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Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways

Layla J. M. Kily[1], Yuka C. M. Cowe[1], Osman Hussain[1], Salma Patel[1], Suzanne McElwaine[2], Finbarr E. Cotter[2] and Caroline H. Brennan[1,*]

[1]School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End, London E1 4NS, UK and [2]Centre for Haematology, Institute of Cell and Molecular Science, Barts & The London, Queen Mary's School of Medicine, 4 Newark Street, London E1 2AD, UK

*Author for correspondence (e-mail: C.H.Brennan@qmul.ac.uk)

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SUMMARY

Addiction is a complex psychiatric disorder considered to be a disease of the brain's natural reward reinforcement system. Repeated stimulation of the 'reward' pathway leads to adaptive changes in gene expression and synaptic organization that reinforce drug taking and underlie long-term changes in behaviour. The primitive nature of reward reinforcement pathways and the near universal ability of abused drugs to target the same system allow drug-associated reward and reinforcement to be studied in non-mammalian species. Zebrafish have proved to be a valuable model system for the study of vertebrate development and disease. Here we demonstrate that adult zebrafish show a dose-dependent acute conditioned place preference (CPP) reinforcement response to ethanol or nicotine. Repeated exposure of adult zebrafish to either nicotine or ethanol leads to a robust CPP response that persists following 3 weeks of abstinence and in the face of adverse stimuli, a behavioural indicator of the establishment of dependence. Microarray analysis using whole brain samples from drug-treated and control zebrafish identified 1362 genes that show a significant change in expression between control and treated individuals. Of these genes, 153 are common to both ethanol- and nicotine-treated animals. These genes include members of pathways and processes implicated in drug dependence in mammalian models, revealing conservation of neuro-adaptation pathways between zebrafish and mammals.

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Key words: nicotine, alcohol, conditioned place preference, drug dependency, zebrafish, gene expression.

INTRODUCTION

Nicotine and ethanol are two of the most widely abused addictive drugs, and although the feasibility of pharmacological treatment for either addiction has been demonstrated many alcoholics and chronic smokers relapse, often after prolonged periods of abstinence. The long-lasting neuro-adaptation that is responsible for such relapsing behaviour is thought to be in response to chronic, repeated activation of the brain's natural reward reinforcement circuit. The accepted view of reward is that when an activity increases dopamine transmission in the mesolimbic dopaminergic system the rise in dopamine in the nucleus accumbens is translated into a motivational activity of the animal, such that the behaviour is reinforced and repeated. With few exceptions, addictive drugs are those that enhance dopamine levels in the nucleus accumbens (Kalivas, 2007; Wise, 1996; Wise and Bozarth, 1984). Nicotine leads to elevated dopamine in the nucleus accumbens *via* direct activation of nicotinic acetylcholine receptors present on the neurons of the mesolimbic dopaminergic pathway (Mansvelder and McGehee, 2002). Ethanol exposure has a broader range of effects that include altering activity of glutamatergic, opioid and gamma amino butyric acid (GABA)ergic neurons that interact with the mesolimbic system and ultimately also results in increased levels of dopamine in the nucleus accumbens (Tupala and Tiihonen, 2004).

How dopaminergic transmission and reinforcement is related to addiction is not fully understood. However, from a cellular and molecular perspective it is likely that repeated exposure to addictive drugs causes stable changes in gene expression, posttranslational modification and/or synaptic plasticity that have lasting effects on brain function and thus behaviour. In this context a number of studies have identified lasting neuro-adaptations that are associated with such addiction-related behaviours as compulsive drug taking and persistent tendency to relapse (Kalivas, 2004; Shaham and Hope, 2005; Weiss et al., 2001). These neuro-adaptations include altered basal levels or sensitivity of dopaminergic, serotonergic and glutamate neurotransmission (Kalivas et al., 2003; Tupala and Tiihonen, 2004; Weiss et al., 2001) in addition to dysregulation of neuro-endocrine systems (Lovallo, 2006; Weiss et al., 2001). Similarly, expression analysis has identified components of a number of neurotransmitter (glutamatergic, cannabinoid, monaminergic) and signal transduction pathways [ERK (extracellularly regulated kinase), PI3K (phosphatidylinositol 3-kinase) and NFkappaβ (nuclear factor kappa beta)] that are altered in their levels or domains of expression in the brains of animals demonstrating drug dependency (Lu et al., 2006; Pollock, 2002; Rhodes and Crabbe, 2005; Yuferov et al., 2005). Changes in the gene expression of many of these compounds were identified using a hypothesis-driven or candidate-gene approach, based on results of pharmacological analysis (Koob et al., 2004; Nestler, 2004). However, more recently, microarray analysis has enabled the simultaneous interrogation of expression levels of thousands of genes

in different brain regions of control and drug-treated animals. This approach has identified further candidate molecules and pathways that may be the basis of the neuro-adaptation that underlies drug addiction (Lehrmann et al., 2006; Yuferov et al., 2005).

The primitive nature of reward reinforcement pathways and the near universal ability of drugs of abuse to target the same system allow drug-associated reinforcement to be modelled in non-mammalian species. Indeed, reinforcement pathways are strongly activated by drugs of abuse in several model systems including rodents, fish, insects and nematodes (Bretaud et al., 2007; Darland and Dowling, 2001; Mohn et al., 2004; Ninkovic and Bally-Cuif, 2006; Ninkovic et al., 2006; Wolf and Heberlein, 2003). Conditioned place preference (CPP), where drug exposure is paired with specific environmental cues, is commonly used as a measure of drug reward or reinforcement (Tzschentke, 1998). Persistent CPP that lasts following a period of abstinence or in the face of an adverse stimulus is a model for dependency. Recently, by virtue of its inherent suitability for forward genetic screens, the zebrafish has become established as a valuable animal disease model (Anderson and Ingham, 2003; Berghmans et al., 2005; Shin and Fishman, 2002). With respect to studies of drug-induced reinforcement and addiction, anatomical analyses have demonstrated that neurons expressing tyrosine hydroxylase (the rate limiting enzyme in catecholamine synthesis) project from the posterior tuberal nucleus to the basal forebrain in a manner reminiscent of the ventral tegmental–nucleus accumbens connection of the mesolimbic system in mammals (Rink and Wullimann, 2002). Zebrafish show CPP responses to cocaine (Darland and Dowling, 2001), amphetamine (Ninkovic and Bally-Cuif, 2006) and opiates (Bretaud et al., 2007) and the amphetamine-induced response is modified by pathways known to influence dopamine release in the nucleus accumbens in other systems (Ninkovic et al., 2006). These results demonstrate the existence of a conserved drug-responsive 'reward' or reinforcement pathway in zebrafish and suggest that zebrafish may show adaptive changes and behavioural correlates of addiction after prolonged exposure to addictive drugs. We use CPP and microarray analysis to test this hypothesis with regard to nicotine and ethanol exposure.

MATERIALS AND METHODS

Animals and maintenance

Zebrafish (*Danio rerio*) were maintained according to established protocols (Westerfield, 1995). They were kept on a constant 14 h:10 h light:dark cycle at 28°C. The animals used in these experiments were 0.5–1 g, 4-month-old, sex and age matched Tuebingen wild-type stock, bred in house.

Behavioural assays

Fish were subject to treatment regimes as detailed in Table 1.

Assessing the reinforcing properties of ethanol or nicotine using conditioned place preference

Experiment 1: conditioned place preference assay following a single drug exposure

A balanced conditioning paradigm modified from Darland and Dowling (Darland and Dowling, 2001) was used to assess the reinforcing properties of ethanol or nicotine in zebrafish. The testing apparatus was a 2 l rectangular tank (Aquatic Habitats, Apopka, FL, USA) that could be divided in half with a Perspex divider. Each end of the tank had distinct visual cues (1.5 cm diameter black spots uniformly distributed on all sides *versus* vertical 0.5 cm wide black and white stripes). After an initial 5 min settling period each fish was tested for baseline preference by determining the time spent on a given side of the tank over a 2 min period. Each fish was then restricted first to the preferred side for 20 min using a Perspex divider so that the fish was surrounded by either spots or stripes and then the fish was restricted to the least preferred side and either nicotine, ethanol or fish-water added in a volume of 10 ml so as to give the desired final drug concentration. Drug concentrations used ranged from 0–300 μmol l^{-1} for nicotine (0–50 mg l^{-1}) and 0–264 mmol l^{-1} [0–1.5% (v/v)] for ethanol. After 20 min the fish were removed to fresh water in clean tanks and returned to the aquarium. To determine the reinforcing effects of ethanol or nicotine, the place preference of each fish was determined the following day by again, after a 5 min settling period, determining the percentage time spent on each side of the tank over a 2 min test period. Any change in place preference was determined by subtracting the baseline time spent on the drug-treatment side from the final time spent on the drug-treatment side expressed in seconds. Fish that showed a greater than 70% baseline preference for either side of the tank, approximately 10% of fish tested, were not used further. Each drug concentration was tested on 15–24 fish and two parallel groups of 20 control fish received fish-water only. All fish tracking was performed manually with assessment of place preference performed by an observer blinded to the treatment conditions.

Conditioned place preference following repeat exposure to nicotine or ethanol

Experiment 2: place preference following three consecutive conditioning sessions

Following determination of baseline preference, each fish was restricted first to the preferred side for 20 min and then to its least preferred side where it was exposed to either nicotine or ethanol for 20 min. Fish were exposed to tank concentrations of nicotine ranging from 0–300 μmol l^{-1} (0–50.0 mg l^{-1}) for 20 min each day for 3 days before determination of their place preference. Each drug concentration was tested on 10–12 fish. As the results of these experiments and others (Ninkovic and Bally-Cuif, 2006) suggested that repeat exposure to the apparatus leads to a slight change in the baseline preference that stabilizes over three consecutive exposures, in all subsequent experiments fish were subject to three conditioning sessions in the absence of any drug prior to the determination of their baseline preference.

Experiment 3: place preference following 4 weeks of daily conditioning

Groups of 35 sex and age matched fish were subject to the conditioning paradigm on the consecutive days in the absence of any drug to allow familiarization to the apparatus and protocol. Baseline place preference for each fish was then determined as described above. Any fish showing greater than 70% baseline preference for either side of the tank was not used further; 5–10% of fish were excluded on this basis. Following determination of baseline preference each fish was restricted first to the preferred side for 20 min and then to its least preferred side where it was exposed to either 30 μmol l^{-1} nicotine or 175 mmol l^{-1} ethanol for 20 min. Conditioning sessions were repeated each day over a 4 week period.

Conditioned place preference despite an adverse stimulus

Adverse stimulus test

Following determination of their basal preference, individual fish were placed in the testing apparatus, allowed to settle for 5 min and then each time the fish entered its preferred side it was punished by removal from the tank to the air for 3 s. On return to the tank the fish was restricted to its non-preferred side for 30 s to allow

Table 1. Treatment regimes

Experiment	Treatments					
Experiment 1 Conditioned place preference in response to a single exposure	Day 1 (i) Determine baseline preference over a 2 min period (ii) 20 min conditioning ± nicotine (0–300 μmol l^{-1}) or ethanol (0–264 mmol l^{-1})	Day 2 Determine conditioned place preference over a 2 min period				
Experiment 2 Conditioned place preference following three consecutive conditioning sessions	Day 1 (i) Determine baseline preference over a 2 min period (ii) 20 min conditioning ± nicotine (0–300 μmol l^{-1})	Days 2 and 3 (i) 20 min conditioning ± nicotine (0–300 μmol l^{-1})	Day 4 Determine conditioned preference over a 2 min period			
Experiment 3 Conditioned place preference following 4 weeks of daily conditioning with either 30 μmol l^{-1} nicotine or 175 mmol l^{-1} ethanol. *N*=35 for each treatment group	Day 1 (i) Determine baseline preference over a 2 min period (ii) 20 min conditioning to apparatus in absence of any drug	Days 2 and 3 (i) 20 min conditioning in absence of any drug	Days 4–31 (i) 20 min conditioning ± either 30 μmol l^{-1} nicotine or 175 mmol l^{-1} ethanol	Day 32 (i) Determine conditioned place preference over a 2 min period		
Experiment 4 Conditioned place preference despite an adverse stimulus	Day 1–32 as for experiment 3 above. *N*=18–20 for each treatment group	Day 33 (i) Determine place preference despite an adverse stimulus				
Experiment 5 Conditioned place preference following a period of abstinence	Day 1–31 as for experiment 3 above. *N*=35 for each treatment group	Day 32 (i) Determine place preference for each fish over a 2 min period (ii) 10–12 fish selected randomly from each group and assessed for place preference despite an adverse stimulus prior to sacrifice	Days 32–37 No treatment. Fish maintained in aquarium	Day 38 (i) Determine place preference for each fish over a 2 min period (ii) 10–12 fish selected randomly from each group and assessed for place preference despite an adverse stimulus prior to sacrifice	Days 39–52 No treatment. Fish maintained in aquarium	Day 53 (i) Determine place preference for each fish over a 2 min period (ii) Remaining 10–12 fish from each group assessed for place preference despite an adverse stimulus prior to sacrifice. Brains dissected and stored at minus 70°C prior to preparation of RNA for microarray analysis

recovery. As a control, separate fish were subject to the same procedure but without the 3 s punishment: they were restricted to their least preferred side for 30 s each time they entered the preferred side. After this time the divider was removed and the fish allowed free access to the entire tank. In each case the number of returns to the preferred side over a 10 min period was determined.

Experiment 4: place preference despite an adverse stimulus

Following 4 weeks of conditioning, the effect of punishment compared with restriction on the number of returns made to the drug treatment side over a 10 min period was determined. Single fish were placed in the conditioning apparatus, allowed a 5 min settling period and then each time the fish entered the drug-treatment side it was restricted to the non-drug-treatment side for 30 s using a Perspex divider. After 30 s the divider was removed and the fish allowed free access to the whole tank. The number of returns made over a 10 min period was determined. An hour later each fish was returned to the testing apparatus, allowed 5 min to settle and then each time the fish entered the drug treatment side it was removed from the tank to the air for 3 s. On return to the tank, the fish was restricted to the non-drug-treatment side for 30 s to allow recovery. After this time the divider was removed and the fish allowed free access to the tank. Again the number of returns made over a 10 min period was determined. Tests were carried out on 18–20 fish for each treatment group with two parallel control groups.

Conditioned place preference following a period of abstinence

Experiment 5: groups of 35 sex and age matched fish were used for each drug treatment with two parallel control groups

Following determination of their baseline preference, fish were exposed to either 30 μmol l^{-1} nicotine or 175 mmol l^{-1} ethanol for 20 min each day over a 4 week period. The day after the last drug treatment each fish was tested for a change in place preference by, following a 5 min settling period, determining the time spent on each side of the tank over a 2 min test period. The change in place preference was calculated as final time minus baseline time spent on the drug-treatment side as previously. An hour later 10–12 fish from each group were also tested for place preference in the face of an adverse stimulus (see experiment 4) before being sacrificed. The remaining fish were then returned to the aquarium for a period of 1 or 3 weeks where they experienced no further drug treatment. At 1 or 3 weeks following the last drug treatment the fish were again tested for their place preference and 10–12 fish from each group also tested for place preference despite an adverse stimulus before being sacrificed.

RNA extraction and microarray analysis

Brains from control fish or fish that had been conditioned to ethanol or nicotine for 20 min each day over a 4-week period followed by 3 weeks of withdrawal were homogenized using an Ultra Turrax T25 polytron homogenizer in Trizol (Invitrogen, Carlsbad, CA, USA) and RNA extracted according to the manufacturer's instructions. Total RNA (5 μg) from the zebrafish brain tissue was used to synthesize double stranded cDNA according to the one-cycle protocol from Affymetrix (www.affymetrix.com/support/technical/manual/expression_manual.affx). Eight cDNA synthesis reactions were performed, two for each drug treatment and two for each parallel set of control animals. RNA from two brains was pooled for each cDNA synthesis. An *in vitro* transcription was performed for 16 h at 37°C to generate biotinylated cRNA. Biotinylated cRNA (20 μg) was fragmented at 94°C for 35 min and 15 μg of fragmented cRNA was added to the hybridization cocktails. Zebrafish expression arrays were hybridized for 16 h at 42°C and subsequently stained and scanned according to the manufacturer's instructions. All microarray images were analysed by Microarray Suite 5.0 (MAS 5, Affymetrix; www.affymetrix.com). Each microarray was initially multiplied by a scaling factor to make its mean intensity equal to an arbitrary target intensity value (100 was used in our experiment). The scaling factor for each array must be within threefold of each other or they are not suitable for comparison. Following scaling, microarray data were imported into GeneSpring 6.1 (Agilent Technologies, Stockport, UK). Normalization of all imported data was performed in GeneSpring according to the manufacturer's recommendations. Imported files were normalized using the 'per chip' (normalizes to a median or percentile) and 'per gene' (normalizes to median) function. GeneSpring first divides each raw intensity value by the median of the chip. Then each value is further divided by the median value of each gene across samples, resulting in the final normalized value. The normalized data were then filtered to identify differentially expressed genes between control and drug-treated zebrafish. Data were initially 'filtered on flags' eliminating genes called 'absent' in all samples. Subsequently genes called either present or marginal in 70% of the arrays were used in statistical or fold-change comparisons. We used ANOVA comparing control *versus* ethanol-treated and control *versus* nicotine-treated animals to identify genes with statistically different levels of expression in control and drug-treated groups. We also generated lists of genes that were 1.5-fold increased or decreased in control *versus* ethanol-treated, or control *versus* nicotine-treated animals. Venn diagram analysis of the merged fold change and statistically significant lists was then performed to identify genes showing at least a 1.5-fold significant different change in expression in both ethanol- and nicotine-treated animals.

Quantitative real-time PCR

Microarray results for each cDNA were validated for selected genes, chosen from different groups when genes were sorted according to biological process, using quantitative real-time PCR (Q-RT-PCR). *Gria2* [α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) ionotropic glutamate receptor subunit GluR2] was chosen for validation as this gene has been consistently reported to be upregulated in models of drug dependency. Other genes were selected at random as we aimed to identify changes in the expression of genes not previously associated with drug addiction. Primers used for PCR were based on the array sequences and are given in Table 2.

Table 2. Primers used for PCR

Gene name/symbol	5′-primer	3′-primer
CalcineurinB	5′-atattcgacacagacggaaac-3′	5′-ccaccatcatcttcagcac-3′
GRIA2a (AMPA GluR2)	5′-ctctaaatccctcctcttcctc-3′	5′-actgcccgttatagacaacc-3′
AMMECR1	5′-gggaccacattcagaccatag-3′	5′-gctcatcgtcatcttctcac-3′
pBDZR	5′-ttgatgagtggcacagtgg-3′	5′-gttagctggaatagtgttggg-3′
β-actin	5′-aagcaggagtacgatgagtc-3′	5′-tggagtcctcagatgcattg-3′

Parallel 25 μl PCRs were set up, each containing 1 μl (25 ng) cDNA and 300 ng each primer. PCR was performed (50 cycles) at 55°C on a MX3000P QPCR system (Stratagene, Cedar Creek, TX, USA) followed by a thermal dissociation step to allow analysis of the product for purity. DNA synthesis was monitored using SYBR green (Stratagene, Cedar Creek, TX, USA) and normalization of expression against β-actin

permitted comparison between cDNAs. Each measurement was performed in duplicate from two different animals on each of three separate days with reverse transcriptase-free samples for each treatment acting as negative controls.

Statistical analysis

CPP was analyzed using ANOVA followed by Tukey's *post-hoc* comparison and by paired or two sample *t*-test as appropriate. Conditioned place preference despite an adverse stimulus data were analyzed using two-way ANOVA with a repeat measure over condition (restricted *versus* punished) using Graphpad Prism 5, Instat (GraphPad, San Diego, CA, USA), followed by *post-hoc* two-sample or paired *t*-test, as appropriate, with Bonferroni adjustment. Microarray data were analyzed using ANOVA parametric tests without multitask correction, variances not assumed equal (Welch *t*-test). A *P*-value of 0.05 was considered significant. This restriction tested 9201 genes. Approximately 460 genes would be expected to pass the restriction by chance.

RESULTS

Nicotine and ethanol induce dose-dependent conditioned place preference in zebrafish

Central to current theories of drug addiction is the idea that repeated stimulation of the brain's reward reinforcement circuit leads to lasting adaptations that underlie changes in behaviour. In order to enable the use of zebrafish as a model system in which to test this hypothesis with regard to nicotine or ethanol we first determined whether zebrafish show a CPP reinforcement response on exposure to these drugs. 20 min exposure to either nicotine or ethanol induced a dose-dependent change in preference for the site of drug exposure (Fig. 1). Nicotine at concentrations between 3–300 μmol l^{-1} (0.5 mg l^{-1} and 50 mg l^{-1}) induced a significant (ANOVA $P<0.05$) increase in preference for the treatment side (Fig. 1A). The maximum change in preference was seen at a tank concentration of 30 μmol l^{-1}. In 10 fish tested, a tank concentration of 600 μmol l^{-1} (100 mg l^{-1}) nicotine induced signs of toxicity (vibration, rapid breathing) and caused a decrease in place preference (results not shown). CPP in response to a single exposure to ethanol at tank concentrations of 88, 175 and 264 mmol l^{-1} (0.5, 1 and 1.5% v/v) was determined. Only exposure to 175 mmol l^{-1} ethanol induced a significant (ANOVA $P<0.05$; Fig. 1B) change in place preference (73±8 s increase, mean ± s.e.m., $N=12$). In this set of experiments control, water-treated, fish also showed a significant increase in place preference after treatment compared with before treatment (paired *t*-test $P<0.05$; Fig. 1B).

The aim of our study was to assess behaviour and gene expression changes in zebrafish following chronic exposure to nicotine or ethanol. As high concentrations of nicotine induced signs of toxicity in zebrafish and the rate of metabolism of nicotine in zebrafish is unknown, we were concerned that repeated exposure may lead to the toxic build up of the drug in the fish and influence the CPP response, or tolerance to the effects of nicotine may develop. We therefore tested the CPP response following 3 days of drug treatment. We detected a significant increase in preference for the treatment side in control fish after 3 days of treatment compared with before treatment (paired *t*-test, $P<0.05$; Fig. 2) suggesting that the place preference changes slightly as the fish become familiarized or habituated to the apparatus and handling procedure. Despite this habituation effect, fish exposed to either 6 or 30 μmol l^{-1} nicotine induced a significant increase in preference for the treatment side compared with the reaction of control, water-treated fish (two-sample *t*-test $P<0.05$; Fig. 2). Three repeat exposures to 300 μmol l^{-1} nicotine led to a significant decrease in place preference compared

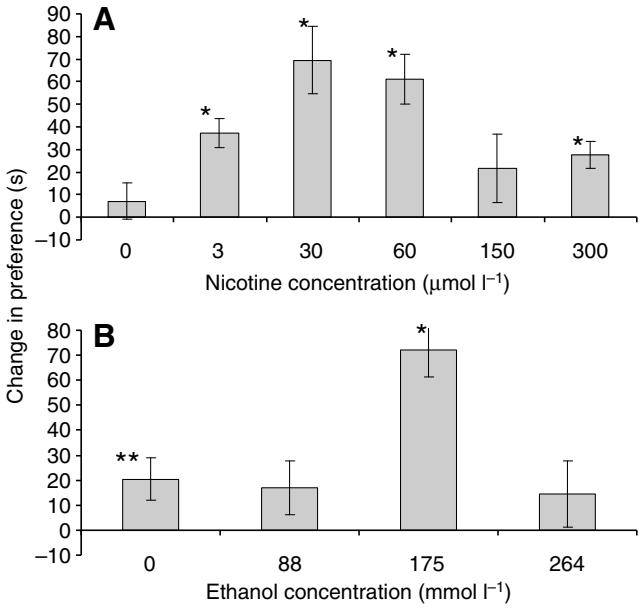

Fig. 1. Conditioned place preference following a single 20 min treatment with nicotine or ethanol. (A) Exposure to 3–300 μmol l^{-1} (0.5–50 mg l^{-1}) nicotine induced a significant change in preference compared with the control treatment (ANOVA, *$P<0.05$). (B) 175 mmol l^{-1} (1% v/v) ethanol induced a significant change in preference (ANOVA, *$P<0.05$) compared with the control. Water-treated control fish also showed a significant change in preference after treatment compared with before treatment (paired *t*-test, **$P<0.05$). Change in preference (s) is calculated as time spent on treatment side after drug exposure minus 'baseline' time spent on treatment side before drug exposure. Place preference was determined over a 120 s period.

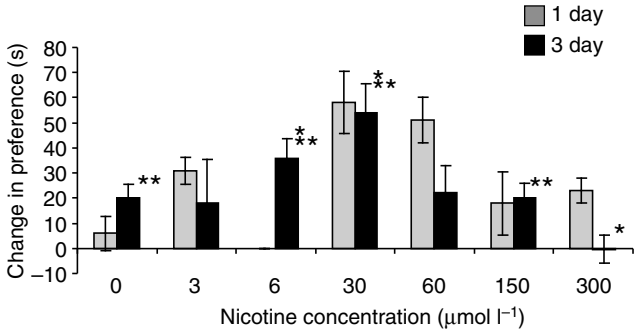

Fig. 2. Conditioned place preference (CPP) following a single exposure or three consecutive exposures to nicotine. Fish showed a concentration-dependent change in preference for the treatment side following both a single exposure (grey bars) and three repeat exposures to nicotine on each of three consecutive days (black bars). The CPP response to 6 μmol l^{-1} nicotine after a single exposure was not determined. Following exposure to 0, 3, 6, 30 and 150 μmol l^{-1} nicotine for 20 min on each of three separate days fish showed a significant increase in place preference for the treatment side compared with before treatment (**$P<0.05$). Fish subject to three treatments with 6 or 30 μmol l^{-1} nicotine showed a significantly greater change in place preference for the treatment side than control, water-treated fish (*$P<0.05$). Three exposures to 300 μmol l^{-1} nicotine induced a significant decrease in place preference compared with water-treated controls (*$P<0.05$).

with either control fish, or to fish exposed to a single treatment of 300 μmol l^{-1} nicotine (two-sample *t*-test and paired *t*-test, respectively, $P<0.05$; Fig. 2).

These results demonstrate that zebrafish show a dose-dependent acute reinforcement response to both nicotine and ethanol, consistent with the hypothesis that they may show lasting behavioural and gene expression adaptations following continued, repeated exposure to these drugs. Concentrations of 30 μmol l^{-1} nicotine and 175 mmol l^{-1} ethanol were chosen for such repeated drug treatments.

Repeat exposure to nicotine or ethanol induces conditioned place preference that persists despite prolonged drug abstinence

Following 4 weeks of repeated 20 min daily exposure to either 30 μmol l^{-1} nicotine or 175 mmol l^{-1} ethanol, zebrafish showed a significant (two-sample *t*-test, $P<0.05$) increase in time spent in the treatment side: 50±6 s and 72±11 s for nicotine and ethanol respectively, compared with 7±7 and 3±6 s (mean ± s.e.m.) for each of the control groups. This CPP response persisted for 3 weeks following the last drug exposure for both nicotine-treated and ethanol-treated fish. However, after 7 or 21 days of drug abstinence the nicotine-treated fish showed a significant reduction (two-sample *t*-test, $P<0.05$) in preference for the treatment side when compared with the day after the last drug treatment (Fig. 3A).

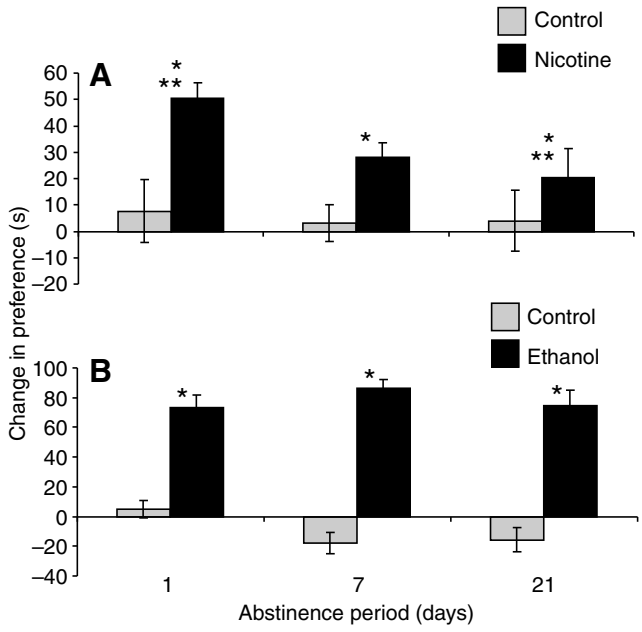

Fig. 3. Conditioned place preference persists over a 3-week period of abstinence from nicotine or ethanol. (A) Following 4 weeks of daily 20 min exposure to 30 μmol l^{-1} nicotine (black bars) fish showed a significantly greater change in place preference for the treatment side compared with control water-treated fish (grey bars; paired *t*-test **P*<0.05). The change in preference exhibited by nicotine-treated fish was significantly greater than the change in preference exhibited by control, water-treated fish 24 h, 7 or 21 days after last drug exposure (two-sample *t*-test, *P*<0.05): control, water-treated fish showed no significant change in preference. The place preference for the treatment side after 21 days of abstinence was significantly less than the preference after 24 h of abstinence (two-sample *t*-test, ***P*<0.05). (B) 4 weeks of daily 20 min exposure to 175 mmol l^{-1} ethanol (black bars) induced a significant change in preference compared with control, water treatment (grey bars; **P*<0.05, two-sample *t*-test). This preference persisted over 3 weeks of abstinence.

Conditioned place preference persists despite adverse consequences

Drug seeking, despite adverse consequences, is an accepted model of drug dependence in animal studies. Here we used a 3 s removal from the tank each time the fish entered the drug treatment side as an adverse stimulus or punishment for drug seeking. To establish the aversive effect of removal from the tank we determined the number of returns separate control fish made to their initially preferred side of the tank over a 10 min period in the face of either 30 s restriction or 3 s removal from the tank followed by 30 s restriction. 3 s removal from the tank led to a significant reduction (two-sample *t*-test, $P<0.05$) in the number of returns control fish made to the initially preferred side (Fig. 4A). Following 4 weeks of conditioning, 3 s removal from the tank significantly reduced the number of returns made by control, water-treated fish, and ethanol-conditioned fish but did not significantly alter the number of returns made by nicotine-conditioned fish (Fig. 4B,C). There was a significant interaction between drug and treatment (repeat-measures two-way ANOVA; Fig. 4) such that 3 s removal from the tank had a significantly reduced effect on decreasing the number of returns made by nicotine- or ethanol-conditioned fish compared with controls (*post-hoc* paired *t*-test, $P<0.01$; Fig. 4B,C). Furthermore, nicotine- or ethanol-conditioned fish continued to demonstrate increased drug seeking despite punishment up to 21 days following the last drug exposure (Fig. 4D,E). Thus zebrafish show persistent dependency-related behaviour following a 4-week daily exposure to either 30 μmol l^{-1} nicotine or 175 mmol l^{-1} ethanol.

Microarray analysis of brain samples from nicotine- and ethanol-treated fish

Although the nature of the neuro-adaptations underlying addiction-related behaviour is not fully understood, a number of lines of evidence suggest that long-lasting changes in gene expression contribute to changes in behaviour. To test whether zebrafish demonstrating dependency-related behaviour show long-lasting changes in gene expression similar to those seen in mammals we performed microarray analysis of brain samples from ethanol-treated and nicotine-treated fish 21 days after the last drug exposure. We screened the Affymetrix zebrafish microarray that contains probe sets for 16 000 zebrafish ESTs for differences in expression in whole brain samples from ethanol-treated, nicotine-treated and control fish. Sets of genes were identified for which the expression was significantly altered by each of the drug treatments compared with controls: 647 for ethanol-treated and 868 for nicotine-treated fish (ArrayExpress Accession number: E-MEXP-1301). Significant change in expression between control and treated individuals was found for 1362 genes. Of these, 545 had a 1.5-fold or greater change in expression compared with controls. When cluster analysis was performed using these 545 genes the samples clustered according to treatment group (Fig. 5A) indicating that specific reproducible changes in expression occur as a result of the different treatments. Of the 1362 genes that showed significant changes in expression, 153 were common to both nicotine-treated and ethanol-treated brains. In addition, 128 genes that showed a twofold or greater change in expression in treated animals compared with controls were common to both nicotine- and ethanol-treated groups. These shared genes include components of neurotransmitter and signalling pathways implicated in drug dependence in mammalian models (Table 3; see supplementary material Tables S1 and S2 for complete lists).

Changes in gene expression for selected genes were confirmed by Q-RT-PCR of cDNA generated from the original RNA used for

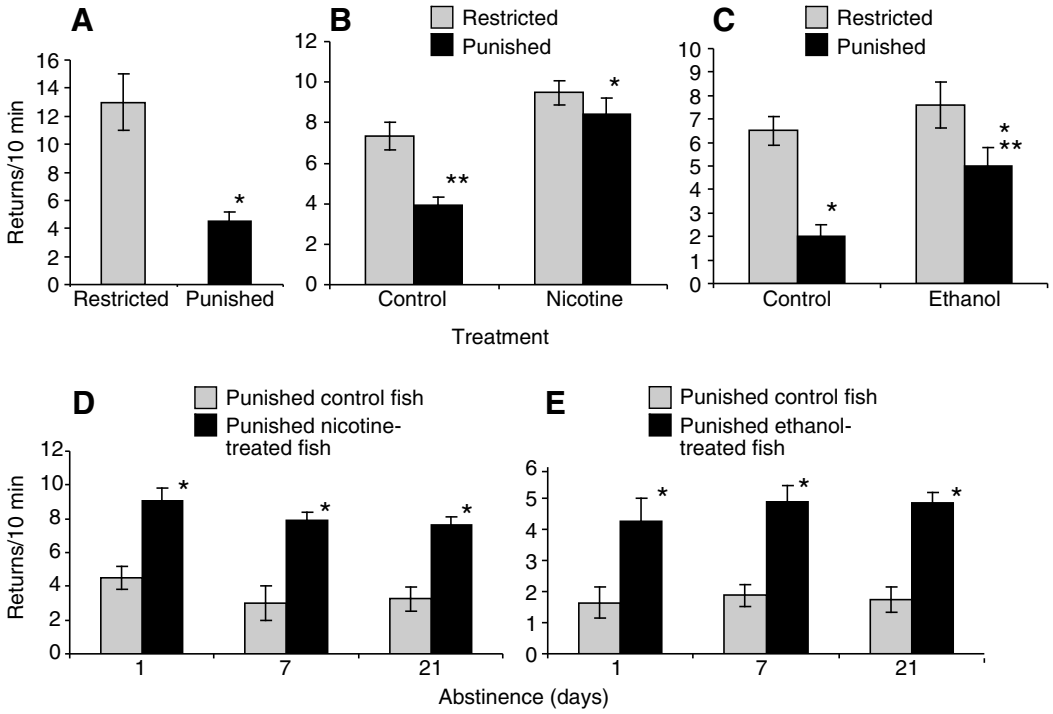

Fig. 4. Conditioned place preference despite an adverse stimulus. Fish were punished by 3 s removal from the tank each time they entered the treatment-paired side: (A) punished *versus* unpunished/restricted control fish; (B,D) nicotine-treated and paired control fish; (C,E) ethanol-treated and paired control fish. (A) Fish that were punished by removal from the tank for 3 s made significantly fewer returns to the treatment side compared with unpunished/restricted fish (two-sample *t*-test **P*<0.01). (B,C), Number of returns made to the drug-paired side in the face of restriction or punishment. Data were subject to two-way repeat-measures ANOVA analysis followed by *post-hoc*, paired or two-sample, *t*-test, as appropriate, followed by Bonferroni adjustment. Following Bonferroni adjustment comparisons were significant at the *P*<0.01 level. (B) Fish that had been conditioned for 4 weeks with 30 μmol l^{-1} nicotine made more returns to the drug-paired side than control fish when either restricted (two-sample *t*-test, *P*=0.03) or punished (two-sample *t*-test, **P*<0.01). 3 s removal from the tank caused a significant reduction in returns made by control fish (paired *t*-test, restricted compared with punished, ***P*<0.01) but not nicotine-treated fish. Repeat-measures two-way ANOVA analysis showed there to be a significant interaction between drug treatment and punishment (punishment plus drug interaction $F_{1,34}$=8.74, *P*=0.006). (C) 3 s removal from the tank caused a significant reduction (paired *t*-test, restricted compared with punished, **P*<0.01) in number of returns made by both control fish and fish that had been conditioned for 4 weeks with 175 mmol l^{-1} ethanol. Fish that had been conditioned for 4 weeks with 175 mmol l^{-1} ethanol made significantly more returns to the drug-paired side when punished (two-sample *t*-test ***P*<0.01) but not restricted. Repeat measures two way ANOVA analysis showed there to be a significant interaction between drug treatment and punishment (punishment plus drug interaction $F_{1,34}$=7.24, *P*=0.011). (D,E) Significantly increased drug seeking despite punishment persisted over 21 days of abstinence (two-sample *t*-test, **P*<0.05 drug-treated compared with control).

the microarray (Fig. 5B). The microarray results for ionotropic glutamate receptor subunit 2a (gria2a), Alport syndrome, mental retardation, midface hyperplasia and elliptocytosis chromosome region 1 (AMMECR1), calcineurin B (CalB) and peripheral benzodiazepine receptor (pBDZR) were validated by Q-RT-PCR.

DISCUSSION

One of the most debilitating characteristics of drug addiction is the persistent tendency to relapse despite even prolonged periods of drug abstinence. This tendency is thought to result, in part, from lasting adaptations that alter brain function and thus behaviour. Here we address the possibility of using zebrafish as a model system for the identification of candidate molecules and pathways that underlie neuro-adaptation to addictive drugs using nicotine and ethanol as examples. We demonstrate that adult zebrafish show a dose-dependent CPP response to ethanol or nicotine. Repeated exposure to either drug leads to robust CPP responses that persist despite extended periods of abstinence and in the face of an adverse stimulus, consistent with the establishment of dependency. Furthermore, our microarray analysis identified changes in gene expression that suggests the conservation of adaptive mechanisms between zebrafish and mammals. These studies support the use of zebrafish as a model system for the identification of molecular mechanisms underlying persistent drug-seeking behaviour.

Acute effects of ethanol treatment on zebrafish development and behaviour in terms of swim behaviour and the startle response have been described (Damodaran et al., 2006; Dlugos and Rabin, 2003; Gerlai et al., 2000; Lockwood et al., 2004). Dlugos and Rabin (Dlugos and Rabin, 2003) and Gerlai et al. (Gerlai et al., 2006) have also demonstrated adaptation of adult zebrafish after chronic exposure to ethanol such that tolerance to the acute effects of the drug develops. By contrast, studies of the effect of nicotine on zebrafish development and behaviour are limited (Levin et al., 2007; Levin and Chen, 2004; Levin et al., 2006; Matta et al., 2007; Svoboda et al., 2002). Levin et al. have shown that 3 min exposure to low doses of nicotine (38–77 μmol l^{-1} nicotine) improves memory function in zebrafish (Levin and Chen, 2004) and that acute exposure to similar concentrations has an anxiolytic effect (Levin et al., 2007). Despite the emerging use of zebrafish for the study of reinforcing effects of drugs of abuse (Bretaud et al., 2007; Darland and Dowling, 2001; Ninkovic and Bally-Cuif, 2006) this is the first report of reinforcing properties of ethanol or nicotine in this species.

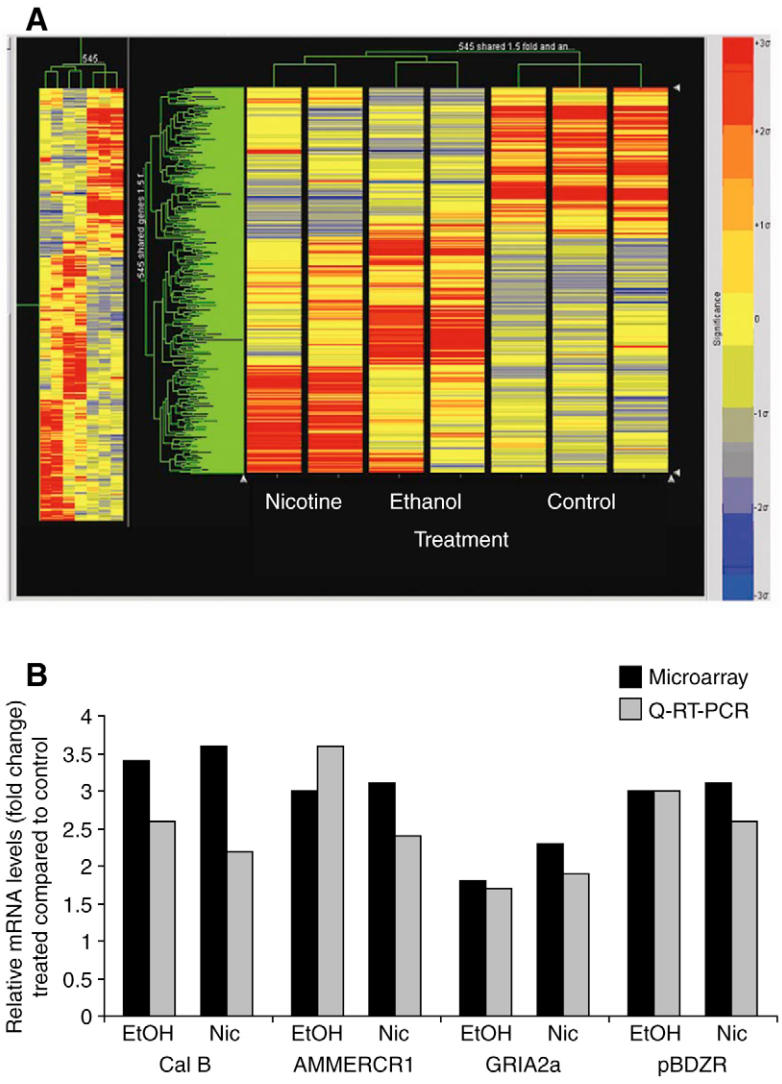

Fig. 5. Microarray analysis. (A) Cluster analysis of genes identified as differentially expressed in brains from control, nicotine- and ethanol-treated fish. All Zebrafish data were imported into GeneSpring 6.1, analytical software for microarray analysis. There was a significant 1.5-fold or greater change in expression of 545 genes between control and treated animals. Using these 545 genes an experiment tree was generated using a Spearman correlation. Subsequently, a gene tree was produced using a Pearson correlation. The resulting tree is shown. Data are coloured based on how far the gene is over- or underexpressed (relative to a normalized expression level of 1), in terms of the standard error of the measurement. The colour bar ranges from +3σ to –3σ. The standard error is based on the standard deviation of the replicate data for a particular gene and condition. Note that the samples cluster according to their experimental treatment either control, ethanol or nicotine treated. (B) Quantitative real-time PCR (Q-RT-PCR) was used to validate the microarray data. Individual genes with different cellular roles (see Table 3) were selected for validation. The four genes selected showed similar expression changes when assessed by Q-RT-PCR as determined by microarray analysis. EtOH, ethanol; Nic, nicotine; Cal B, calcineurin B; AMMECR1, Alport syndrome, mental retardation, midface hyperplasia and elliptocytosis chromosome region; GRIA2a, AMPA glutamate receptor 2a; pBDZR, peripheral benzodiazepine receptor.

In mammals, reinforcing effects are seen at blood concentrations of around 30 mmol l^{-1} for ethanol and 0.05–0.6 μmol l^{-1} for nicotine (Lewis and June, 1990; Matta et al., 2007; Rimondini et al., 2002; Roberts et al., 2000). Here, we obtained reproducible reinforcing effects at tank concentrations of 175 mmol l^{-1} for ethanol and 30 μmol l^{-1} for nicotine. Although we did not determine brain ethanol or nicotine concentrations in our study, previous work of others suggests that these tank concentrations are considerably higher than the brain concentrations that would have been reached. Dlugos and Rabin (Dlugos and Rabin, 2003) have shown that a 15 min exposure of zebrafish to 88 mmol l^{-1} (0.5% v/v) ethanol in the tank water led to a brain ethanol concentration of approximately 20 mmol l^{-1}. Assuming a linear relationship between tank concentration and brain concentration this suggests that the brain alcohol concentration in our experiment may have reached 40 mmol l^{-1}, which is somewhat higher than the brain alcohol level reached in mammals after consumption of alcohol doses that are reinforcing. However, the precise relationship between tank concentration and brain alcohol concentration and the nature of factors that may influence the rate of uptake, such as temperature, age and activity, has yet to be established. Furthermore, several additional factors, including the rate of metabolism and excretion, influence the final brain concentration reached. Alcohol dehydrogenase is the principle enzyme responsible for ethanol metabolism in mammals. Although a zebrafish alcohol dehydrogenase has been identified, the details of its distribution and kinetics have not been established. Thus, although further work is required to establish the pharmacodynamics of ethanol in zebrafish, the available data is consistent with the reinforcing effect of exposure to a tank concentration of 175 mmol l^{-1} ethanol seen here.

We also obtained reproducible reinforcing effects following exposure to 30 μmol l^{-1} nicotine in the tank water. In mammals reinforcing effects of nicotine are observed at a blood nicotine concentration of 0.05–0.6 μmol l^{-1}. Again, although no data on the pharmacodynamics of nicotine in zebrafish has been published, brief (3–5 min) exposure of zebrafish to 40–80 μmol l^{-1} nicotine in the tank water has a similar anxiolytic and memory enhancing effect to that seen in humans with treatments that result in a blood nicotine concentration in the range of 0.1–0.2 μmol l^{-1} (Marchant et al., 2007; Rusted et al., 2005). These results are consistent with the reinforcing effect of exposure to a tank concentration of 30 μmol l^{-1} nicotine seen here. As discussed by Matta et al. (Matta et al., 2007), the rate at which nicotine reaches the central nervous system and the concentration achieved in specific regions of the brain, are determinant factors in eliciting reward and dependence in humans.

Table 3. Shared genes showing significant changes in expression between control and drug treated fish

Function	Accession number	Gene name/symbol	Fold change	Published link to drug dependency	Reference
Neurotransmission	NM_131894	**GRIA2**	1.9 (ethanol) 2.3 (nicotine)	AMPA glutamate receptors implicated in stimulus-induced relapse	1, 2 see 3 for review
	XM_001340391	NMDAR1	3.9 (ethanol) 4.7 (nicotine)	Elevated NMDA R1 protein and mRNA expression associated with cocaine/ ethanol dependence and withdrawal. NMDA R1 mRNA expression associated with opiate withdrawal	3–6
	XM_684668	Hypocretin receptor 2	0.6 (ethanol) 0.7 (nicotine)	Hypocretin receptors implicated in regulation of motivational behaviour, regulation of NMDA and AMPA receptor levels in VTA and stress induced relapse	7,8
Signal transduction	NM_001004553	**Calcineurin B**	3.4 (ethanol) 3.6 (nicotine)	Role in short-term memory and reward induced CPP	9
	NM_131398	Protein phosphatase type 2C beta	1.5 (ethanol) 1.4 (nicotine)		
Steroid metabolism	BC083388	**Peripheral benzodiazepine receptor**	2.8 (ethanol) 3.0 (nicotine)	Regulate steroid metabolism. Indirect regulators of GABA-A receptors. Implicated in mechanism of melatonin reversal of opiate dependence	10,11
	NM_199872	Oxysterol binding protein	2.0 (ethanol) 2.6 (nicotine)		
Cell adhesion/neural plasticity	NM_131830	NCAM2	2.4 (ethanol) 2.3 (nicotine)	Roles in synaptic plasticity. Changes in cell adhesion molecule expression associated with drug dependence	12-14
	NM_212571	Protocadherin 10a	3.2 (ethanol) 3.3 (nicotine)		
	AF506734	Glial fibrillary acidic protein (GFAP)	1.5 (ethanol) 1.7 (nicotine)	Chronic morphine exposure leads to increased GFAP expression. Factors that inhibit morphine dependence and relapse prevent GFAP upregulation	15
Other	NM_201346	Lis 1A (Lissencephaly 1)	2.6 (ethanol) 2.7 (nicotine)		
	BC067667	**AMMECR1** (Alport syndrome, mental retardation, midface hyperplasia and elliptocytosis chromosome region 1)	2.9 (ethanol) 3.0 (nicotine)		
	XM_001333947	Coatomer protein subunit beta 2	0.4 (ethanol) 0.4 (nicotine)	Coatomer complex regulates D1 receptor transport	16

Selected examples of genes that showed a significant change in expression in brain tissue from fish treated with either nicotine (30 μmol l^{-1}, 20 min day^{-1}) or ethanol (175 mmol l^{-1}, 20 min day^{-1}) following 3 weeks of withdrawal. Only genes that showed similar changes in expression in both sample groups are included. Potential link to dependency, where known, are given. Changes in expression of genes highlighted in bold were validated by Q-RT-PCR (Fig. 5B).

References: (1) (Mead and Stephens, 2003), (2) (Sanchis-Segura et al., 2006), (3) (Kalivas, 2004), (4) (Follesa and Ticku, 1995), (5) (Noda and Nabeshima, 2004), (6) (Ahmed et al., 2005), (7) (Boutrel, 2005), (8) (Borgland et al., 2006), (9) (Biala et al., 2005), (10) (Sanna et al., 2004), (11) (Raghavendra and Kulkarni, 1999), (12) (Weber et al., 2006), (13) (Miller et al., 2006), (14) (Abrous et al., 2002), (15) (Alonso et al., 2007), (16) (Bermak et al., 2002).

Factors influencing the pharmacodynamics of nicotine (or ethanol) in individual species include the rate of uptake, efficiency of metabolism, potential physiological effects of metabolites and the rate of excretion. In mammalian species, nicotine is extensively and rapidly metabolised by the liver, with 70–80% of nicotine being converted to cotinine by the action of specific cytochrome P450 enzymes, and approximately 5% being excreted unchanged. Although several zebrafish cytochrome P450 enzymes have been characterized, a zebrafish equivalent of the human CYP2A6 enzyme, the enzyme that is primarily responsible for the metabolism of nicotine to cotinine in humans, has not been identified. Further work is required to establish the rate of uptake, metabolism, and clearance of nicotine or ethanol in zebrafish and how the route of administration may effect final brain concentrations.

As 20 min exposure to high doses (600 μmol l^{-1}; 100 mgl l^{-1}) of nicotine induced signs of toxicity in zebrafish (data not shown) and the rate of metabolism of nicotine in zebrafish is not known, we assessed whether exposure to nicotine on three consecutive days significantly altered the results. Although zebrafish continued to show a dose-dependent CPP response to nicotine exposure with maximal effect seen at 30 μmol l^{-1}, there were important differences in the results obtained. Control-treated fish showed a significant increase in preference for the site of drug (in this case water) exposure after three treatments compared with either their basal

preference or following a single treatment. The increase in preference shown by control fish suggests that there was some habituation to the apparatus over the three test periods. Similar habituation was seen by Ninkovic and Bally-Cuif (Ninkovic and Bally-Cuif, 2006) when using a biased paradigm to study amphetamine induced CPP in zebrafish. In the hands of Ninkovic changes in basal place preference as a result of habituation to the apparatus stabilized after three exposures suggesting that the results of our 3-day treatment may give a more reliable measure of the basal preference of zebrafish to the conditioning apparatus used. Thus consecutive conditioning sessions over 3 days in the absence of any drug was performed prior to determination of basal preference in all subsequent experiments. Importantly three exposures to tank concentrations of either 6 or 30 μmol l^{-1} nicotine induced a significant increase in time spent in the treatment side compared with control water-treated fish, indicating a consistent reinforcement response to these nicotine concentrations. Following three exposures to concentrations of 60 μmol l^{-1} or greater, nicotine-treated fish no longer showed a significant increase in preference for the treatment side compared with controls. Indeed after 3 days of exposure to 300 μmol l^{-1} nicotine, zebrafish showed a significant decrease in preference for the site of drug treatment compared with controls. This result may reflect an inability of the fish to effectively clear such high doses of nicotine from their systems between treatments. On binding nicotine, the receptors responsible for many of the central effects of nicotine, including activation of the 'reward' circuit, are rapidly desensitized. In humans at least 8 h of abstinence (overnight) may be required in order for nicotine levels and associated tolerance to decline sufficiently to be able to detect many of the physiological effects of nicotine. As neither the blood concentration reached during the course of these experiments, nor the clearance rate in zebrafish is known, the lack of reinforcement is consistent with persistent desensitization and tolerance to the effects of acute administration of the drug.

There are a number of criteria (see DSM-IV 1994) that need to be met before CPP is considered a model of dependence rather than reinforcement (O'Brien and Gardner, 2005). These include the persistence of the response despite prolonged abstinence and CPP in the face of adverse consequences. We examined our model against these criteria using 3 weeks as a period of abstinence and 3 s removal from the tank as an adverse consequence. Removal from water has been shown previously to induce stress in fish: cortisol levels are increased when trout are removed from water for 30 s (Demers and Bayne, 1997). We confirmed that 3 s removal from the tank was an adverse stimulus for zebrafish by comparing the number of returns previously un-treated control fish made to a given region of the tank when punished or not (Fig. 4). Both ethanol-treated and nicotine-treated fish showed persistent CPP despite punishment. This CPP despite punishment persisted for 3 weeks after the last drug treatment consistent with it being a dependency-related behaviour.

To determine whether the drug-associated CPP persisted following a period of abstinence, CPP responses were determined after 7 and 21 days of abstinence. As can be seen from Fig. 3B the CPP shown by ethanol-treated fish did not alter significantly over this time. However, nicotine-treated fish showed a significant decrease in CPP after 3 weeks of abstinence compared with 24 h after the last drug treatment. As all fish were tested for their place preference on each occasion, the decline may reflect a tendency towards extinction of the preference by exposure to the conditioning cues in the absence of drug treatment. The basis for the difference in behaviour of ethanol-treated fish was not explored. Nonetheless, zebrafish treated with either ethanol or nicotine for 4 weeks showed the dependence-related behaviour of drug-induced CPP that persists over prolonged periods of abstinence and in the face of an adverse stimulus. Although these behaviours are consistent with the establishment of drug dependency in zebrafish, it is also possible that the establishment of new memories, extinction memory or the reversal of existing memories is impaired in fish pretreated with ethanol or nicotine. Further studies are required to address this possibility. As discussed below, the expression of several genes associated with synaptic plasticity, memory and learning, such as calcineurin identified here, were found to be altered following chronic exposure to alcohol or nicotine.

Homeostatic theories of drug dependence and relapse suggest that long-lasting neuro-adaptations occur that underlie the change in behaviour. We used microarray analysis and Q-RT-PCR to determine whether exposure to nicotine or ethanol that induced dependence-related behaviour in zebrafish induced similar changes in gene expression in this species as in mammalian models of drug dependence. We focused on changes that were seen in common in both treatment groups rather than in individual groups as these changes may reflect conserved adaptations underlying dependency rather than a specific response to the individual drug. We identified 153 genes that showed a significant, 1.5-fold or greater, change in expression in the brains of both nicotine-treated and ethanol-treated fish compared with controls. Several of these shared genes are components of pathways that also show lasting adaptation in the brains of mammalian models of dependence. These include glutamate receptors [AMPA and NMDAR1 (*N*-methyl-D-aspartate receptor 1) (Kalivas, 2004; Noda and Nabeshima, 2004; Sanchis-Segura et al., 2006)] and the peripheral BDZR (Sanna et al., 2004), and molecules associated with synaptic plasticity such as NCAM (Abrous et al., 2002; Miller et al., 2006; Weber et al., 2006). Although neuro-adaptations related directly to dopamine stimulation are thought to be critical for the development of addiction, alterations in glutamatergic neurotransmission are thought to be key to the relapsing nature of drug addiction (Chao et al., 2002; Gao et al., 2006; Kalivas, 2004). In this regard, repeated intermittent exposure to cocaine, amphetamine or ethanol (as used here) has been reported to cause alterations in levels of AMPA and NMDA glutamate receptor subunits in the ventral tegmental area (Churchill et al., 1999; Fitzgerald et al., 1996; Nestler, 2001; Nestler, 2004; Ortiz et al., 1995). However, at least in terms of cocaine-increased gria1 (AMPA GluR1) expression, protein levels do not result from increased mRNA but seem to be due to posttranscriptional mechanisms including trafficking to the cell surface (Beitner-Johnson et al., 1992; Borgland et al., 2006; Gao et al., 2006; Lu et al., 2002; Ungless et al., 2001). We detected an increase in whole brain *gria2* mRNA expression. Such a whole brain change in *gria2* mRNA expression has not been reported for mammalian models of dependency although *gria2* mRNA is increased in the nucleus accumbens both in animal models of dependency (Boudreau and Wolf, 2005; Lu et al., 2003) and in brains of human cocaine users (Crespo et al., 2002). The majority of brain AMPA receptors are either gria1–gria2 (GluR1–GluR2) or gria2–gria3 (GluR2–GluR3) oligomers although other subunit compositions also occur (Wenthold et al., 1996; Wolf et al., 2004). Interestingly, the gria3 subunit, which forms a complex with gria2 in calcium impermeable AMPA receptors, is upregulated in rats during alcohol abstinence. Furthermore targeted *gria3* gene knock out leads to a blunted cue-induced reinstatement response to alcohol implying a role for this subunit/AMPA receptor subtype in alcohol relapse (Sanchis-Segura et al., 2006). *Gria2* loss-of-function mice display multiple behavioural abnormalities (Gerlai et al., 1998; Mead and Stephens, 2003) that have limited the use of this line in

addiction studies. Nonetheless, *Gria2* loss-of-function mice show reduced amphetamine-induced conditioned reinforcement of reward seeking (Mead and Stephens, 2003). Thus our finding that gria2 receptors are altered in their level of expression in the brains of zebrafish showing persistent alcohol (or nicotine)-induced CPP is consistent with the generally accepted model that alterations in glutamate neurotransmission are critical for the expression of addiction related behaviour.

In addition our microarray identified a number of genes that are implicated in drug dependency but have not previously been reported to have altered levels of expression. These include calcineurin B and the hypocretin receptor. The calcineurin B gene was significantly increased (3.5-fold) in the brain of both nicotine- and ethanol-treated fish. Although increased expression of calcineurin in brain tissue from mammalian models of addiction has not been reported previously, the involvement of calcineurin in synaptic plasticity and neurotransmission related to drug dependence has been suggested. A line of memory-deficient mice that overexpress calcineurin fail to demonstrate amphetamine-induced CPP (Biala et al., 2005). Additionally calcineurin regulates the release of dopamine from presynaptic terminals such that high levels of calcineurin activity inhibit dopamine release (Iwata et al., 1997). This suggests that calcineurin levels may be increased in nicotine- or ethanol-treated fish as an adaptive response to repeated dopamine release. As elevated calcineurin also appears to have a negative effect on short-term memory and learning (Genoux et al., 2002; Malleret et al., 2001; Mansuy et al., 1998), the enhanced level of expression of this gene in the brains of the drug-treated fish may have contributed to their continued drug seeking in the face of punishment.

There have been a number of microarray analyses of gene expression changes following either acute or chronic exposure to drugs of abuse (e.g. Boudreau and Wolf, 2005; Hemby, 2006; Li et al., 2004; Rimondini et al., 2002; Toda et al., 2002; Walker et al., 2004). Direct comparison between these is difficult because of variation in the treatment paradigms used and the length of time after drug exposure. Nonetheless microarray analyses consistently report changes in expression of factors associated with altered synaptic plasticity as well as components of the dopaminergic and glutamate neurotransmitter and signal transduction pathways as seen here (supplementary material Tables S1 and S2). Although we found a number of changes in gene expression reminiscent of those seen in mammalian models suggesting conservation of adaptive pathways, a number of novel genes were also identified. The majority of published microarray analyses compare frontal cortex or nucleus accumbens in control and drug-treated animals (Li et al., 2004; Rimondini et al., 2002) (for reviews, see Pollock, 2002; Rhodes and Crabbe, 2005; Sommer et al., 2005; Yuferov et al., 2005). The rationale for this approach is twofold: (1) these are the primary brain regions shown to be involved in mammalian reward responses and (2) the complexity of the mammalian brain may lead to subtle differences being obscured if whole brain tissue were used. We chose not to limit our study in this way. This decision was based, in part, on the premise that the reduced complexity of the zebrafish brain may allow pathways to be identified that are obscured by the complexity of the mammalian brain or that had been excluded by the choice of tissue. Additionally, the small size of the zebrafish brain would have necessitated either the pooling of tissue from a large number of animals, or a pre-amplification step in order to obtain enough cDNA for array analysis.

In summary, we have demonstrated that zebrafish show the dependency-related behaviour of persistent CPP despite an adverse stimulus on repeated exposure to two of the most commonly abused drugs, nicotine and ethanol, and identified conserved changes in gene expression that may contribute to the change in behaviour. These findings add to the body of evidence validating the use of zebrafish as a model system for the study of the genetic basis of reward and addiction.

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