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**Abstract**

Cellular and molecular processes that mediate individual variability in impulsivity, a key behavioural component of many neuropsychiatric disorders, are poorly understood. Zebrafish heterozygous for a nonsense mutation in *Ache* (ache$^{sb55/+}$) showed lower levels of impulsivity in a 5-choice serial reaction time task (5-CSRTT) than wild type and ache$^{+/+}$. Assessment of expression of cholinergic (nAChR), serotonergic (5-HT) and dopamine (DR) receptor mRNA in both adult and larval (9dpf) ache$^{sb55/+}$ revealed significant downregulation of *Chrna2*, *Chrna5* and *Drd2* mRNA in ache$^{sb55/+}$ larvae, but no differences in adults. Acute exposure to cholinergic agonist/antagonists had no effect on impulsivity, supporting the hypothesis that behavioural effects observed in adults were due to lasting impact of developmental alterations in cholinergic and dopaminergic signalling. This shows the cross-species role of cholinergic signalling during brain development in impulsivity, and suggests zebrafish may be a useful model for the role of cholinergic pathways as a target for therapeutic advances in addiction medicine.

## Introduction

The identification of endophenotypes, as quantifiable, core components of complex behavioural traits and disease phenotypes makes genetic analysis of the pathogenesis of neuropsychiatric disease more tractable in both humans and model organisms (Burmeister et al., 2008b). One such potential endophenotype is impulsivity (Urcelay and Dalley, 2012). Impulsivity not only is the hallmark symptom of a number of neuropsychiatric disorders (ADHD, addiction) but, in the case of addiction, has been shown to predict patterns of relapse and compulsive drug seeking in rats (Belin et al., 2008).

Despite the well-established role in a number of neuropsychiatric disorders, the cellular and molecular mechanisms that underlie impulsivity are not well understood. The cholinergic system, in particular cholinergic projections from the PFC, has long been implicated in sustained attention (Sarter et al., 2001). For example, IgG-saporin lesions of cholinergic neurons in the basal forebrain reduce sustained attention (McGaughy and Sarter, 1998), while systemic administration of the nAChR agonist nicotine improves performance accuracy and reduces omissions on the 5-CSRTT (Blondel et al., 2000;Hahn and Stolerman, 2002;Young et al., 2004). In addition, infusions of scopolamine (mAchR antagonist) into the medial pre-frontal cortex (mPFC), and systemic mecamylamine (nAchR antagonist) reduce response accuracy (Robbins et

66 al., 1998). The effects of chronic elevation of ACh, however, are less clear, although
67 Grottick and Higgins (2000) found that improved performance accuracy is apparent
68 with chronic nicotine exposure. The effects of genetic alteration of ACh activity have not
69 previously been tested, particularly with respect to premature responding on the 5-
70 CSRTT.

71 Notwithstanding their small size, low housing costs and prolific breeding, there
72 now exists a number of genetic tools for zebrafish research, including ENU mutagenized
73 lines, extensive sperm libraries and a number of GFP/RFP lines. Despite anatomical
74 differences between the fish and their mammalian counterparts, key neurochemical
75 pathways are well conserved between the species (Guo, 2004); for example, the
76 ascending and descending midbrain catecholeminergic pathways (Guo et al., 1999).

77 Here, we tested the performance of in *Ache* deficient ($ache^{sb55/+}$) zebrafish, for
78 performance characteristics on the 5-CSRTT, a task designed to test aspects of impulse
79 control through examination of anticipatory responding. $ache^{sb55}$ contain a point
80 mutation close to the catalytic site of the enzyme resulting in a replacement of Ser226
81 by an Asn. Ser226 is conserved in all ache gene family members, and is important for
82 catalytic activity (Behra et al., 2002). Chronic alterations in cholinergic signalling with
83 the acetylcholinesterase (AChE) inhibitor chlorpyrifos has previously been
84 demonstrated to increase impulsivity, make cholinergic signalling an interesting target

for inquiry into the molecular mechanisms underlying impulse control (Middlemore-Risher et al., 2010;Cardona et al., 2011;Oca et al., 2012). Zebrafish have previously been shown to respond well on the 5-CSRTT (Parker et al., 2012a;Parker et al., 2013a;Parker et al., 2014)

## Materials and Methods

### Ethics statement

All experimental procedures, including drug dosing and behavioural testing, were carried out under the Animals (Scientific procedures) Act (1984). The procedures carried out conformed both to local ethical guidelines and to the terms of a project licence from the UK Home Office. In addition, all experiments were approved by the Queen Mary Animals Welfare and Ethical Review Board.

### Subjects

Twenty-nine (n=10 ache$^{sb55/+}$(Ninkovic et al., 2006), n = 19 Tubingen wild-type [*w/t*]) adult zebrafish (age = 6 months; mixed sex) were selected for the first part of the study (5-sec fixed interval PSI), and 12 adult zebrafish (age = 5 months; mixed sex; n = 5 ache$^{sb55/+;}$ n = 7 ache$^{+/+}$) were selected for the second part (Variable PSI). All were sourced initially from the Sanger Institute (Cambridge, UK), and bred and reared in the

aquarium facility at Queen Mary University of London according to standard protocols (Westerfield, 1993). During the entire experimental period, fish were fed artemia/bloodworm mix during testing trials, and this was supplemented with flake food/artemia in the evenings and at weekends.

**Apparatus**

[FIGURE 1 HERE]

Figure 1 displays the 5-CSRTT tanks used in the study. The shell of the testing tanks was constructed from opaque acrylic, as were the central gates. The lights were LEDs (magazine light green, stimulus aperture lights yellow). The reinforcer used was artemia liquidised with bloodworm, suspended in aquarium-treated water (R-O water with added salts). The food was delivered via a plastic syringe fitted with a 1mm diameter rubber catheter tube, which was driven by a linear stepper motor (Figure 1).

**General Procedure**

The main procedure is an extension and modification of the commonly used rodent 5-CSRTT, and has been described in detail elsewhere (Parker et al., 2012a;Parker et al., 2013b;Parker et al., 2014).

**Pre-training**

Prior to commencing training, all subjects were habituated to the test room for one week to acclimate to the conditions. All pre-training, training and testing was carried out Monday-Friday (0800-1800), with the exception of the final stage (Stage 8, see Table 1), which was also carried out Saturday and Sunday. Training was divided into eight distinct stages (see Table 1).

[TABLE 1 HERE]

During stages 1-3 (pre-training) data were collected and examined to ensure that all animals were receiving food during training. Any that did not perform the task (e.g., froze in the tank or did not approach the lights; $n < 2$ on any given session) had their food supplemented immediately after the session. During acclimation (Stage 1), fish were placed individually into the test tanks for 30-mins. During this all lights were illuminated and the gate was open. Immediately after acclimation, the fish were trained to enter the food magazine (Stage 2). During this stage, the gate remained closed at all times. The magazine light was illuminated for 30-sec intervals, during which entry to the magazine resulted in the light turning off, and a small delivery (~20µl) of artemia/bloodworm mix. In Stage 3 the fish were trained to approach the response apertures. Here, the gate opened to reveal all of the response apertures illuminated, and entry to any one of the apertures was conditionally reinforced with illumination of the

magazine light. Subsequent entry to the food magazine was reinforced with artemia/bloodworm mix. During Stage 3 (response aperture orientation) only fish that completed 20 or more correct trials were taken forward to 5-CSRTT training.

**Five-Choice Serial Reaction Time Task: Phase 1**

After a 2-min habituation period, the magazine light was illuminated, and entry to the food magazine initiated the trial sequence after an inter-trial interval (ITI) of 20-secs[1]. This ITI always followed food delivery, and allowed the fish time to consume the reinforcer ration. After 20-secs, the gate was raised, and one of the stimulus apertures was illuminated after a pre-stimulus interval (PSI). Entry to the correct aperture during the stimulus illumination, or during a brief pause thereafter (limited hold; LH), were conditionally reinforced by illumination of the magazine light, and the trial ended when the fish collected the food. All training sessions lasted 30-mins. For the first four weeks (Stage 4) the fish were trained with 30-sec stimulus duration, a PSI of 1-sec and a 1-sec limited hold period. At all times during training and testing, the magazine light remained illuminated for 30-secs following a correct response, after which magazine entry was not reinforced. During the second stage of 5-CSRTT training (Stage 5) the

[1] Note that in the rodent version of the 5-CSRTT, there is no gate lifted, and as such the pause prior to the stimulus presentation is an inter-trial interval. In our version of the task, the trial is initiated by the opening of the gate, and as such we refer to this as pre-stimulus interval (PSI).

stimulus duration was reduced to 10-sec, the PSI was increased to 5-sec and limited hold remained at 1-sec. The criterion for moving from each stage to the next was that the fish had reached a steady-state response, operationalized as completing >20 trials per session over 5-consecutive sessions. Any fish not meeting this criterion were excluded from the subsequent stage.

**Long PSI stage**

There were three long PSI sessions, during which the PSI was increased to 7-sec. All other test parameters remained the same as during Stage 5 (stimulus duration = 10-sec, limited hold = 1-sec). The three long PSI sessions were interspersed by two baseline sessions (Stage 5; PSI = 5-sec, stimulus duration = 10-sec, limited hold = 1-sec). During the long PSI sessions, the length of the session was increased to 35 min. The criterion for a fish progressing to the long PSI phase of the experiment was that they reached steady state responding, again, operationally defined as having completed five sessions of >20 trials prior to testing. Any fish that did not meet this criterion were excluded from the testing phase.

**Five-Choice Serial Reaction Time Task: Phase 2**

For the second phase of the experiment, we trained a group of experimentally naïve fish (n = 5 $ache^{sb55/+;}$ n = 7 $ache^{+/+}$) in an identical manner to that described above for stages 1-4. For Stage 5, we introduced 5-second variable interval (VI) PSI. All other timings were the same as in Phase 1, Stage 5 (stimulus duration = 10-sec, limited hold = 1-sec). There was no Long-PSI stage in Phase 2.

**Acute exposure to AChE antagonist, and nAChR and mAChR agonists**

Trained fish (*w/t* from Phase 1) were selected for the drug administration phase. The exposure schedule was organised according to a full crossover design, with each fish receiving each of the drugs over a 1-week period. Fish were initially re-trained (2-weeks) in the absence of drug to establish steady-state baseline performance (>20 reinforced trials/session, for 5 sessions). The 5-CSRTT was as before in Stage 5 (see above: stimulus-duration = 10-sec, PSI = 5-sec, LH = 1-sec), except that in this phase we employed a variable interval (VI) 5-second PSI. During the first experiment, there was no difference between the strains during the long PSI trials, but there was a difference during the earlier stages of training. As such, we chose to increase the complexity of the task by using a VI-PSI during the entire training period. Immediately prior to training, fish were immersed in a pre-treatment tank (1L) either in the drug solution or in aquarium-treated $H_2O$ for 20-mins. Drugs (nicotine: 1.54μM [Sigma-Aldrich, UK];

197 pilocarpine [Sigma-Aldrich, UK]: 8.64μM; Donepezil [Sigma-Aldrich, UK]: 2.63μM) were
198 dissolved in aquarium-treated $H_2O$. Doses of Donepezil, nicotine and pilocarpine were
199 selected based on previous work on attention/impulsivity (Day et al., 2007;Brembs,
200 2009;Cardona et al., 2011). The dose of Donepezil was also based on an initial
201 assessment of brain levels of ACh and AChE following drug administration to determine
202 a dose that best reflected the ACh and AChE levels in ache $^{sb55/+}$ (Ninkovic et al., 2006).
203 Brain levels of AChE and ACh were assessed in *w/t* fish exposed to 2.63μM
204 Donepezil or aquarium-treated $H_2O$ for 20 mins using a fluorescence-based approach
205 (George et al., 1961). Following exposure to drug fish were placed in a recovery tank for
206 5-mins, and then killed by immersion in ice water. Brains were immediately removed,
207 weighed and homogenized in ice-cold Tris-HCl (pH 8). Samples were then centrifuged
208 (20-min at 13,000 RPM) and AChE and ACh was assessed from the resulting
209 supernatant using Amplex Red Acetylcholine/Acetylcholinesterase assay kit (Molecular
210 Probes, Invitrogen Detection Technologies, Paisley, UK) according to manufacturer's
211 instructions. Briefly, AChE converts ACh into choline, which is then oxidized by choline
212 oxidase to betaine and $H_2O_2$. Brain levels of AChE and ACh were measured using 10-
213 acetyl-3, 7-dihydroxyphenoxazine, a flourogenic probe for $H_2O_2$. All ACh and AChE
214 samples were examined in duplicate against standards and fluorescence was measured
215 on a fluorescence microplate reader (FLUOstar OPTIMA, BMG LABTECH, Cary, NC).

Following exposure to 2.63μM Donepezil, the levels of ACh were found to be higher in the drug group (11.8nM/g vs. 7.1nM/g; $t$ (8) = 2.81, $P$ = 0.02), which was directly comparable to levels seen in the ache$^{sb55/+}$ thus validating the dose used (Ninkovic et al., 2006).

The exposure schedule was as follows: Week 1: drug A, Week 2: recovery (no drug), Week 3: drug B, Week 4: recovery, Week 5: drug C. As stated, each fish was tested in the presence of each of the three drugs, the order of which was counterbalanced across weeks.

**Gene expression changes in ache$^{sb55/+}$**

[TABLE 2 HERE]

We collected embryos from 4 x ache$^{sb55/+}$ in-crosses. All homozygous individuals were removed at 72hpf (easily identifiable by morphological features and lack of motor activity) leaving petri dishes with ~2/3 heterozygous individuals. We also collected embryos from 4 x ache$^{+/+}$ in-crosses for comparison. Reference genes used were β-actin, ef1α and rpl13α based on previous findings findings (Tang et al., 2007). Target genes used are listed in Table 2. All embryos were manually sorted to ensure all were at the same developmental stage over the first 72hpf, and grown to 9dpf in petri dishes (~40/dish) in an incubator (28$^{o}$C). At 9dpf embryos were terminally anesthetized in

MS-222, and placed in RNAlater until assay (4ºC). Eight batches of n = 3 embryos per strain (ache $^{sb55/+}$ and ache$^{+/+}$) were lysed in 200µl Lysis buffer with 2µl Proteinase K for 30-45min (55ºC). mRNA was isolated using 40µl Dynabeads® Oligo$(dT)_{25}$ according to manufacturer's instructions. Ten adult (6 months) brains (n = 5 ache$^{sb55/+}$; n = 5 ache$^{+/+}$) were homogenized in 400µl Lysis buffer with 4µl Proteinase K for 30-min (55ºC). mRNA was isolated using 80µl Dynabeads® Oligo$(dT)_{25}$ according to manufacturer's instructions. All qPCR reactions were carried out in triplicate. 1µl of cDNA and 1.5µl each of forward and reverse primers (see Table 2) were added to 5µl SYBR® Green PCR Master mix and run in a 384-well plate format (Roche Diagnostics). Method reported in full elsewhere (Gemenetzidis et al., 2010) (Teh et al., 2013).

**Data analysis**

5-CSRTT data were fitted to general linear models (fit by REML), with time (5-CSRTT phases 1-5) and strain (either ache$^{sb55/+}$ vs. ache$^{+/+}$ or ache$^{sb55/+}$ vs. *w/t*) as fixed effects. In the drug administration phase, drug (4-levels, nicotine, pilocarpine, Donepezil and control) was added as a fixed factor, with ID and day as random effects. In each case, the dependent measure was calculated from performance in the 5-CSRTT:

- Correct; calculated as: $\frac{correct}{(correct + incorrect)}$
- Omissions; calculated as: $\frac{omissions}{(correct + incorrect + omissions)}$

- Premature; calculated as: $\frac{premature}{(correct + incorrect + omissions + p\square ematurе)}$

Post-hoc Tukey tests were carried out to examine main effects and interactions of 5-CSRTT data.

Finally, to test the difference between levels of mRNA expression in larvae and adult ache$^{sb55/+}$ and ache$^{+/+}$ siblings, we carried out a series of Mann-Whitney U tests, with strain (ache$^{sb55/+}$ vs ache$^{+/+}$) as the independent variable and target gene expression, relative to reference genes, as the dependent variables. For mRNA expression data, *P* values were estimated following Bonferroni correction for multiple comparison. Effect sizes for all differences in expression were also calculated using the Grissom and Kim (2012) method. Descriptive statistics are reported as mean ± SEM unless otherwise stated. A type-1 error rate of $\alpha = 0.05$ was adopted for all statistical tests. All data were analysed using IBM SPSS Statistics v.21 for Macintosh.

## Results

[FIGURE 2 HERE]

### ache$^{sb55/+}$ show higher levels of responding during pre-training

The ache $^{sb55/+}$ heterozygotes were selected by systematic in-crosses, the mutation being homozygous-lethal. There was a main effect for day, $F(4,35) = 3.42$, $P < 0.02$. Post-hoc

pairwise comparisons revealed that there was a significant increase after Day 1 (*P*s ≤ 0.05), but no change thereafter (*P*s > 0.6). There was also a significant main effect for strain, $F(1,85) = 5.61$, $P < 0.01$, with the ache$^{sb55/+}$ making significantly more response than the *w/t* (Figure 2a). There was no day × strain interaction ($F < 1$). Of the original 39 fish, 3 of the ache$^{sb55/+}$ (30%) and 8 of the 19 *w/t* (42%) failed to meet criteria (i.e., < 20 reinforcers were received).

**ache$^{sb55/+}$ show lower levels of premature responding in long fixed-interval and variable-interval PSIs**

The rates of correct responses, omissions and premature responding were comparable with our previously published work with zebrafish (Parker et al., 2012a;Parker et al., 2013a;Parker et al., 2014). There was a significant main effect of phase for correct responses, $F$ (4,24) = 23.61, $P$ < 0.01. Post-hoc tests revealed that the proportion of correct responses increased after phase 1 (phase 1 < phases 3, 4 and long-PSI, $P$s < 0.01, but not phase 2, $P$ = 0.06) and phase 2 (phase 2 < phases 3, 4 and long-PSI, $P$s > 0.01), but there was no difference between phases 3, 4 and long-PSI ($P$s > 0.14). There was no main effect of strain (ache$^{sb55/+}$ = 0.52 ± 0.02, *w/t*= 0.52 ± 0.02), $F$ < 1, nor a significant phase × strain interaction, $F$ < 1.

The rates of premature responding were comparable with our previous studies (Parker et al., 2012a;Parker et al., 2013b;Parker et al., 2014). There was a significant effect of phase, $F$ (4, 20) = 37.17, $P$ < 0.01. Post-hoc test revealed that phase 1 < phases 2, 3, 4 and long-PSI ($P$s < 0.01), phase 2 < phases 3, 4 and long-PSI ($P$s < 0.01), phase 3 = phase 4 ($P$ = 0.3), and subjects performed more premature responses in the long-PSI phase than phases 3 and 4 ($P$s < 0.05). There was also a significant main effect of strain (Figure 2b), $F$ (1,28) = 5.07, $P$ = 0.03, with the ache$^{sb55/+}$ performing a lower proportion

298 of premature responses than the *w/t*. There was no significant phase × strain
299 interaction, $F$ (4,20) = 2.11, $P$ = 0.12.
300 Rates of omissions were again comparable with our previous study (Parker et al.,
301 2012a;Parker et al., 2013b;Parker et al., 2014). There were significant main effects of
302 phase, $F$ (4,27) = 22.02, $P$ < 0.01. Post-hoc tests revealed that phase 1 < phases 2, 3, 4
303 and long-PSI ($P$s < 0.01), and phase 2 > phases 3 and 4 ($P$s < 0.04), but not long-PSI ($P$ =
304 0.3). Phase 3 was not significantly different from phase 4 ($P$ = 0.14) but was significantly
305 lower than long-PSI ($P$ < 0.03). There was no significant effect of strain ($ache^{sb55/+}$ = 0.32
306 ± 0.02, *w/t* = 0.31 ± 0.01), $F$ < 1, nor was there a significant phase × strain interaction, $F$
307 (4,27) = 1.85, $P$ = 0.14.
308 There was a significant effect of phase on the latency to approach the stimulus
309 for correct responses, $F$ (4,23) = 26.91, $P$ < 0.01, with subjects taking longer to approach
310 the stimulus in Phase 1 (12.69 ± 0.77 s) than in phases 2 (4.51 ± 0.27 s), 3 (5.31 ± 0.21
311 s), 4 (5.45 ± 0.19 s) or the long PSI phase (6.0 ± 0.18 s). There was no significant effect
312 of strain, $F$ < 1, nor was there a phase × strain interaction, $F$ (4,23) = 1.18, $P$ = 0.35.
313 The number of trials completed in each session during 5-CSRTT training changed
314 significantly according to phase, $F$ (4,30) = 7.96, $P$ < 0.01, characterised as fish
315 completing the most trials in phase 3, and fewer trials in the long-PSI phase than in

316 phase 4 (Figure 6). There was no main effect of strain, $F < 1$ nor a phase × strain
317 interaction, $F < 1$.

318 Finally, we carried out a replication with ache$^{sb55/+}$ heterozygotes and ache$^{+/+}$
319 wild-type siblings. First, fish were trained for 20 sessions (1-sec fixed interval PSI), and
320 finally with six, 5-second variable-interval (VI) PSI trials included. ache$^{sb55/+}$ showed a
321 significantly lower proportion of premature responses during the VI-PSI trials, $F$ (1, 18)
322 = 10.48, $P$ = 0.03 (Figure 2c). There were no differences in correct responses (ache$^{sb55/+}$
323 = 0.66 ± 0.03; ache$^{+/+}$ = 0.61 ± 0.02; $P$ = 0.13), nor omissions (ache$^{sb55/+}$ = 0.34 ± 0.05;
324 ache$^{+/+}$ = 0.24 ± 0.03; $P$ = 0.1).

325

326 **Acute manipulation of cholinergic activity increases performance accuracy but**
327 **has no effect on anticipatory responding in adult wild-type zebrafish**

328

329 Figure 2d shows the results of drug administration on 5-CSRTT performance in wild-
330 type fish. There was a significant main effect of drug on correct responses, $F$ (3,75) =
331 4.01, $P$ = 0.01. Post-hoc pairwise comparisons (α-adjusted for multiple tests) revealed
332 that there was a significant increase from control in correct responses during the
333 nicotine ($P$ = 0.02) but not pilocarpine ($P$ = 0.19) or Donepezil ($P$ = 0.85). There were no
334 differences between nicotine and Donepezil ($P$ = 0.07), nicotine and pilocarpine ($P$ =

335 0.68) or pilocarpine and Donepezil ($P$ = 0.53). There were no differences between the
336 drugs' effects in terms of premature response rates (control = 0.126 ± 0.02; nicotine =
337 0.104 ± 0.03; pilocarpine = 0.103 ± 0.03; Donepezil = 0.13 ± 0.03; $F$ < 1), nor in terms of
338 omissions (control = 0.08 ± 0.03; nicotine = 0.1 ± 0.04; pilocarpine = 0.1 ± 0.04;
339 Donepezil = 0.13 ± 0.04; $F$ (3, 79) = 1.22, $P$ = 0.3). There were no differences in the total
340 number of trials completed in each session (control = 21.4 ± 0.52; nicotine = 19.2 ± 0.94;
341 pilocarpine = 21.7 ± 0.94; Donepezil = 21.4 ± 0.94; $F$ (3, 80) = 1.77, $P$ = 0.16). Finally,
342 there was no effect of drug on approach latency (control = 8.6 ± 1.3; nicotine = 8.8 ± 1.5;
343 pilocarpine = 9.1 ± 1.5; Donepezil = 9.1 ± 1.5; $F$ < 1).
344
345 **ache$^{sb55/+}$ have down regulation of *chrna2*, *chrna5* and *drd2* mRNA at 9dpf, but no**
346 **detectable differences in adult expression**
347 [TABLE 3 HERE]
348 Finally, to help understand the mechanisms by which developmental reduction in AChE
349 affected the observed reduction in anticipatory responding, we characterized the gene
350 expression profile of ache$^{sb55/+}$ focussing on neural circuits known to be involved in
351 impulse control. Table 3 summarises the differences in mRNA expression for ache$^{sb55/+}$
352 heterozygotes vs ache$^{+/+}$ wild-type siblings. We found that in the ache$^{sb55/+}$
353 heterozygotes, there was robust downregulation in *chrna2*, *chrna5*, and *drd2* mRNA, the

genes that code for the alpha-2, alpha-5 receptor subunits (nAChRa2, nAChRa5), and the dopamine d2 receptor subunit (DRD2), respectively. In the adults, there was no difference in expression of any of the genes we observed.

## Discussion

The aim of this experiment was to test the hypothesis that developmental alterations in cholinergic signalling affect impulse control using a zebrafish model of the commonly used 5-CSRTT with a strain heterozygous for a missense mutation in *Ache* ($ache^{sb55/+}$). We found that $ache^{sb55/+}$ showed a lower proportion of premature responding than $ache^{sb55/+}$ siblings and *w/t* zebrafish. There were no significant differences in either the number of correct responses, latency to respond, number of trials or the number of omissions, although the $ache^{sb55/+}$ appeared to learn faster, collecting more reinforcers during pre-training. Acute reductions of AChE (donepezil) had no significant effects on premature responding, or other 5-CSRTT parameters, and acute administration of a nAChR agonist significantly increased performance accuracy, while having no effect on premature responding. Finally, $ache^{sb55/+}$ have a down regulation of *chrna2, chrna5,* and *drd2* mRNA expression at 9dpf, but no difference in expression in any of the genes we examined in adulthood. Previous studies have shown that high levels of AChE inhibition during development (e.g., with the organophosphate weedkiller chlorpyrifous

373 (Middlemore-Risher et al., 2010;Cardona et al., 2011;Oca et al., 2012)) increase
374 impulsivity in later life. Collectively, these data provide the first evidence that variation
375 in AChE during development has a J-shaped effect on impulse control, potentially
376 through downstream effects on cholinergic and dopaminergic pathways.
377 Lesion, neuropsychological and pharmacological studies have demonstrated that
378 cortical cholinergic projections to mid-brain regions are strongly implicated in
379 sustained attention and in general top-down cognitive control (Sarter et al., 2001). In
380 particular, during 5-CSRTT performance rats display elevated ACh release from the
381 medial pre-frontal cortex (mPFC), and phasic increases in ACh release when a visual
382 distracter was introduced to increase task complexity (Passetti et al., 2000). We did not
383 see any differences in the number of correct responses in our version of the task, but
384 more of the ache$^{sb55/+}$ met criteria to move to the 5-CSRTT stage of training, and of those
385 that met criteria, overall performance in terms of reinforcers gained was significantly
386 greater than the *w/t*. This finding replicates assessment of this strain's learning
387 previously demonstrated in a T-maze task (Ninkovic et al., 2006). During this initial
388 training stage, despite the strain difference, there was no day × strain interaction,
389 suggesting that ache$^{sb55/+}$ learnt at the same rate. It may be that the ache$^{sb55/+}$ were
390 more motivated to perform, or habituated faster than the *w/t*. This effect was transient,
391 however, disappearing once training started on the 5-CSRTT. We did, however, find

392 evidence for the role of nAChR in task performance, with acute exposure to nicotine
393 (nAChR agonist) increasing the proportion of correct responses in the task. This
394 supports previous data from rodents (Blondel et al., 2000;Hahn and Stolerman,
395 2002;Young et al., 2004).

396 A potential mechanism for the observed differences in premature responding
397 may relate to the role of nAChR during early brain development and patterning. nAChR
398 subtypes, in particular $\alpha$4, $\alpha$5, $\alpha$7, $\beta$2 and $\beta$4, are found early in brain development, and
399 have been suggested to play a role in modulating and mediating early patterning,
400 dendritic outgrowth and synaptogenesis (Hellström-Lindahl et al., 1998).It is possible
401 therefore that reduction in AChE levels, as is characteristic of the $ache^{sb55/+}$
402 heterozygotes, during early brain development alter the distribution of nAChRs thus
403 causing differences in patterning and dendritic morphology. Indeed, in zebrafish, AChE
404 enzymatic activity has been shown to be important for both axon outgrowth and
405 synapse stability, albeit within the neuromuscular projections of the nervous system
406 (Behra et al., 2002;Downes and Granato, 2004).

407 Chronic reductions of AChE in adult rats with donepezil increases expression of
408 $\alpha$4 and $\alpha$7 nAChR (Kume et al., 2005), and ACh-modulated reductions in impulsive
409 action in the 3-CSRTT are mediated by $\alpha$4 nAChR (Tsutsui-Kimura et al., 2010).
410 Although we did not observe differences either in *chrna4* or *chrna7* here, we did

observe robust down regulation of *chrna2* and *chrna5* mRNA expression in the *ache*$^{sb55/+}$ heterozygotes at 9dpf, but no differences in adulthood. CHRNA2 and CHRNA5 variants have been shown to predict impulsive responding in response-inhibition in humans (Rigbi et al., 2008), and transgenic mice overexpressing the Chrna3, Chrna5, Chrnb4 gene cluster show a reduction in impulsivity (Viñals et al., 2012). However, the differences in behaviour observed in the ache$^{sb55/+}$ heterozygotes demonstrate haploinsufficiency of the AChE gene, and thus has implications for the impact of AChE mutations within the human population. Although we are yet to understand the mechanism, this may inform our exploration of potential targets for therapeutics in the future.

The functional properties of nAChRs on catecholaminergic (in particular, dopaminergic) axonal terminals alter during development, highlighting their role in the development of the dopamine system (Azam et al., 2007). It is clear that over-activation of nAChR during early development, e.g., from maternal smoking during pregnancy, can result in an increased risk for impulse control disorders (Button et al., 2007). In addition, as discussed above, excessive inhibition of AChE during development, resulting from exposure to the organophosphate insecticide chlorpyrifos, results in higher impulsivity (Middlemore-Risher et al., 2010;Cardona et al., 2011;Oca et al., 2012). Although this shows a clear link between developmental effects of cholinergic-

system disruption and impulsivity, it is not clear at this stage the mechanisms by which subtle alterations, such as are seen with ache$^{sb55/+}$, subsequently reduces impulsivity. It is possible that this reflects species-specific differences in patterning during early brain ontogeny, although this seems unlikely based on documented similarities between fish and mammalian cholinergic system development (Xie et al., 2000;Behra et al., 2002).

During development, AChE is transiently involved with aspects of neural patterning and hodological development. For example, during cortical synaptogenesis and development of thalamo-cortical pathways, AChE activity is recorded in various brain regions (Button et al., 2007). The cholinergic system interacts with mid-brain dopamine activity in a number of ways. First, the nucleus accumbens (NAc) is densely innervated by cholinergic projection neurons (Meredith et al., 1989;Woolf, 1991). Second, cholinergic receptors (both muscarinic [mAChR] and nicotinic [nAChR]) are found on ventral tegmental area (VTA) dopamine neurons, suggesting dopaminergic control of cholinergic activity (Clarke and Pert, 1985). Third, mesolimbic cholinergic projection neurons are abundant with dopamine receptors, suggesting cholinergic mediation of dopamine activity (Gronier et al., 2000), creating a feedback loop. Rats characterised as high trait impulsivity based on baseline performance on the 5-CSRTT show a greater tendency for elevated cocaine self-administration (Dalley et al., 2007), increased compulsive cocaine seeking (Belin et al., 2008) and increased relapse to

449 compulsive cocaine seeking following punishment-induced abstinence (Economidou et
450 al., 2009). In addition, high impulsive rats show a reduction in DRD2/DRD3 receptors in
451 the ventral striatum, suggesting a potential biomarker for the addiction phenotype
452 (Dalley et al., 2007). Interestingly, ache$^{sb55/+}$ have previously been characterised as
453 showing a decrease in conditioned place preference (CPP) for amphetamine (Ninkovic
454 et al., 2006). It is well established, through the therapeutic efficacy of dopamine agonists
455 such as methylphenidate in reducing impulsivity in ADHD patients (Barkley, 1997), that
456 impulsivity is, at least in part, related to a reduction in availability of dopamine (Li et al.,
457 2006). As such, it seems possible that as genetic impairment of AChE in ache$^{sb55/+}$, which
458 results in higher levels of circulating ACh and as such, desensitization of AChRs
459 (Ninkovic et al., 2006), may act to stabilise dopamine activity (Zhou et al., 2001), thus
460 decreasing impulsive responding. However, although we observed downregulation in
461 *drd2* mRNA in 9dpf ache$^{sb55/+}$ embryos, there was no significant differences in the
462 adults. As such, this requires further exploration in order to elucidate the mechanism.
463 In rodents, low levels of premature responding in the 5-CSRTT are predictive of
464 animals that show resistance to developing compulsive drug seeking (Belin et al., 2008)
465 and relapse following abstinence (Economidou et al., 2009), and this has been
466 interpreted as these animals showing low levels of trait impulsivity affecting top-down
467 cognitive control (Dalley et al., 2011). The neural circuits of impulsivity are currently

not well understood (Brown et al., 2006;Chang et al., 2012), but these findings suggest that zebrafish, an established genetic model system, offer a means for exploration of this.

Gaining a better understanding of the aetiology of psychiatric disease is currently a priority area of research (Campbell, 2010), and with current advances in neuroimaging and huge increases in genetic sequencing power this aim is beginning to be realised. For example, genome-wide association studies (GWAS) are making progress in this regard (Sullivan, 2010), but are limited by uncontrollable factors such as environmental influences and heterogeneity of diseases (Burmeister et al., 2008a). As such, animal models have proved useful in terms of identifying molecular mechanisms of many psychiatric diseases, as symptoms consistent with DSM-IV (APA, 2000) diagnoses of psychiatric disorder have been characterised in many models (Gould and Gottesman, 2006). A better understanding of the molecular mechanisms will be helpful in tailoring treatment options for patients, but also for early identification of at-risk individuals to allow preventative measures to be adopted in the early stages of the disorder (Uhl et al., 2008). Progress in identifying molecular mechanisms, however, has remained slow. This study shows more evidence that zebrafish may be very useful in expediting this process.

In conclusion, this study has found that alterations in *Ache* reduce premature responding in zebrafish on the 5-CSRTT. This effect appears to relate specifically to developmental effects of reduced AChE, as acute exposure to an AChE antagonist had no effect on premature responding in the task. Molecular analyses suggest that the route of action may be through cholinergic interactions with midbrain dopamine systems during development. This study opens the door for potential large-scale forward genetic population screening of mutagenized lines of zebrafish to identify novel alleles for phenotypes such as impulsivity, which is crucial in the search for novel therapeutics and individualised medicine (Jain et al., 2011).



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713 Figure/Table legends
714
715 *Figure 1*. Testing unit and the constituent parts. A) The pneumatic gate mechanism. B)
716 The stimulus light area. The stimuli were 5 white LEDs. C) The food delivery area and
717 magazine. This comprised a green LED to act as a stimulus to signal food availability. D)
718 Food was delivered via activation of a linear stepper motor driving the plunger of a
719 1.5ml plastic syringe, E). The food (liquidized bloodworm and brine-shrimp) was
720 delivered to the fish through 1mm latex catheter tubing. Adapted from (Parker et al.,
721 2012b)
722
723 *Figure 2*. Five-choice serial reaction time task data. A) *ache* $^{sb55/+}$ receive more
724 reinforcers in the stimulus-light training session that TU wild-type fish; B) *ache* $^{sb55/+}$
725 perform a lower proportion of anticipatory responses during 5-CSRTT training than TU
726 wild-type; C) *ache* $^{sb55/+}$ perform a lower proportion of anticipatory responses in 5-
727 CSRTT thank *ache* $^{+/+}$; D) 1.54uM nicotine increases proportion of correct responses
728 during 5-CSRTT in TU wild-type fish. Note: * $P < 0.05$; ** $P < 0.01$
729
730

731 *Table 1.* Procedure for pre-training and training during 5-CSRTT.

732

| Stage | Procedure | Description | Timecourse |
|---|---|---|---|
| **Pretraining** | 1. Acclimation | All apparatus lights on, barrier raised | Day 1-5 |
| | 2. Magazine training | Barrier down. Magazine light on 30-sec. Food available on entry to magazine. 10-sec ITI. | Day 6-10 |
| | 3. Response aperture orientation | All stimulus lights illuminated. Barrier lifted, all stimulus lights illuminated. Entry to any hole reinforced with illumination of magazine light. Food delivered on entry to magazine. Barrier down after correct response. 10-sec ITI (stimulus lights off, barrier down) | Day 11-15 |
| **5 CSRTT** | 4. 30-sec stimulus training | Trial commences with barrier lifted, followed by 1-sec pause (ITI). Stimulus lights illuminated in random order (30-sec), followed by 1-sec limited hold period (stimulus light off). Responses during the stimulus or the limited hold conditionally reinforced with illumination of magazine light. Food delivered on entry to magazine. Barrier down after correct response. Ten second pause following magazine entry (stimulus lights off, barrier down). Subsequent trial initiated following next magazine entry following this pause | Day 16-35 |
| | 5. 10-sec stimulus training | As above (4), but stimulus light illuminated for 10-sec | Days 36-45 |
| | 6. 5-sec stimulus light, 2-sec ITI | As above (4), but stimulus light illuminated for 5-sec, and ITI increased to 2-sec | Day 46-55 |
| | 7. 5-sec stimulus light, 5-sec ITI (Baseline) | As above (6), but ITI increased to 5-sec. | Day 56-60 |
| **Testing** | 8. Long ITI training | Day 1 - Long ITI (as above (7; baseline), but ITI increased to 7-sec). Days 2-3 - Baseline (as above(7). Day 4 - Long ITI, Days 5-6 - Baseline. Day 7 - Long ITI | Day 61 - 68 |

734

735 *Table 2.* Primer pairs for all reference and target genes examined in quantitative real-
736 time PCR analysis.

| Gene name | Primers |
|---|---|
| Reference genes | |
| *β-actin-F* | CGA GCT GTC TTC CCA TCC A |
| *β-actin-R* | TCA CCA ACG TAG CTG TCT TTC TG |
| *rpl13α-F* | TCT GGA GGA CTG TAA GAG GTA TGC |
| *rpl13α-R* | AGA CGC ACA ATC TTG AGA GCA G |
| *eF1α-F* | CTG GAG GCC AGC TCA AAC AT |
| *eF1α-R* | ATC AAG AAG AGT AGT ACC GCT AGC ATT AC |
| Target genes | |
| *adora2aa-F* | CTT GAG CGC AGG AAC CAG AG |
| *adora2aa-R* | CGC GCA CTG AGA GAT GAC AG |
| *chrna2-F* | GCG GAA AAC CGG ATA AAA ACA CTC |
| *chrna2-R* | AGT TTG TCC TCT GCG TGT GCA T |
| *chrna3-F* | TGT ACA TCC GCC GAT TAC CGC T |
| *chrna3-R* | TCC GCA GTC GGA GGG CAG TA |
| *chrna4-F* | TTA CAA GAG GTT TGG GCG CT |
| *chrna4-R* | ACA GAC CAG TAG ATC ATC ACT CC |
| *chrna5-F* | GGC TCC CAG GTC GAC ATT |
| *chrna5-R* | AAC CCC GGT TAC CAG TGG CCT |
| *chrna6-F* | CTT TGG GCC TCT TCC TGC AA |
| *chrna6-R* | TCA GAG TCT TGA TGT AGT GAC GG |
| *chrna7-F* | ACC GTG TCA CAT TGT TCA TTC TC |
| *chrna7-R* | ACA GGT CTC TCC AGT GGG TTA |
| *chrnb2-F* | GGC TGC CTG ATG TTG TTC TT |
| *chrnb2-R* | TGG TGG CAA CCA GAA GAC ACT T |
| *chrnb3-F* | CAG GAG TCA ACC TCC GCT TT |
| *chrnb3-R* | TGA ATC TGA ACG CAC TGG CT |
| *chrnb4-F* | TGA TCA CAT GAT GGG GAA TGA CG |
| *chrnb4-R* | CAC CAC ACA CAC GAT CAC AAA G |
| *drd1-F* | TGG TTC CTT TCT GCA ACC CA |
| *drd1-R* | AGT GAT GAG TTC GCC CAA CC |
| *drd2-F* | TCC ACA AAA TCA GGA AAA GCG T |
| *drd2-R* | CAG CCA ATG TAA ACC GGC AA |
| *drd3-F* | ATC GAG TTT CGC AGA GCC TT |
| *drd3-R* | TCC ACA GTG TCT GAA AGC CG |
| *htr1aa*-F | GGA GCC CGC CAT GCG TCT T |
| *htr1aa*-R | CGT CGC GTT CCC GCT CCA A |
| *oprm1-F* | CCG TAT GTG ACA GGA CGC CA |
| *oprm1-R* | TTT CCC ACC AGT CCC ATC ACA |
| *slc6a2-F* | AGG TGA CAT TGT TTG AGA TGT CTT |
| *slc6a2-R* | TGT CTT GGT AGT GTC AAG TTG T |
| *slc6a3-F* | TAT GTG GTC CTG ACC GTG CT |
| *slc6a3-R* | CAC ATG TGT AGG CGC AGG AA |
| *slc6a4*-F | GCC ACA GGC CCC GCT GTT A |
| *slc6a4*-R | ACC AGG GGC GAA GCC AAG CA |

737

738 *Table 3.* mRNA expression for *ache*$^{sb55/+}$ vs. *ache*$^{+/+}$ at 9dpf and 6 months of age. All
739 expression ratios are reported relative to *Bact*, *Rpl13a* and *eF1a*.
740

| Gene | U | N(a) | N(b) | Uncorrected P-value | Corrected P-value | Effect size (Grissom & Kim, 2012) | Direction of change in mRNA expression |
|---|---|---|---|---|---|---|---|
| | | | **Adult (6 months)** | | | | |
| *adora2aa* | 9 | 4 | 5 | 0.9 | 1 | 0.45 | - |
| *chrna2* | 17 | 5 | 5 | 0.42 | 1 | 0.68 | - |
| *chrna3* | 17 | 5 | 5 | 0.42 | 1 | 0.68 | - |
| *chrna4* | 12 | 4 | 5 | 0.73 | 1 | 0.6 | - |
| *chrna5* | 18 | 5 | 5 | 0.31 | 1 | 0.72 | - |
| *chrna6* | 11 | 4 | 5 | 1 | 1 | 0.55 | - |
| *chrna7* | 14 | 4 | 5 | 0.41 | 1 | 0.7 | - |
| *chrnb2* | 9.5 | 4 | 5 | 0.9 | 1 | 0.475 | - |
| *chrnb3* | 8 | 4 | 5 | 0.73 | 1 | 0.4 | - |
| *chrnb4* | 10 | 4 | 5 | 1 | 1 | 0.5 | - |
| *drd1* | 9 | 4 | 5 | 0.9 | 1 | 0.45 | - |
| *drd2* | 11 | 4 | 5 | 1 | 1 | 0.55 | - |
| *drd3* | 10 | 5 | 5 | 0.69 | 1 | 0.4 | - |
| *htr1aa* | 12 | 4 | 5 | 0.73 | 1 | 0.6 | - |
| *optm1* | 13.5 | 4 | 5 | 0.41 | 1 | 0.675 | - |
| *slc6a2* | 14 | 4 | 5 | 0.41 | 1 | 0.7 | - |
| *slc6a3* | 14 | 5 | 5 | 0.85 | 1 | 0.56 | - |
| *slc6a4* | 16 | 5 | 5 | 0.55 | 1 | 0.64 | - |
| | | | **9 dpf** | | | | |
| *adora2aa* | 51 | 8 | 8 | 0.05 | 0.9 | 0.797 | - |
| ***chrna2*** | **47** | **8** | **6** | **0.001** | **0.02** | **0.979** | ***ache***$^{sb55/+}$ ↓ |
| *chrna3* | 33.5 | 8 | 8 | 0.9 | 1 | 0.523 | - |
| *chrna4* | 46 | 8 | 8 | 0.16 | 1 | 0.719 | - |
| ***chrna5*** | **94.5** | **8** | **8** | **0.003** | **0.05** | **1.477** | ***ache***$^{sb55/+}$ ↓ |
| *chrna6* | 50 | 8 | 8 | 0.065 | 1 | 0.781 | - |
| *chrna7* | 50 | 8 | 8 | 0.065 | 1 | 0.781 | - |
| *chrnb2* | 52 | 8 | 8 | 0.038 | 0.68 | 0.813 | - |
| *chrnb3* | 28 | 8 | 8 | 0.72 | 1 | 0.438 | - |
| *chrnb4* | 50 | 8 | 8 | 0.065 | 1 | 0.781 | - |
| *drd1* | 54 | 8 | 8 | 0.02 | 0.36 | 0.844 | - |
| ***drd2*** | **53** | **8** | **7** | **0.002** | **0.036** | **0.946** | ***ache***$^{sb55/+}$ ↓ |
| *drd3* | 57 | 8 | 8 | 0.007 | 0.126 | 0.891 | - |
| *htr1aa* | 54 | 8 | 8 | 0.02 | 0.36 | 0.844 | - |
| *optm1* | 53 | 8 | 8 | 0.03 | 0.54 | 0.828 | - |
| *slc6a2* | 55.5 | 8 | 8 | 0.01 | 0.18 | 0.867 | - |
| *slc6a3* | 45 | 8 | 7 | 0.054 | 0.972 | 0.804 | - |
| *slc6a4* | 25 | 8 | 6 | 1 | 1 | 0.521 | - |

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Figure 1.TIFF

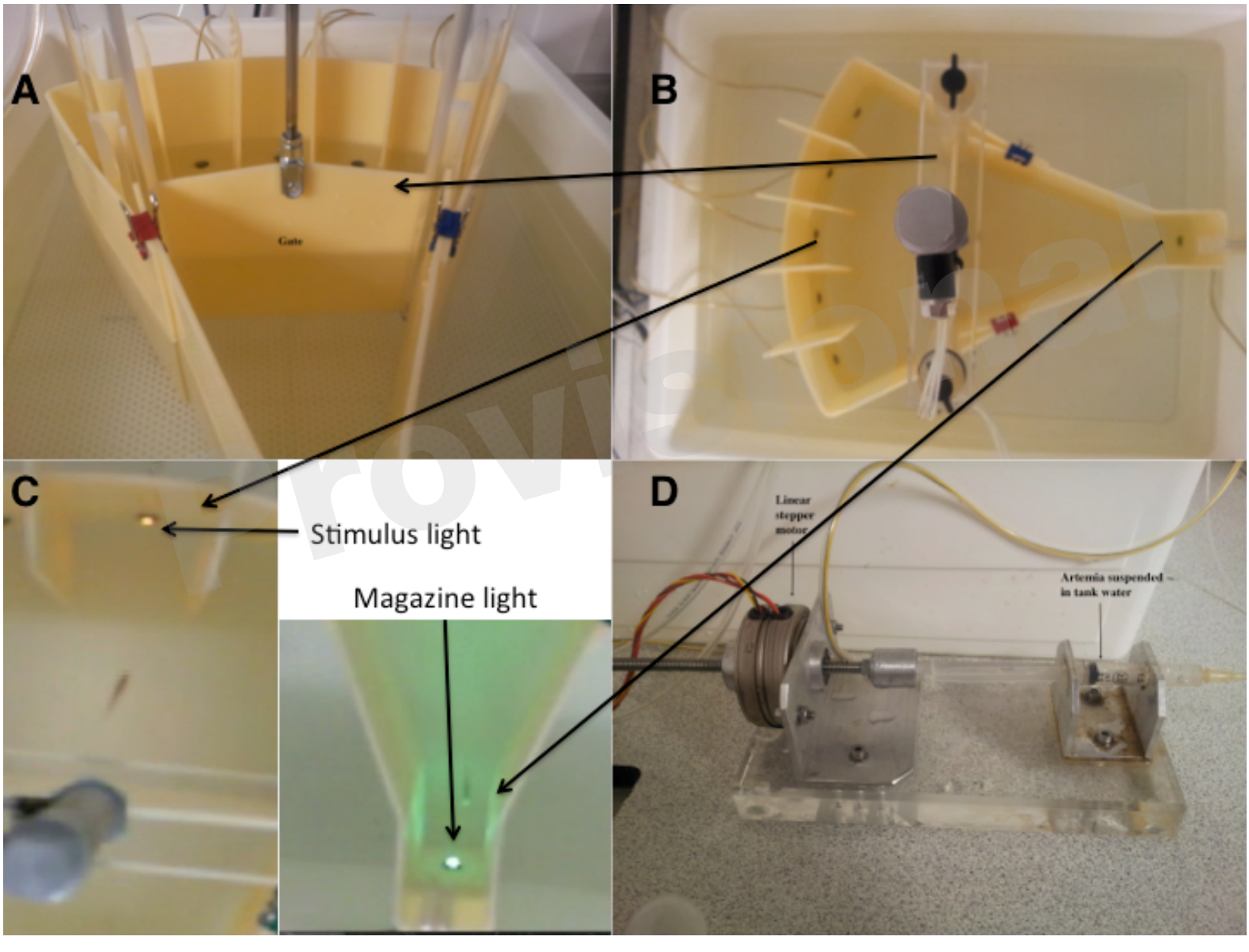

Figure 2.TIFF