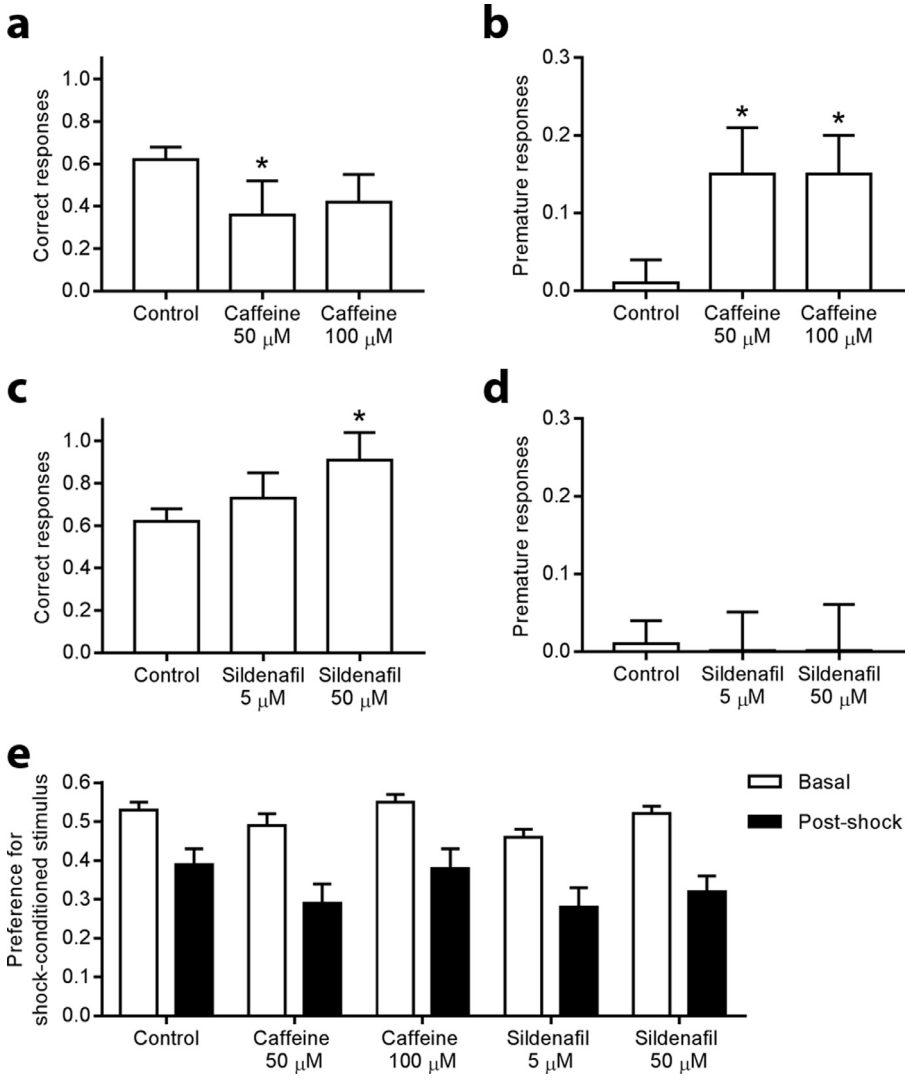


Fig. 4 5-choice serial reaction time task and fear conditioning following caffeine or sildenafil application. a-d) 5-choice serial reaction time task. (a) 50 μM caffeine decreases correct responses in the 5-CSRTT. (b) 50 μM and 100 μM caffeine increases premature responses in the 5-CSRTT. (c) 5 μM sildenafil increases correct responses in the 5-CSRTT. (d) 5 μM and 50 μM sildenafil does not affect premature responses in the 5-CSRTT. (e) Aversive fear conditioning. Correct responses in this test were significantly affected by drug dose (linear mixed effects model, fixed effect = dose, random effect = ID nested in test-day; F (4, 65) = 3.28, $p = 0.02$). There was a significant reduction in correct responses from control following exposure to 50 μM ($p = 0.05$) but not 100 μM caffeine ($p = 0.15$; pairwise comparisons). Conversely, application of 50 μM sildenafil significantly increased correct responses ($p = 0.05$), but 5 μM sildenafil did not ($p = 0.44$; pairwise comparisons). Regarding premature responses, drug dose had a significant main effect (linear mixed effects model, fixed effect = dose, random effect = ID nested in test-day; F (4, 65) = 2.48, $p = 0.05$). Exposure to 50 μM ($p = 0.02$) or 100 μM ($p = 0.03$) caffeine caused a significant increase in premature responses compared to control (pairwise comparisons). There were no significant effects of sildenafil on premature responses ($p > 0.8$). Aversive conditioning, revealed a significant reduction in preference for the shock-paired stimulus (2-way mixed-design analysis of variance (ANOVA), F (1, 43) = 65.76, $p < 0.001$), but there was no main effect of dose (F (4, 43) = 1.97, $P = 0.12$), and no dose × conditioning interaction (F (4, 43) = 0.27, $p = 0.9$). This suggests that impaired learning cannot explain our findings in the 5-CSRTT. $n = 12$ control, $n = 12$ 100 μM caffeine and $n = 12$ 10 μM sildenafil.

4.1. Caffeine

Acute immersion in caffeine caused a decrease in the mean level of both juvenile (Fig. 1) and adult zebrafish aggression (Fig. 3). Low doses of caffeine can also reduce agonistic behaviour in humans and rats (Cherek et al., 1984; Wilson et al., 2000) indicating that the action of this drug may be conserved across species. Treatment of zebrafish with caffeine also decreased the number of correct responses and increased impulsivity (Fig. 4(a) and (b)) in the 5-choice serial reaction time task (5-CSRTT). Locomotion (Fig. 1(a)), anxiety-like behaviour (Fig. 3(g)-(j)) and fear-conditioning (Fig. 5(e)) were unaffected at most of the concentrations that we tested. This suggests that the decrease

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

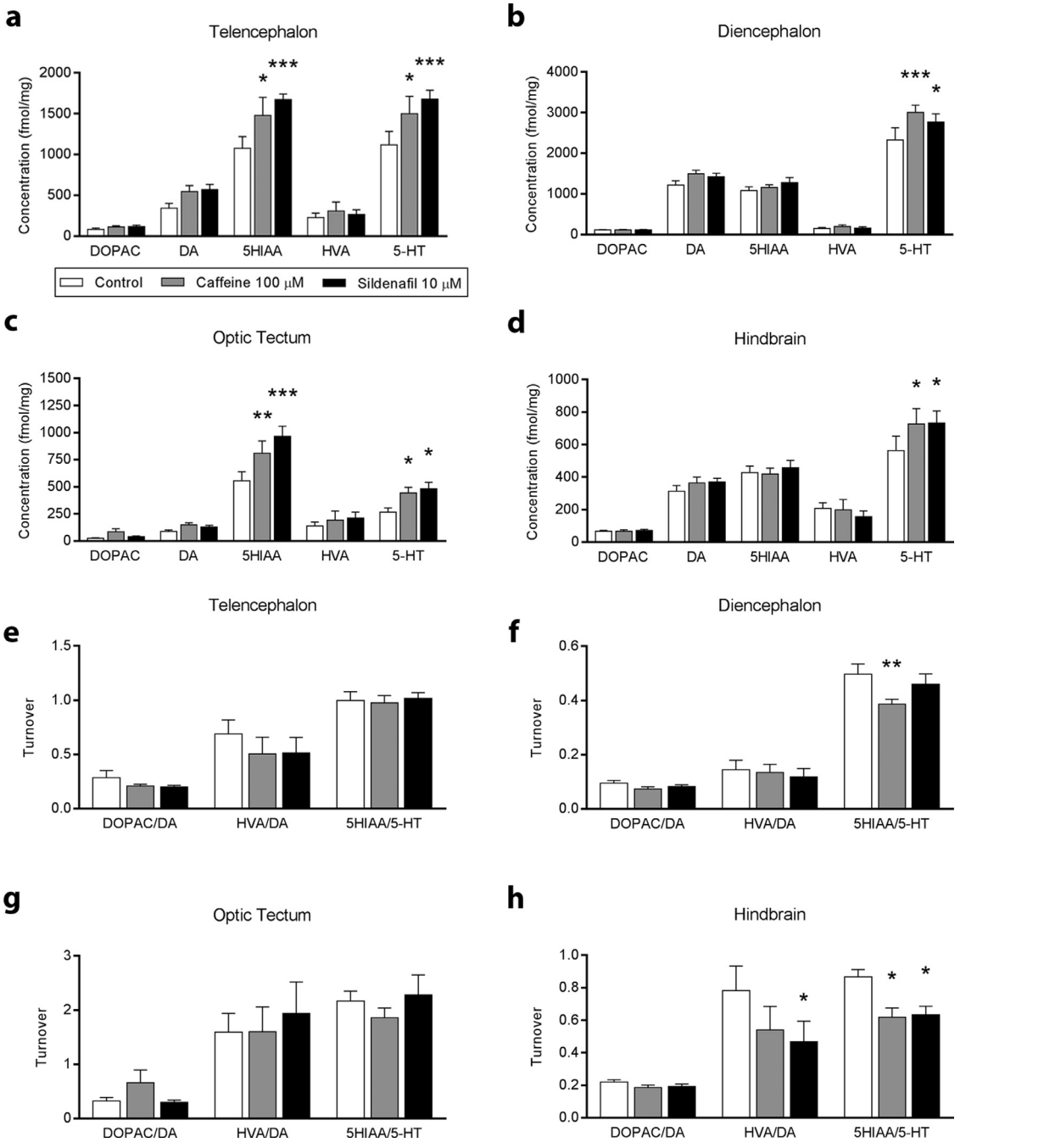


Fig. 5 Neurochemistry following caffeine or sildenafil application. (a–d) Basal level of monoamines. 5-HT levels are increased by caffeine or sildenafil application in the (a) telencephalon, (b) diencephalon, (c) optic tectum and (d) hindbrain. 5HIAA levels are increased by caffeine in the (a) telencephalon and (c) optic tectum. (e–h) Utilisation ratio of monoamines. (h) The utilisation ratio of dopamine to HVA is decreased by sildenafil in the hindbrain. The utilisation ratio of 5-HT to 5HIAA is decreased by (f) caffeine in the diencephalon and (h) both caffeine and sildenafil in the hindbrain. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$: significant difference from control (Dunnett's multiple comparison test, based on significant main effects of drug in two-way ANOVA in telencephalon (F (2, 99) = 8.48, $p < 0.001$), optic tectum (F (2, 99) = 9.90, $p < 0.001$) and diencephalon (F (2, 99) = 3.81, $p = 0.026$). There was no significant main effect of drug in hindbrain (F (2, 99) = 1.10, $p = 0.338$); $n = 8$ control, $n = 8$ 100 μM caffeine and $n = 8$ 10 μM sildenafil.

in aggression relative to control treatment could be due to a reduction in attention towards the mirror stimulus.

At dietary levels caffeine acts as a competitive antagonist of adenosine A1 and A2A receptors (A1R and A2AR) with a similar affinity for both targets (Fredholm et al., 1999). At nonpharmacological doses caffeine can also inhibit monoamine oxidase and phosphodiesterases and mobilise calcium stores (Cappelletti et al., 2015; Fredholm et al.,

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

1999; Petzer et al., 2013). However, since A2AR knock-out mice are more aggressive than WT (Ledent et al., 1997) it seems likely that adenosine signalling might underpin the decrease in aggression that we observed.

The behavioural effect of caffeine appears to be concentration dependent (El Yacoubi et al., 2000; Wilson et al., 2000). Previous studies have shown that low doses of caffeine (between 1 μM and 5 μM) do not alter adult zebrafish locomotion whereas high doses (100 μM to 1280 μM) decrease locomotion (Ladu et al., 2015; Tran et al., 2017) and increase anxiety-like behaviour (520 μM, Egan et al., 2009; Wong et al., 2010). In our setup we only observed decreased locomotion when we applied 200 μM caffeine. The inconsistency of results across studies could be due to the genetic background used. Caffeine has a different behavioural effect in short-fin wild-type and *leopard* zebrafish (Rosa et al., 2018); and genetic polymorphisms in the *ADENOSINE A2A RECEPTOR* can predict the anxiogenic profile of caffeine in humans. Alternatively, the dose-dependent modulation of aggression might be explained by the activation of different targets at higher concentrations.

Caffeine readily passes the blood-brain barrier and has both central and peripheral effects (Watson et al., 2002). Acute application of 100 μM caffeine caused widespread activation of a large number of larval brain areas that did not reach significance compared to control-treated animals. This finding agrees with a study of humans in which a moderate dose of caffeine increases resting brain entropy across the brain (Chang et al., 2018). Caffeine's ability to alter behaviour might be due to dynamic small-scale changes in the activity of multiple brain areas rather than a significant alteration in one nucleus. Live-imaging identified a significant increase in peak intensity in the anterior commissure, diencephalon, caudal lobe of the cerebellum and the eminentia granularis (Fig 2(f)). Long-term caffeine treatment has been shown to increase the number of adenosine receptors in the brainstem and cerebellum of mice (Marangos et al., 1984). Together, these observations suggest that the cerebellum may mediate caffeine's effect, perhaps via reciprocal connections with the telencephalon (Matsui et al., 2014). In adult zebrafish, immersion in 100 mg/L (520 μM) caffeine activates neurons in the central part of the dorsal telencephalon, optic tectum and lateral longitudinal fascicle (Chatterjee et al., 2015). However, our data showed that at 4 days, 10 μM caffeine did not significantly activate these brain areas suggesting that there are stage- or concentration differences in the way in which the zebrafish brain responds to this drug.

4.2. Paraxanthine

Caffeine is metabolised to paraxanthine, theobromine and theophylline by the enzyme Cytochrome P450A1 (Ferré et al., 2013). In humans, around 80% of caffeine is metabolised to paraxanthine although this rate differs in other species (Benowitz et al., 1995). Paraxanthine blocks A1R and A2AR with a higher potency than caffeine and also inhibits Phosphodiesterase 9 (Pde9) (Orru et al., 2013). Since the majority of caffeine is metabolised to paraxanthine it is possible that this compound is responsible for the pharmacological activity of caffeine (Benowitz et al., 1995). However, the acute exposure time used here argues against this idea. Alternatively, both drugs could have a similar effect on A1R and A2AR. Theobromine and theophylline did not reduce aggression in our screen (Fig. 1(d) and (e)) even though they are also non-selective adenosine receptor- and phosphodiesterase inhibitors (Boswell-Smith et al., 2006; Martínez-Pinilla et al., 2015). One explanation for this discrepancy could be that theobromine and theophylline are more active at cAMP-specific phosphodiesterases than paraxanthine which binds to the cGMP-specific Pde9 (Essayan, 2001; Orru et al., 2013). Although paraxanthine reduced aggression it also significantly reduced locomotion when applied at 50 μM and 100 μM concentration. This means that paraxanthine does not fulfil the selection criteria used in our screen design (reduced aggression in the absence of changes to locomotion), making it a less interesting compound for future study.

4.3. Sildenafil

Sildenafil treatment decreased both juvenile and adult zebrafish aggression (Figs. 1(b) and 3(d)). Conversely, the structurally-related drug vardenafil did not alter aggression or locomotion (Fig. 1(f)). Both sildenafil and vardenafil are potent phosphodiesterase 5 (PDE5) inhibitors that can prolong nitric oxide (NO) signalling (Essayan, 2001). Vardenafil binds PDE5 ten to forty fold more tightly than sildenafil making it much more potent and selective (Bischoff, 2004; Rotella, 2002). This difference in potency might explain the inability of vardenafil to reduce aggression if the concentration that we tested in this study was too high. Sildenafil has already been linked to the control of aggression. Withdrawal of the drug decreased attack latency and increased the frequency and duration of aggression in mice (Hotchkiss et al., 2005). In a separate study, sildenafil was shown to increase aggression, sexual behaviour, exploration and anxiety in subordinate mice without changing the social hierarchy. This suggests that the motivation to be aggressive had been altered (Dadomo et al., 2013, 2011).

Sildenafil also increased the number of correct responses and decreased impulsivity in the 5-CSRTT (Fig. 4(c) and (d)) whereas learning was not affected (Fig. 4(e)). This supports previous work demonstrating the cognitive enhancing effect of PDE inhibition in macaques (Rutten et al., 2008). Interestingly, sildenafil has a different effect in macaques depending upon the difficulty of the task. While the PDE4 and PDE5 inhibitors increased performance on the more complex version of an object-retrieval protocol, no effects were observed on the simpler iterations. This strongly supports our data, whereby performance on the cognitively demanding 5-CSRTT was improved by sildenafil.

As well as relaxing peripheral blood vessels, sildenafil can cross the blood brain barrier and act centrally to enhance nitric oxide (NO) signalling (Kouvelas et al., 2009). In rat, mice and human brains Pde5 is restricted to the hippocampus and Purkinje cells of the cerebellum (Giordano et al., 2001; Kotera et al., 2000; Lakics et al., 2010; Teich et al., 2016). Imaging of the larval zebrafish brain showed that acute application of 10 μM sildenafil causes a broad increase in AUC fluorescence in the diencephalon and decreases in several forebrain and hindbrain regions. Importantly

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

the increase in AUC fluorescence reached significance in the eminentia thalami, an ambiguous structure located above the preoptic area and ventral to the prethalamus (Mueller, 2012). The eminentia thalami disappears during mammalian development, whereas it is maintained as the ventral entopeduncular nucleus (vENT) in adult fish and amphibians (Mueller, 2012; Mueller and Guo, 2009). The BNSM sends efferent projections to the habenula, optic tectum and pallial forebrain. Afferent projections have been mapped from the cerebellar Purkinje cells to the thalamus (Matsui et al., 2014). Although the function of the eminentia thalami/vENT is not known, the connection between the cerebellum and eminentia thalami could form part of a cerebellar-thalamus-forebrain loop that controls emotional behaviour (e.g. Tsai et al., 2012). Further research, such as direct manipulation of the eminentia thalami using optogenetic tools, would be required to test this hypothesis.

4.4. Convergence of signalling pathways activated by caffeine and sildenafil

Although both caffeine and sildenafil reduced both juvenile and adult aggression, they had an opposite effect on impulsivity (Fig. 4(a) and (c)) and produced a different neural signature in the 4 day-old brain. This makes it difficult to know whether they decrease aggression via the same neural network or signalling pathway. A global reduction of 5-HT activity has been linked to control of aggression across species; and both caffeine and sildenafil increased the basal level of 5-HT throughout the adult brain (Fig. 5), as well as decreasing its utilisation ratio in the diencephalon. However, the mechanism by which these drugs can alter monoamine signalling is not yet clear. At high concentrations caffeine can inhibit monoamine oxidase (Fredholm et al., 1999) providing one possible explanation for the heightened levels of 5-HT observed in this study. Further research, such as measuring MAO activity using an enzymatic assay after caffeine application (Carreno Gutierrez et al., 2017) would be required to address this issue. Another potentially important signalling pathway is nitric oxide. Inhibition of Pde5 by sildenafil stabilises cGMP levels to enhance NO signalling; and adenosine signalling (a target of caffeine) can activate both Nos1 and Nos3 via the adenosine receptor 2b (Wen and Xia, 2012). However, caffeine and sildenafil activate different areas of the brain, with sildenafil having a broader profile than caffeine. Even though both drugs reduce aggression the neural substrate underlying this behaviour appears to be different. Future studies are required to investigate whether caffeine and sildenafil modify aggression via NO or 5-HT signalling.

In summary, we have used a biased approach to search for novel drugs that can reduce zebrafish aggression. We demonstrated that both caffeine and sildenafil can decrease aggression across the lifespan, although they have the opposite effect upon impulsivity. By combining a range of techniques we have identified some of the brain areas and neurotransmitter systems that may alter aggression. A major drawback of this approach is that because of technical limitations we compared neural activity at larval stages to neurochemistry in the adult brain and behaviour across the lifespan. Future work would benefit from identifying the mature neural circuits activated by these drugs. The next step for this research would be to further test these compounds in rodent models to investigate whether caffeine and sildenafil reduce aggression across vertebrate species (Norton, 2019).

Role of the funding source

The research leading to these results received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 602805. Hector Carreno is funded by the NC3Rs (NC/R001049/1).

Contributors

H.C.G. and I.V. performed the drug screen and analysed the data. G.D. and A.A.V. helped with statistical analysis of results. H.C.G. carried out all adult zebrafish experiments apart from fear conditioning/5-CSRTT and analysed the data. B.O.C. and A.M.J.Y performed the HPLC measurements and analysis. M.C. and M.O.P. performed the aversive learning and 5-CSRTT and analysed the data. A.T. and M.J.W. imaged elavl3:GCaMP6s and analysed the data. W.H.J.N. analysed the data and wrote the first version of the manuscript. All authors improved the manuscript before submission.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We are grateful to Carl Breaker and members of the preclinical research facility for zebrafish care, Ceinwen Tilley for technical support and all members of the Norton lab for discussions about this data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.10.005.

References

Baxendale, S., Holdsworth, C.J., Meza Santoscoy, P.L., Harrison, M.R., Fox, J., Parkin, C.A., et al., 2012. Identification of compounds with anti-convulsant properties in a zebrafish model of epileptic seizures. Dis. Model Mech. 5 (6), 773–784.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B (Methodological) 57 (1), 289–300.

Benowitz, N.L., Jacob 3rd, P., Mayan, H., Denaro, C., 1995. Sympathomimetic effects of paraxanthine and caffeine in humans. Clin. Pharmacol. Ther. 58 (6), 684–691.

Bischoff, E., 2004. Vardenafil preclinical trial data: potency, pharmacodynamics, pharmacokinetics, and adverse events. Int. J. Impot. Res. 16 (Suppl 1), S34–S37.

Blair, R.J., 2010. Neuroimaging of psychopathy and antisocial behavior: a targeted review. Curr. Psychiatry Rep. 12 (1), 76–82.

Boswell-Smith, V., Cazzola, M., Page, C.P., 2006. Are phosphodiesterase 4 inhibitors just more theophylline. J. Allergy Clin. Immunol. 117 (6), 1237–1243.

Cappelletti, S., Piacentino, D., Sani, G., Aromatario, M., 2015. Caffeine: cognitive and physical performance enhancer or psychoactive drug. Curr. Neuropharmacol. 13 (1), 71–88.

Carreno Gutierrez, H., O'Leary, A., Freudenberg, F., Fedele, G., Wilkinson, R., Markham, E., et al., 2017. Nitric oxide interacts with monoamine oxidase to modulate aggression and anxiety-like behaviour. Eur. Neuropsychopharmacol. doi:10.1016/j.euroneuro.2017.09.004, pii:S0924-977X(17)30906-9. [Epub ahead of print].

Carreno Gutierrez, H., Vacca, I., Pons, A.I., Norton, W.H.J., 2018. Automatic quantification of juvenile zebrafish aggression. J. Neurosci. Methods 296, 23–31.

Chang, D., Song, D., Zhang, J., Shang, Y., Ge, Q., Wang, Z., 2018. Caffeine caused a widespread increase of resting brain entropy. Sci. Rep. 8 (1), 2700.

Chatterjee, D., Tran, S., Shams, S., Gerlai, R., 2015. A simple method for immunohistochemical staining of zebrafish brain sections for c-fos protein expression. Zebrafish 12 (6), 414–420.

Cherek, D.R., Steinberg, J.L., Brauchi, J.T., 1984. Regular or decaffeinated coffee and subsequent human aggressive behavior. Psychiatry Res. 11 (3), 251–258.

Connors, S.L., Matteson, K.J., Sega, G.A., Lozzio, C.B., Carroll, R.C., Zimmerman, A.W., 2006. Plasma serotonin in autism. Pediatr. Neurol. 35 (3), 182–186.

Copmans, D., Meinl, T., Dietz, C., van Leeuwen, M., Ortmann, J., Berthold, M.R., et al., 2016. A KNIME-based analysis of the zebrafish photomotor response clusters the phenotypes of 14 classes of neuroactive molecules. J. Biomol. Screen. 21 (5), 427–436.

Cunliffe, V.T., 2016. Building a zebrafish toolkit for investigating the pathobiology of epilepsy and identifying new treatments for epileptic seizures. J. Neurosci. Methods 260, 91–95.

Dadomo, H., Parmigiani, S., Nicolini, Y., Freschini, S., Gioiosa, L., Patrelli, T.S., et al., 2013. Repeated and chronic administration of Vardenafil or Sildenafil differentially affects emotional and socio-sexual behavior in mice. Behav. Brain Res. 253, 103–112.

Dadomo, H., Volpi, R., Ferrari, M., Vignali, A., Bartolomucci, A., Palanza, P., et al., 2011. Sildenafil counteracts the inhibitory effect of social subordination on competitive aggression and sexual motivation in male mice. Behav. Brain Res. 216 (1), 193–199.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., et al., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205 (1), 38–44.

El Yacoubi, M., Ledent, C., Menard, J.F., Parmentier, M., Costentin, J., Vaugeois, J.M.., 2000. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A(2A) receptors. Br. J. Pharmacol. 129 (7), 1465–1473.

Essayan, D.M., 2001. Cyclic nucleotide phosphodiesterases. J. Allergy Clin. Immunol. 108 (5), 671–680.

Ferré, S., Orrú, M., Guitart, X., 2013. Paraxanthine: connecting caffeine to nitric oxide neurotransmission. J. Caffeine Res. 3 (2), 72–78.

Fraser, T.W.K., Khezri, A., Lewandowska-Sabat, A.M., Henry, T., Ropstad, E., 2017. Endocrine disruptors affect larval zebrafish behavior: testing potential mechanisms and comparisons of behavioral sensitivity to alternative biomarkers. Aquat. Toxicol. 193, 128–135.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51 (1), 83–133.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67 (4), 773–782.

Giordano, D., De Stefano, M.E., Citro, G., Modica, A., Giorgi, M., 2001. Expression of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in mouse tissues and cell lines using an antibody against the enzyme amino-terminal domain. Biochim. Biophys. Acta 1539 (1-2), 16–27.

Hotchkiss, A.K., Pyter, L.M., Gatien, M.L., Wen, J.C., Milman, H.A., Nelson, R.J., 2005. Aggressive behavior increases after termination of chronic sildenafil treatment in mice. Physiol. Behav. 83 (5), 683–688.

Jones, L.J., Norton, W.H., 2015. Using zebrafish to uncover the genetic and neural basis of aggression, a frequent comorbid symptom of psychiatric disorders. Behav. Brain Res. 276, 171–180.

Jordi, J., Guggiana-Nilo, D., Bolton, A.D., Prabha, S., Ballotti, K., Herrera, K., et al., 2018. High-throughput screening for selective appetite modulators: a multibehavioral and translational drug discovery strategy. Sci. Adv. 4 (10), eaav1966.

Kempes, M., Matthys, W., de Vries, H., van Engeland, H., 2005. Reactive and proactive aggression in children–a review of theory, findings and the relevance for child and adolescent psychiatry. Eur. Child Adolesc. Psychiatry 14 (1), 11–19.

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C.Y., Mateus, R., et al., 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat. Chem. Biol. 6 (3), 231–237.

Kotera, J., Fujishige, K., Omori, K., 2000. Immunohistochemical localization of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in rat tissues. J. Histochem. Cytochem. 48 (5), 685–693.

Kouvelas, D., Goulas, A., Papazisis, G., Sardeli, C., Pourzitaki, C., 2009. PDE5 inhibitors: in vitro and in vivo pharmacological profile. Curr. Pharm. Des. 15 (30), 3464–3475.

Laan, A., Iglesias-Julios, M., de Polavieja, G.G., 2018. Zebrafish aggression on the sub-second time scale: evidence for mutual motor coordination and multi-functional attack manoeuvres. R. Soc. Open Sci. 5 (8), 180679.

Ladu, F., Mwaffo, V., Li, J., Macri, S., Porfiri, M., 2015. Acute caffeine administration affects zebrafish response to a robotic stimulus. Behav. Brain Res. 289, 48–54.

Lakics, V., Karran, E.H., Boess, F.G., 2010. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology 59 (6), 367–374.

Ledent, C., Vaugeois, J.M., Schiffmann, S.N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J.J., et al., 1997. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. Nature 388 (6643), 674–678.

Loy, J.H., Merry, S.N., Hetrick, S.E., Stasiak, K., 2012. Atypical antipsychotics for disruptive behaviour disorders in children and youths. Cochrane Database Syst. Rev. 8, CD008559. doi:10.1002/14651858.CD008559.pub3.

MacRae, C.A., Peterson, R.T., 2015. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 14 (10), 721–731.

Marangos, P.J., Boulenger, J.P., Patel, J., 1984. Effects of chronic caffeine on brain adenosine receptors: regional and ontogenetic studies. Life Sci. 34 (9), 899–907.

Martínez-Pinilla, E., Oñatibia-Astibia, A., Franco, R., 2015. The relevance of theobromine for the beneficial effects of cocoa consumption. Front. Pharmacol. 6, 30.

Matsui, H., Namikawa, K., Köster, R.W., 2014. Identification of the zebrafish red nucleus using Wheat Germ Agglutinin transneuronal tracing. Commun. Integr. Biol. 7 (6), e994383.

Montoya, E.R., Terburg, D., Bos, P.A., van Honk, J., 2012. Testosterone, cortisol, and serotonin as key regulators of social aggression: a review and theoretical perspective. Motiv. Emot. 36 (1), 65–73.

Mueller, T., 2012. What is the Thalamus in Zebrafish. Front. Neurosci. 6, 64.

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005

Mueller, T., Guo, S., 2009. The distribution of GAD67-mRNA in the adult zebrafish (teleost) forebrain reveals a prosomeric pattern and suggests previously unidentified homologies to tetrapods. J. Comp. Neurol. 516 (6), 553–568.

Nelson, R.J., Trainor, B.C., 2007. Neural mechanisms of aggression. Nat. Rev. Neurosci. 8 (7), 536–546.

Nevels, R.M., Dehon, E.E., Alexander, K., Gontkovsky, S.T., 2010. Psychopharmacology of aggression in children and adolescents with primary neuropsychiatric disorders: a review of current and potentially promising treatment options. Exp. Clin. Psychopharmacol. 18 (2), 184–201.

Norton, W.H., 2013. Toward developmental models of psychiatric disorders in zebrafish. Front. Neural Circuits 7, 79.

Norton, W.H., Stumpenhorst, K., Faus-Kessler, T., Folchert, A., Rohner, N., Harris, M.P., et al., 2011. Modulation of Fgfr1a signaling in zebrafish reveals a genetic basis for the aggression-boldness syndrome. J. Neurosci. 31 (39), 13796–13807.

Norton, W.H.J., 2019. Screening for drugs to reduce aggression in zebrafish. Neuropharmacology 156, 107394. doi:10.1016/j.neuropharm.2018.10.023, Epub 2018 Oct 16.

Orru, M., Guitart, X., Karcz-Kubicha, M., Solinas, M., Justinova, Z., Barodia, S.K., et al., 2013. Psychostimulant pharmacological profile of paraxanthine, the main metabolite of caffeine in humans. Neuropharmacology 67, 476–484.

Pappadopulos, E., Woolston, S., Chait, A., Perkins, M., Connor, D.F., Jensen, P.S., 2006. Pharmacotherapy of aggression in children and adolescents: efficacy and effect size. J. Can. Acad. Child Adolesc. Psychiatry 15 (1), 27–39.

Petzer, A., Pienaar, A., Petzer, J.P., 2013. The interactions of caffeine with monoamine oxidase. Life Sci. 93 (7), 283–287.

Polzer, J., Bangs, M.E., Zhang, S., Dellva, M.A., Tauscher-Wisniewski, S., Acharya, N., et al., 2007. Meta-analysis of aggression or hostility events in randomized, controlled clinical trials of atomoxetine for ADHD. Biol. Psychiatry 61 (5), 713–719.

Rennekamp, A.J., Huang, X.P., Wang, Y., Patel, S., Lorello, P.J., Cade, L., et al., 2016. sigma1 receptor ligands control a switch between passive and active threat responses. Nat. Chem. Biol. 12 (7), 552–558.

Rihel, J., Prober, D.A., Arvanites, A., Lam, K., Zimmerman, S., Jang, S., et al., 2010. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327 (5963), 348–351.

Rihel, J., Schier, A.F., 2012. Behavioral screening for neuroactive drugs in zebrafish. Dev. Neurobiol. 72 (3), 373–385.

Rosa, L.V., Ardais, A.P., Costa, F.V., Fontana, B.D., Quadros, V.A., Porciuncula, L.O., et al., 2018. Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. Pharmacol. Biochem. Behav. 165, 1–8.

Rotella, D.P., 2002. Phosphodiesterase 5 inhibitors: current status and potential applications. Nat. Rev. Drug Discov. 1 (9), 674–682.

Rutten, K., Basile, J.L., Prickaerts, J., Blokland, A., Vivian, J.A., 2008. Selective PDE inhibitors rolipram and sildenafil improve object retrieval performance in adult cynomolgus macaques. Psychopharmacology 196 (4), 643–648.

Scotto Rosato, N., Correll, C.U., Pappadopulos, E., Chait, A., Crystal, S., Jensen, P.S., 2012. Treatment of maladaptive aggression in youth: CERT guidelines II. Treatments and ongoing management. Pediatrics 129 (6), e1577–e1586.

Tcheremissine, O.V., Lieving, L.M., 2006. Pharmacological aspects of the treatment of conduct disorder in children and adolescents. CNS Drugs 20 (7), 549–565.

Teich, A.F., Sakurai, M., Patel, M., Holman, C., Saeed, F., Fiorito, J., et al., 2016. PDE5 exists in human neurons and is a viable therapeutic target for neurologic disease. J. Alzheimer's Dis.: JAD 52 (1), 295–302.

Tran, S., Fulcher, N., Nowicki, M., Desai, P., Tsang, B., Facciol, A., et al., 2017. Time-dependent interacting effects of caffeine, diazepam, and ethanol on zebrafish behaviour. Prog. Neuropsychopharmacol. Biol. Psychiatry 75, 16–27.

Tsai, P.T., Hull, C., Chu, Y., Greene-Colozzi, E., Sadowski, A.R., Leech, J.M., et al., 2012. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. Nature 488 (7413), 647–651.

Vliet, S.M., Ho, T.C., Volz, D.C., 2017. Behavioral screening of the LOPAC(1280) library in zebrafish embryos. Toxicol. Appl. Pharmacol. 329, 241–248.

Watson, J., Deary, I., Kerr, D., 2002. Central and peripheral effects of sustained caffeine use: tolerance is incomplete. Br. J. Clin. Pharmacol. 54 (4), 400–406.

Wen, J., Xia, Y., 2012. Adenosine signaling: good or bad in erectile function. Arterioscler. Thromb. Vasc. Biol. 32 (4), 845–850.

Wernicke, J.F., Faries, D., Girod, D., Brown, J., Gao, H., Kelsey, D., et al., 2003. Cardiovascular effects of atomoxetine in children, adolescents, and adults. Drug Saf. 26 (10), 729–740.

Wilson, J.F., Nugent, N.R., Baltes, J.E., Tokunaga, S., Canic, T., Young, B.W., et al., 2000. Effects of low doses of caffeine on aggressive behavior of male rats. Psychol. Rep. 86 (3 Pt 1), 941–946.

Winter, M.J., Windell, D., Metz, J., Matthews, P., Pinion, J., Brown, J.T., et al., 2017. 4-dimensional functional profiling in the convulsant-treated larval zebrafish brain. Sci. Rep. 7 (1), 6581. doi:10.1038/s41598-017-06646-6, Erratum in: Sci Rep. 2018 Oct 23;8(1):15903.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., et al., 2010. Analyzing habituation responses to novelty in zebrafish (Danio rerio). Behav. Brain Res. 208 (2), 450–457.

Please cite this article as: H.C. Gutiérrez, I. Vacca and G. Schoenmacker et al., Screening for drugs to reduce zebrafish aggression identifies caffeine and sildenafil, European Neuropsychopharmacology, https://doi.org/10.1016/j.euroneuro.2019.10.005