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Assessing the value of the zebrafish conditioned place preference model for predicting human abuse potential

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Running Title Page

Assessing zebrafish CPP for predicting human abuse potential

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List of abbreviations:

CPP – Conditioned Place Preference

FN – False Negative

FP – False Positive

HSE – human subjective effects

NHPs – Non-Human Primates

NPV – Negative Predictive Value

PPV – Positive Predictive Value

SA – Self-Administration

TN – True Negative

TP – True Positive

TU – Tubingen Wildtype

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Abstract

Regulatory agencies recommend that centrally-active drugs are tested for abuse potential prior to approval. Standard preclinical assessments are conducted in rats or non-human primates (NHPs). This study evaluated the ability of the zebrafish conditioned place preference (CPP) model to predict human abuse outcomes. Twenty-seven compounds from a variety of pharmacological classes were tested in zebrafish CPP, categorized as positive or negative, and analyzed using standard diagnostic tests of binary classification to determine the likelihood that zebrafish correctly predict robust positive signals in human subjective effects studies (+HSE) and/or DEA drug scheduling. Results were then compared with those generated for rat self-administration and CPP, as well as NHP self-administration, using this same set of compounds. The findings reveal that zebrafish concordance and sensitivity values were not significantly different from chance for both +HSE and scheduling. Although significant improvements in specificity and negative predictive values were observed for zebrafish relative to +HSE, specificity without sensitivity provides limited predictive value. Moreover, assessments in zebrafish provided no added value for predicting scheduling. By contrast, rat and NHP models generally possessed significantly improved concordance, sensitivity, and positive predictive values for both clinical measures. While there may be predictive value with compounds from specific pharmacological classes (e.g. μ-opioid receptor agonists, CNS stimulants) for zebrafish CPP, altogether these data highlight that using the current methodology, the zebrafish CPP model does not add value to the preclinical assessment of abuse potential.

Introduction

Zebrafish are one of the most widely used model systems in developmental biology. Owing to the numerous benefits of using this vertebrate system, including their small size, inexpensive housing costs, short life span and unparalleled genetic tractability, they are also now becoming widely used in other areas of biological sciences including pharmacological and behavioral screening (Guo, 2004; Stewart et al., 2015). The zebrafish system can provide a relatively high throughput *in vivo* option, bridging the gap between *in vitro* cell based models and *in vivo* rodent models; therefore, zebrafish may contribute to the refinement of animal use in research. Mounting evidence implicates zebrafish as a promising model species for reward and addiction research (Guo, 2004; Ninkovic et al., 2006; Mathur and Guo, 2010; Klee et al., 2012; Stewart et al., 2015). Thus, there may be a potential role for zebrafish abuse models in the drug discovery process, where centrally-active compounds are typically assessed for their abuse potential in animal models prior to regulatory approval.

Two of the main animal models used to assess the rewarding properties of compounds are self-administration and conditioned place preference (CPP) (Panlilio and Goldberg, 2007; Tzschentke, 2007). Although self-administration has greater face and predictive validity to human drug-seeking behavior than CPP, the simplicity of CPP has led to its widespread use in the study of reward processes (Bardo and Bevins, 2000; Tzschentke, 2007). Based on the principles of Pavlovian conditioning, CPP is proposed to reflect the rewarding properties of drugs through examination of their association with contextual stimuli (Tzschentke, 1998; Tzschentke, 2007). CPP protocols are typically conducted in an apparatus with a retractable partition that can divide the apparatus into two compartments, each side with distinct visual and/or tactile cues. CPP usually comprises three phases: 1) the animal is allowed to explore the entire apparatus, and the time spent in each

compartment is used as a measure of baseline preference; 2) the animal is sequentially restricted to each compartment for a period of time in which they are exposed to either drug or control, thus forming a Pavlovian association between the drug and the environment; 3) the animal is once again allowed access to the entire apparatus in the absence of drug, and final place preference for the compartments is measured. If a significant change in preference towards the drug-paired compartment is observed, CPP is considered to have been established, reflecting rewarding properties of the drug.

Previous work has shown that cocaine (Darland and Dowling, 2001), amphetamine (Ninkovic et al., 2006), nicotine, ethanol (Kily et al., 2008; Brennan et al., 2011), and morphine (Lau et al., 2006) induce place preference in zebrafish; however, the number of compounds assessed in this model has been too limited to quantitatively analyze the predictive value of the model for effects in humans. The aim of the present study was to expand the number of compounds tested in zebrafish CPP using a standardized study design in order to determine the ability of the model to predict the outcome of robust positive human subjective effects (+HSE) and/or DEA scheduling status in the USA. We selected scheduling status in addition to +HSE, because a number of factors other than the pharmacology of a drug can influence whether a drug is scheduled (e.g., medicinal uses, overall safety profile, as well as societal and economic factors). Compounds from a variety of pharmacological classes were tested, and the list included drugs that are considered to have positive outcomes in the clinic (including both strong and weak reinforcing drugs), as well as drugs that have negative clinical outcomes (i.e., do not exhibit +HSE or are unscheduled). The confidence of zebrafish CPP to predict positive clinical outcomes was determined using binary classification to categorize the selected drugs as true positives, true negatives, false positives or false negatives in the model. Overall concordance and standard diagnostic tests of binary classifications were used to

objectively and statistically quantify the predictive validity of zebrafish CPP. Similar analyses have been utilized to determine the predictive validity of other preclinical abuse assessments, including self-administration, drug discrimination, and locomotor activity in rats (O'Connor et al., 2011; Horton et al., 2013). As such, outcomes from zebrafish CPP were then compared with those reported in the literature with rat self-administration and CPP models, as well as NHP self-administration, using the same set of compounds.

Methods

Subjects

Wild type zebrafish (*Danio rerio*) were either bred in house or obtained from a commercial supplier (Wades Tropical Import Ltd, UK) at 4 weeks of age (Tubingen wildtype (TU) or Wades Singapore strain, respectively) and raised in the Queen Mary University London Fish Facility according to standard protocols. Based on power analysis of previous studies using nicotine and ethanol, we aimed to use approximately 20 fish for each concentration of each drug (including vehicle) in CPP experiments and 10 fish for each concentration of each drug (including vehicle) for locomotion analysis. All analyses were performed using 3-4-month-old (0.4-0.8g) male and female fish with as close to 50% distribution of sexes across the concentration groups as possible. All behavioral experiments were carried out in a 28°C room with water changes between each test subject to ensure water temperature was as consistent as possible for each fish and was similar to standard housing conditions. During experimental days, fish were fed in the morning (8.00-9.00am) as well the evening but not during an experiment. Fish were tested over an entire day (~8 hours, 10am – 6pm) with an equal number of fish from each concentration tested at the same time to minimize any effect that time of day may have on experimental outcomes. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, under local ethical guidelines from the Queen Mary Animal Care and Use Committee and under UK Home Office project license.

CPP experimental design

CPP was conducted as previously published (Parker et al., 2013; Parker et al., 2014). Fish were singly housed for one week before being habituated to the conditioning tank over 2 consecutive days. The conditioning tank consisted of an opaque tank measuring 20 cm (w) x 15cm (h) x 30cm

(l) containing 2.5L of aquarium water, with distinct visual cues (spots or stripes) on walls at each end of the tank (Supplementary Figure 1). A ceiling mounted camera and Noldus Ethovision XT 9 software (TrackSys, Nottingham, UK) were used to automatically track the behavior of the fish. After two days of habituation sessions, each drug was tested over 5 days, consisting of 1 baseline day, 3 conditioning days, followed by a day of probe trials. During habituation, each individual fish was placed in the conditioning apparatus for 20 min with free access to both compartments and then returned to its home tank. On the day following the final habituation session, baseline assessments were carried out as follows: fish were placed in the conditioning tank for 10 min. Basal preference was determined by recording time spent in either compartment of the apparatus during the second 5 min of this period (% time spent in each side). Any fish with basal preference greater than 75% was excluded from the study. The number of fish excluded from the study by this criterion ranged widely, with 5% to 40% of the habituated fish showing a basal preference greater than 75%.

Each fish with basal preference less than 75% was then conditioned to the test drug (or its vehicle) over three consecutive days. Specifically, fish were placed in the conditioning tank containing 2.5 litres of water and were restricted first to their preferred side for 20 min in the absence of drug (i.e., conditioned to vehicle) and then to their least preferred side for 20 min in the presence of test drug (or its vehicle in the case of the vehicle controls). The test drug (or vehicle) was added to the tank as a concentrated stock in a volume of 50 ml aquarium water. Each fish was conditioned to the same concentration of drug for 3 consecutive days. A total of 5 concentrations were used for each drug (including vehicle) with ~20 fish at each dose (dependent on how many fish were excluded from baseline). No data were recorded during the conditioning sessions. On the day after the final conditioning day, a 10 min probe trial was conducted, where the fish were allowed free access to

both sides of the tank in the absence of drug to measure the time spent on both sides. Place preference was calculated taking basal preference and preference for the drug-paired side into account, again using only the second 5 min period for data collection. The last 5 minutes were used for analysis because fish often display an initial 'freezing' behavior as a stress response when first added to the test tank, particularly during baseline measurements. Omission of the first 5 minutes allows for a habituation period where the fish are able to acclimate to the tank and ensures that this potential freezing behavior does not contribute towards calculations in change of preference scores. This was repeated in the probe trial to be consistent with the measurements being used for calculations in probe and baseline comparisons.

Locomotor activity

All drugs were tested for their effects on locomotion (distance travelled) independent of the place preference assessment. This was included as a proxy measure to give an indication of whether the 20 min exposure time used for CPP was sufficient to allow uptake of the test drug into the CNS. Each drug was tested at the same five concentrations as in the CPP assessment (using approximately 10 fish per concentration per drug). The order of drug exposure was pseudo-randomized between-subjects. Fish were pre-exposed for 20 min in 1 litre of aquarium water plus test drug in a tank measuring 11cm (w) x 10cm (h) x 20cm (l). Following drug exposure, the fish were netted into locomotor activity assay tanks (22cm (w) x 16 cm (h) x 27cm (l)) containing fresh aquarium water. Locomotor activity was recorded for 20 min using a ceiling mounted camera and Noldus Ethovision XT 9 software (TrackSys, Nottingham, UK). These data were sorted into 2-min time bins to allow temporal as well as spatial analysis.

Drugs and doses

Twenty-seven compounds were analyzed in this study; 26 drugs were tested at four different concentrations plus vehicle, and one drug (ethanol) was tested at three different concentrations plus vehicle. The compounds that were tested included drugs that had been previously used in zebrafish CPP studies, including nicotine, ethanol, cocaine, morphine, caffeine and amphetamine (Darland and Dowling, 2001; Lau et al., 2006; Ninkovic and Bally-Cuif, 2006; Ninkovic et al., 2006; Kily et al., 2008; Brennan et al., 2011; Collier et al., 2014), and 21 previously untested compounds (see Table 1 for drugs tested, supplier, concentration range and vehicle).

Concentration ranges were based on concentrations previously found to be rewarding in zebrafish in other studies or using a concentration that corresponded to doses that were positive in either rodent or NHP CPP or self-administration studies. We employed a maximum final tank concentration [mg/L, administered as μM or mM solution in the tank water] equivalent to 2x the mammalian effective dose [mg/kg]. Compounds with no known rewarding properties were tested at similar ranges (mg/L, administered as a μM or mM solution in the tank water, equivalent to 2x the mg/kg dose used in mammals). All drugs were made up as stock solutions in either water or DMSO (see Table 1) and stored frozen where applicable. All drugs were used at a pH of between 6.95 and 7.5 and were assessed for toxic effects prior to use; starting at the lowest concentration, 3 fish were placed individually in a volume of 200 ml of the drug solution in fish water for an hour. Fish were assessed for signs of toxicity (difficulty swimming, exaggerated breathing, haemorrhaging gills, internal bleeding) during this time and at regular intervals for the following 6 hours and the following morning. If no adverse signs were detected, the concentration was increased and the procedure repeated until the maximal intended dose had been assessed.

Concentrations that induced signs of toxicity were not used further. Drugs were diluted from frozen stocks 10 min prior to use. All drugs were added to the conditioning tanks in a volume of 50 ml aquarium water. When used, the final concentration of DMSO did not exceed 0.1%

CPP data analysis

Concentration-response graphs with 95% confidence interval (CI) estimates are included for all drugs assessed in the zebrafish CPP (see Supplementary Figure 2). We assessed each of the compounds tested under the null hypothesis that they would not induce a change in preference for the drug-paired stimulus following conditioning. A change in preference was calculated as the proportion of time spent in the drug-paired side in the probe trial minus the proportion of time spent in the drug-paired side during the basal preference trial. To assess whether a drug induced a statistically significant change in preference, a two-stage approach was implemented. First, an overall test for evidence of non-monotonicity was performed. If there was no evidence of non-monotonicity (ie, the response was monotonic or, in other words, followed an increasing or decreasing trend with increasing concentration of drug), a sequential trend test was used. If there was evidence of non-monotonicity (ie, the response did not follow an increasing or decreasing trend with increasing concentration of drug), a Dunnett's post hoc test was performed.

In cases where there was no evidence of non-monotonicity and a sequential trend test was conducted, a trend test across all concentrations was first performed. If this test was not statistically significant, the analysis was stopped and no concentrations were declared statistically significant different from vehicle. If this test was statistically significant, the highest concentration was declared statistically significant, and the process was repeated for all but the highest

concentration. This process continued for the remaining lower concentrations, until the point at which the test was no longer statistically significant. In cases where there was evidence of non-monotonicity, a Dunnett's post hoc test was conducted to compare each concentration to vehicle.

Only drugs that induced a statistically significant increase in preference for the drug-paired side at any concentration were considered a positive in zebrafish CPP. If a drug induced a statistically significant decrease in preference for the drug-paired side (ie, the drug might be considered aversive) or CPP was not observed at any concentration, the drug was deemed a negative in zebrafish CPP. CPP data were analyzed in RStudio (version 0.99.489). For all analyses, statistical significance was set at $\alpha = 0.05$.

Locomotor behavior data analysis

Data were fitted to linear mixed effects models, with distance travelled as the dependent variable, and time and concentration as fixed effects (fish ID as a random effect). Time bins where the fish moved less than 100 cm in 2 minutes were checked for tracking failure, and where tracking failed, were removed from analysis. For each drug, any time bins where the fish moved 2 standard deviations from the mean distance moved across all drug concentrations in a drug group including paired controls were regarded as outliers and were excluded from analysis. Further, any fish that 'froze' (failed to travel 100 cm in any 2 minute time bin over the entire assay) were removed (20 out of 1300 fish randomly distributed across all drugs and concentrations).

Binary classification, concordance and diagnostic tests

The ability of zebrafish CPP to predict clinical outcomes with respect to +HSE and scheduling status was assessed and compared to outcomes calculated with self-administration and CPP models in rats, as well as self-administration in NHPs. Classification of positives and negatives in zebrafish CPP are described above. For all other endpoints (+HSE, scheduling status, rat self-administration, rat CPP, and NHP self-administration), classification was conducted as described in (Horton et al., 2013) and Supplemental Table 3. In brief, PubMed was the primary tool for locating peer-reviewed source documents. Google Scholar search engine was used as a follow-up to obtain additional resources, but only peer reviewed data or government documents were used for classifications. Many of the classifications were captured originally in (Horton et al., 2013). A positive in rat/NHP self-administration or rat CPP was defined as a drug maintaining a higher level of responding under a fixed ratio (FR) schedule or inducing place preference for the drug-paired side, respectively, than the drug's vehicle. In cases where there were differences between data published in the literature, a drug was considered positive if any studies revealed a positive result. A drug was considered to induce +HSE if >50% of participants (volunteers with a history of drug use) reported positive 'euphoric', 'drug-liking', or 'high' effects after drug administration using scales such as the Visual Analog Scale of global drug effects, the Addiction Research Center Inventory, the Drug Class Questionnaire, or the Profile of Mood States. If a drug has been classified with a scheduling status of I-V by the Controlled Substances Act, it was considered a positive for scheduling status. Drugs were considered negative in the clinical outcomes if they did not induce +HSE or were unscheduled.

For each preclinical model (zebrafish CPP, rat self-administration, rat CPP, NHP self-administration), drugs were classified relative to +HSE and scheduling status as either a true positive (TP; positive in both the preclinical model and clinical measure), true negative (TN;

negative in both the preclinical model and clinical measure), false positive (FP; positive in the preclinical model but negative in the clinical measure), or false negative (FN; negative in the preclinical model but positive in the clinical measure). The overall concordance of each preclinical model was calculated by dividing the number of compounds for which the model correctly predicted the clinical outcome (total of TPs and TNs) divided by the total number of compounds. Standard diagnostic tests of binary classifications (eg, sensitivity and specificity) and estimation of diagnostic value and level of uncertainty (eg, positive and negative predictive value [PPV and NPV, respectively], value added PPV and NPV, and proportionate reduction in uncertainty [PRU] for positive and negative findings) were calculated as described in (Coulthard, 2007; Horton et al., 2013) (Table 2). Pre-test prevalence, or the overall probability of a drug being abused, was set as described previously in Horton et al., 2013. Briefly, the pre-test prevalence was set at ~0.3, or 30%. This estimate was determined by dividing the number of scheduled drugs (II-V) in the USA in 2013 (total of 217) by the total number of approved drugs in the USA as described in the Orange Book (total of 1468), which gave a ratio of 0.15, or 15%. Given that many of the approved drugs are not centrally active and would therefore inherently lack abuse liability, this ratio was doubled, giving a final pre-test prevalence of 0.3, or 30%, to estimate the likely prevalence in a data set with CNS-active compounds. For concordance, sensitivity, and specificity, pre-test probability was set at 50%, or chance. To determine the confidence or reliability of the statistical estimates used in this study, 95% CIs were calculated for each measure using R statistical software (version 3.3.0) and, in particular, the *PropCIs* and *pairwiseCI* libraries, as well as StatXact (Cytel Studio version 10; Cytel Inc.). In cases where CIs did not overlap with pre-test probabilities (adjusted for prevalence where appropriate), it was considered that the model (eg, zebrafish CPP, rat self-administration rat CPP or NHP self-administration) provided a statistically significant improvement in predictive value.

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Results

Zebrafish CPP analysis: Positives

As seen in previous studies, nicotine, ethanol, amphetamine, cocaine, and morphine induced statistically significant, concentration-dependent increases in preference for the drug-paired side (Figure 1a-e), with the largest magnitude changes occurring at concentrations of at 5 μM (25.6 ± 7.8%), 171 mM (21.5 ± 3.7%), 50 μM (15.0 ± 2.9%), 29.4 μM (26.9 ± 4.3%) and 7.9 μM (19.8 ± 7.8%), respectively (Table 3, Supplemental Table 1).

Of the previously untested compounds, the μ-opioid agonists fentanyl (Figure 2a) and oxycodone (Figure 2b) resulted in statistically significant, concentration-dependent increases in preference for the drug-paired side. The general anaesthetics tetracaine (Figure 2c) and phencyclidine (Figure 2d), as well as the anti-histamine chlorpheniramine (Figure 2e), also significantly increased preference for the drug-paired side. The largest magnitude changes were observed for these drugs at the following concentrations: fentanyl, 0.076 μM (18.0 ± 4.3%); oxycodone, 1.14 μM (25.0± 5.0%); tetracaine, 6.3 μM (17.0 ± 7.0%); phencyclidine, 3.57 μM (24.3 ± 5.7%); chlorpheniramine, 5.1 μM (22.7 ± 5.9%) (Tables 3-4, Figure 22 a-e). All of these drugs were considered positives in the zebrafish model (Table 3, Supplemental Table 1).

Zebrafish CPP analysis: Negatives

The remaining previously untested compounds did not induce a change in place preference in zebrafish at the concentrations evaluated in this study, including the general anesthetics ketamine (Figure 3a) and procaine (Figure 3b), the anti-depressants atomoxetine (Figure 3c), bupropion

(Figure 3d), citalopram (Figure 3e), and fluoxetine (Figure 3f), the anti-histamine diphenhydramine (Figure 3g), the benzodiazepine diazepam (Figure 3h), the cannabinoid 1 (CB1) receptor antagonist rimonabant (Figure 3i), the CB receptor agonists Δ-9 tetrahydrocannabinol (THC) (Figure 3j) and WIN-55,212 (Figure 3k), the potassium channel opener retigabine (ezogabine) (Figure 3l), the metabotropic glutamate receptor 5 (mGluR5) antagonist 2-methyl-6(phenylethynl)pyridine (MPEP) (Figure 3m), and the opioid receptor inverse agonist naloxone (Figure 3n). The barbiturates methohexital and pentobarbital resulted in statistically significant decreases in preference for the drug-paired side, suggesting place aversion (Figure 3o, p). In agreement with previously published data (Collier et al., 2014), caffeine did not induce a statistically significant change in place preference (Figure 3q). All of these drugs were considered negatives in the zebrafish CPP model (Table 3, Supplemental Table 1).

Locomotor activity analysis

Locomotor activity was included as a proxy measure to give an indication of whether the 20 min exposure time used for CPP was sufficient to allow uptake of the test drug into the CNS. All of the compounds tested in this study resulted in a significant concentration-dependent change in distance travelled over the 20 min testing period, with the exception of procaine, phencyclidine and fentanyl (Supplementary Figure 3, Supplementary Table 2). Because phencyclidine and fentanyl resulted in a significant increase in place preference for the drug-paired side (Figure 2, Table 3, Supplemental Table 1), they were assumed to be CNS active.

Predictive value for human subjective effects outcome

Out of the 27 drugs evaluated in zebrafish CPP, HSE data were only available for 20 drugs, so only those 20 were used to determine the predictive value of zebrafish CPP for +HSE (Table 3, Supplemental Table 3). Data for rat CPP, rat self-administration, and NHP self-administration were only available for 19, 18, and 19, respectively, of the 20 drugs with HSE data; therefore, only those drugs with data were used for calculating the predictive value of the models for +HSE using this dataset. Table 4 and Supplemental Table 4 provide a statistical summary of the diagnostic tests of the models to +HSE. The overall concordance of zebrafish CPP relative to +HSE was 65%, compared with 84%, 83%, and 89% for rat CPP, rat self-administration, and NHP self-administration, respectively. Sensitivity values were generally high for +HSE with rat CPP and self-administration (0.93 for both models) and NHP self-administration (1.0); whereas sensitivity for zebrafish CPP was lower (0.53). For both concordance and sensitivity, only the rat and NHP models had values that were significantly greater than chance (or 50%). On the other hand, specificity for zebrafish CPP relative to +HSE was 1.0, while the values were lower for both rat and NHP models (ranging from 0.5 to 0.6). In this instance, only zebrafish had a specificity value that was significantly greater than chance. It should however be noted that the data set for specificity (i.e. clinical negatives) was relatively small in each of these cases (n=5), which limits the ability to draw definitive conclusions. When estimated pre-test prevalence (0.3) was taken into account, rat CPP and NHP self-administration possessed positive predictive values (0.5 and 0.52, respectively) that were significantly greater than the pre-test prevalence. While zebrafish CPP appeared to have the highest positive predictive value (1.0) compared with other models, there was overlap of 95% CIs with the pre-test prevalence value of 0.3; therefore, this did not reach statistical significance. With regard to negative predictive value (i.e., the ability to correctly predict when a drug does not exhibit +HSE), zebrafish and rat CPP, as well as NHP self-administration, models offered negative predictive values that were significantly greater than pre-test prevalence (0.83, 0.95, and 1.0,

respectively). For this data set, although rat self-administration yielded a negative predictive value that was 0.94, which was within the same range as rat CPP and NHP self-administration; however, 95% CIs overlapped with pre-test prevalence values for self-administration, therefore it did not reach statistical significance. Additionally, analysis of PRU values indicated that positive findings in NHP self-administration (PRU+) and negative findings in zebrafish and rat CPP, as well as NHP self-administration (PRU-) significantly reduced the uncertainty of predicting drugs without +HSE. These findings suggest higher confidence for NHP self-administration compared with other preclinical models that a positive result would correctly predict a drug with +HSE; however classification of one drug, caffeine, as a positive in NHP self-administration appears to be what may be differentiating NHP self-administration from rat self-administration. Furthermore, there is lower confidence for zebrafish CPP compared with rat CPP and NHP self-administration that a negative result would result in a drug without +HSE.

Predictive value for scheduling status

Twenty-four of the drugs evaluated in zebrafish CPP in this study have a scheduling designation by the DEA, so only these 24 were used to determine the predictive value (Table 4). Data for rat CPP, rat self-administration, and NHP self-administration were available for 24, 22, and 21, respectively, of the 24 drugs with scheduling status; therefore, only those drugs with scheduling data were used for calculating the predictive value of the rat and NHP models using this dataset. Table 5 and Supplemental Table 4 provide a statistical summary of the diagnostic tests of the models to scheduling status. With regard to scheduling, overall concordance was relatively similar for zebrafish and rat CPP (58% and 52%, respectively) and NHP self-administration (62%), whereas rat self-administration was higher (75%) and was the only model to have a value that was significantly greater than chance (50%). Similar to the data generated for +HSE, sensitivity values

were generally high for scheduling status with rat CPP, rat self-administration, and NHP self-administration (0.82, 1.0, and 1.0, respectively), while sensitivity for zebrafish CPP was lower (0.5). With respect to scheduling status, specificity values were lower than the values generated for +HSE for all 4 models, ranging from 0.2 (rat CPP and NHP self-administration) to 0.44 (rat self-administration) to 0.67 (zebrafish CPP). Taking prevalence into account, rat self-administration provided the highest predictive values for both positive (0.44) and negative (1.0) findings, demonstrating increased absolute added value over pre-test prevalence (0.3). This contrasts with results obtained for the other models, where adjusted predictive values were at or near pre-test prevalence values. As a result, these findings reveal no or limited absolute added value for either positive or negative findings with regard to scheduling status for zebrafish and rat CPP, as well as NHP self-administration, for this dataset. Similar to rat self-administration, NHP self-administration yielded a high adjusted negative predictive value (1.0); however 95% CIs overlapped with pre-test prevalence. Examination of PRU+ and PRU– values shows that with respect to scheduling status, positive and negative results in the rat self-administration model significantly reduce the uncertainty of predicting scheduling by the highest proportions, at 0.19 and 1.0 for PRU+ and PRU–, respectively. Zebrafish CPP models offered lower reductions in uncertainty for positive and negative findings (proportions of 0.13 and 0.19, respectively) that were not statistically significant, and rat CPP models yielded no reductions in uncertainty (proportions of 0.01 and 0.07 for PRU+ and PRU–, respectively). NHP self-administration did not yield any statistically significant reductions in uncertainly for positive (0.07) or negative (1.0) findings.

Discussion

In this work, 27 compounds were evaluated in zebrafish CPP to determine how the model predicts human abuse outcomes. At the concentrations tested, 10 induced CPP and were therefore considered positives, and 17 did not induce CPP and thus were deemed negatives. With the exception of procaine, each of the negatives affected locomotion suggesting brain penetration. As procaine had no effect on locomotion or CPP, one possibility is that its brain penetration may have been impaired. Results from zebrafish CPP were then used to objectively and quantitatively analyze the model's predictive value for +HSE or scheduling. These results were compared with outcomes generated with rat self-administration and CPP, as well as NHP self-administration. The findings show that zebrafish CPP specificity, but not sensitivity, is significantly greater than pre-test probability with respect to HSE; therefore, there is potential value in predicting negative clinical outcomes (drugs without +HSE). However, there is limited value for zebrafish to correctly predict drugs with +HSE. Because zebrafish values were not greater than pre-test prevalence for scheduling status, the data indicate no added value for predicting scheduling. With few exceptions, rat and NHP models generally possessed concordance, sensitivity, and predictive values that were significantly greater than pre-test probabilities for both clinical measures. Overall, while there may be predictive value for zebrafish for some pharmacological classes, such as μ-opioid receptor agonists and psychostimulants, these data highlight that rat and NHP models possess greater predictive value than zebrafish for this limited set of compounds.

This is the first study to demonstrate that fentanyl, oxycodone, tetracaine, phencyclidine, and chlorpheniramine are rewarding in zebrafish CPP. Given that all μ-opioid receptor agonists induced CPP in zebrafish, the data suggests conserved opioid reward pathways with mammals. In contrast, only 2 of the 4 anesthetics tested induced CPP, with no consistency in the mechanism of action and

the observed CPP result. Although both ketamine and PCP act as N-methyl-D-aspartate receptor antagonists, PCP, but not ketamine, induced CPP. Tetracaine and procaine are local anesthetics that inhibit monoamine uptake transporters, but only tetracaine induced CPP. Similarly, both diphenhydramine and chlorpheniramine are histamine receptor H1 antagonists; however, CPP was only produced with chlorpheniramine. The basis for these differences has yet to be determined; however it is possible that the concentrations tested, species-specific drug sensitivities or target expression play a role.

Data from zebrafish CPP were analyzed using standard diagnostic tests of binary classification to determine the model's predictive value relative to +HSE and scheduling. Other abuse-related preclinical models, such as *in-vitro* binding and functional activity, locomotor activity, drug discrimination, and self-administration have been evaluated similarly (Horton et al., 2013). To compare the ability of zebrafish CPP to predict clinical abuse outcomes with other preclinical models, binary classifications of rat and NHP self-administration and rat CPP were generated and analyzed using published literature sources. Because not all drugs assessed in zebrafish have reported HSE data or are approved for medicinal use (and consequently do not have scheduling data), the sample sizes for our analyses were generally small (~18-24 drugs, depending on the model). Therefore, one limitation is that the estimates of variance are relatively high for some comparisons, and data should be interpreted with this in mind.

For both +HSE and scheduling, 6 drugs were considered true positives in zebrafish: phencyclidine, fentanyl, morphine, oxycodone, amphetamine, and cocaine. Four drugs (ethanol, nicotine, tetracaine, and chlorpheniramine) were considered true positives with regard to +HSE, but were deemed false positives with regard to scheduling (Tables 6-7). Classification of ethanol and

nicotine as false positives with respect to scheduling is because both are unscheduled drugs due to their historically-accepted societal use. Neither tetracaine nor chlorpheniramine are scheduled, which may reflect their medicinal use (e.g., tetracaine is typically used as ophthalmic drops) or lack of real-world human abuse. True positives included psychostimulants, μ-opioid receptor agonists, general anesthetics, and anti-histamines. CPP was not induced by drugs from the barbiturate or benzodiazepine classes at the concentrations used here (Tables 6-7). Given that varying results have been reported with the barbiturates methohexital and pentobarbital in other abuse-related preclinical models depending on the route of administration and doses evaluated (Pickens et al., 1981; O'Connor et al., 2011); (Mucha and Iversen, 1984; Lew and Parker, 1998; O'Connor et al., 2011), a negative result in zebrafish model may reflect an inappropriate concentration range or may be due to differences in the procedures used (CPP versus self-administration).

The lack of false positives with regard to +HSE revealed that zebrafish CPP yielded the highest adjusted positive predictive value compared with rats and NHPs; however, because CIs for zebrafish overlapped with pre-test prevalence, the data did not reach statistical significance. Inclusion of more compounds for this analysis could potentially alter the results to suggest significant added value. Although zebrafish CPP possessed significant value added for correctly predicting when a drug would not exhibit +HSE (i.e., adjusted negative predictive value), rat and NHP models were generally higher. Importantly, without confidence in the ability to correctly predict +HSE (i.e., positive clinical outcome), there is minimal value added overall for zebrafish CPP. Additionally, given that none of the diagnostic tests for correctly predicting scheduling with zebrafish CPP were significantly greater than chance or pre-test prevalence, it can be concluded that zebrafish do not provide added value for predicting scheduling.

A few of the drugs that were false negatives with regard to +HSE and scheduling had limited solubility, requiring DMSO or ethanol as a solvent. Thus, poor solubility may have prevented efficient uptake into the fish. However, methohexital induced significant conditioned place aversion, and all of the false negatives induced effects on locomotion. Diazepam also reportedly induces anxiolytic effects in zebrafish using similar concentrations (Bencan et al., 2009; Maximino et al., 2010; Maximino et al., 2011). Thus, failure of these drugs to induce CPP does not appear to be due to inefficient uptake. DMSO could also influence visual acuity (Hull et al., 1969; I., 1972), anxiety status (Hallare et al., 2004; Hallare et al., 2005) or locomotion (Chen et al., 2011). Indeed, it is notable that 'control' group data with drugs formulated in DMSO were more variable than when water was used.

The finding that rat self-administration offers limited added value over pre-test prevalence for HSE is consistent with previous reports using an even larger dataset (56 drugs) (Horton et al., 2013). Interestingly, NHP self-administration does offer significantly greater predictive value over pre-test for +HSE; however this appears to be driven by one drug, caffeine, which was deemed as a positive here due to the criteria used but variable results are reported in the literature. Subjective effects are generally assessed preclinically using drug discrimination procedures and are not always linked to the reinforcing effects of a drug (Ator, 2002; Martelle and Nader, 2009). Thus, it is no surprise that significant predictive value is added for rat drug discrimination when comparing to +HSE (Horton et al., 2013). Furthermore, given that both rat and zebrafish CPP yielded limited value with regard to scheduling, the data suggest a limitation of the model itself, and not the species. CPP and self-administration measure fundamentally different behaviors. It is possible that these differences are important in terms of correctly predicting scheduling or +HSE.

Some significant drawbacks exist for potentially using zebrafish CPP as performed here in the pharmaceutical industry. First, the attrition rate can be high, given that 5-40% of zebrafish fail basal preference requirements and some fish tend to 'freeze'. Second, immersing zebrafish in the tank water containing drug during conditioning can be cost prohibitive. The average drug requirement was 7 grams (ranging from 0.04-68 grams) for the concentrations tested here. Alternatives to immersing zebrafish in the tank water may exist, such as pre-treating zebrafish in a smaller volume containing drug prior to immersing in the conditioning tank or direct injections into zebrafish (Ninkovic et al., 2006; Cadet, 2009). For the latter, both ethical and practical limitations exist, including repeat injections which can cause local trauma at the injection site. Third, water insoluble compounds that require solvents may prove problematic. Administering compounds to zebrafish in food pellets (Zang et al., 2011) has emerged as an option, raising the possibility that use of food pellets may be more cost effective and avoid solubility issues.

The intention of this work was not to propose replacing mammalian abuse potential assessments in drug development prior to approval. Rather, the goal was to determine whether there is potential value of zebrafish CPP earlier in drug discovery, when screening compounds might allow for redirection of resources away from targets or chemical series with high risk. Also, the compounds selected for this assessment were all-inclusive; therefore it is possible that additional data may alter the interpretation of zebrafish CPP predictive value. Even so, by evaluating drugs from a variety of pharmacological classes that are both positive and negative clinically for abuse, this work expands our understanding of the translation of zebrafish CPP and offers some insight about how it might be applied in drug discovery, particularly with drugs from certain classes.

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Authorship Contributions

Participated in research design: Goody, Mead, Parker, Brennan

Conducted experiments: Brock, Sudwarts

Contributed new reagents or analytic tools:

Performed data analysis: Goody, Parker, Sudwarts

Wrote or contributed to the writing of the manuscript: Brock, Goody, Mead, Parker, Brennan

Footnotes

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Footnotes

Susan M. G. Goody is currently employed by Pfizer Drug Safety Research and Development, and Andrew N. Mead was employed by Pfizer Drug Safety Research and Development during the inception of this work and the generation of data. Andrew Mead is currently an employee of AstraZeneca. No information is presented that advocates for or promotes commercial products from any of our organizations. There are no other conflicts of interest.

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Legends for Figures

Figure 1. Confirmation of change in preference (±SEM) following conditioned place preference training in adult zebrafish in drugs previously assessed in this species are, included to confirm reliability of the procedure. There were statistically significant concentration-dependent changes observed for nicotine (a) and ethanol (b); the stimulants amphetamine (c) and cocaine (d); and the opioid agonist morphine (e). *Note:* *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2. Change in preference (±SEM) following conditioned place preference training in adult zebrafish for opioid agonists fentanyl (a) and oxycodone (b); the general anaesthetics tetracaine (c) and phencyclidine (d); and the anti-histamine chlorpheniramine (e). *Note:* *P < 0.05; **P < 0.01.

Figure 3. Change in preference (±SEM) following conditioned place preference training in adult zebrafish for the general anesthetics ketamine (a) and procaine (b); anti-depressants atomoxetine (c), bupropion (d), citalopram (e), and fluoxetine (f); anti-histamine diphenhydramine (g); benzodiazepine diazepam (h); CB1 receptor antagonist rimonabant (i); CB receptor agonists THC (j) and WIN-55,212 (k); potassium channel opener retigabine (l); mGluR5 antagonist MPEP (m); opioid receptor inverse agonist naloxone (n); barbiturates methohexital (o) and pentobarbital (p); caffeine (q). *Note:* *P < 0.05

Tables

Table 1. List of compounds tested by class along with supplier name, catalogue number, concentration range tested and references used to select the concentration range.

COMPOUND	SUPPLIER	CATALOGUE NUMBER	CONCENTRATIONS (μM (mg/L))	VEHICLE	REFERENCES
Atomoxetine HCl	Sequoia	SRP07328a	2 - 8.6 (0.6 - 2.5)	water	(Wee and Woolverton, 2004; Cantilena et al., 2012)
Bupropion HCl	Sequoia	SRP03446b	5.4 - 45.5 (1.5 - 12.5)	water	(O'Connor et al., 2011; Vermoesen et al., 2011)
Caffeine	Johnson Matthey	A10431	26 - 260 (5 - 50)	water	(Wong et al., 2010; O'Connor et al., 2011; Richendrfer et al., 2012)
Chlorpheniramine Maleate	Sequoia	SRP02462c	1.3 - 10.2 (0.5 - 4)	water	(Beardsley and Balster, 1992; Hasenohrl et al., 2001)
Citalopram HBr	Sequoia	SRP03585c	1.2- 14.8 (0.5 - 6)	water	(Howell et al., 2007; Hiranita et al., 2009; Sackerman et al., 2010)
Cocaine HCl	sigma	c5776	5.9 - 29.4 (2 - 10)	water	(Darland and Dowling, 2001; O'Connor et al., 2011)
d-Amphetamine sulphate	Sigma	A5880	5 - 50 (0.92 - 7.36)	water	(Ninkovic and Bally-Cuif, 2006; Irons et al., 2010; O'Connor et al., 2011)
Diazepam	Sigma	D0899	1.76 - 14 (0.5 - 4)	DMSO	(Zhdanova et al., 2001; O'Connor et al., 2011; Richendrfer et al., 2012)
Diphenhydramine	Sequoia	SRP04365d	17 - 137 (5 - 40)	water	(O'Connor et al., 2011)
Ethanol	VWR		100 - 300 (4.6 - 13.8)	water	(Kily et al., 2008; O'Connor et al., 2011)
Fentanyl Citrate	sigma	F3886	0.008 - 0.303 (0.004 - 0.16)	water	(O'Connor et al., 2011)
Fluoxetine HCl	Sequoia	SRP01950f	7.2 - 28.9 (0.6 - 2.5)	water	(Lyness and Smith, 1992; Howell et al., 2007; Maximino et al., 2011)

Ketamine HCl	Sigma	K2753	18.2 - 72.9 (5 - 20)	water	(O'Connor et al., 2011; Riehl et al., 2011)
Methohexital	Sequoia	SRP02643m	3.8 - 38.1 (1 - 10)	DMSO	(O'Connor et al., 2011)
Morphine Sulphate	Sigma	M8777	0.7 -7.9 (0.5 - 6)	water	(Lau et al., 2006; Bretaud et al., 2007; O'Connor et al., 2011)
MPEP	Sequoia	SRP04265m	5.2 - 31 (1 - 6)	DMSO	(Platt et al., 2008; van der Kam et al., 2009)
Naloxone HCl	Sequoia	SRP00860n	3.13 - 25 (1.25 - 10)	water	(O'Connor et al., 2011)
nicotine hemisuphate	Sigma	N1019	0.5 - 10 (0.2 - 4.2)	water	(Bencan and Levin, 2008; Kily et al., 2008; O'Connor et al., 2011)
Oxycodone	Tocris	3958	0.28 - 1.7 (0.1 - 0.6)	water	(O'Connor et al., 2011)
Pentobarbital sodium	sigma	P3761	20 - 120 (5 - 30)	water	(Zhdanova et al., 2001; Renier et al., 2007; O'Connor et al., 2011)
Phencyclindine HCl	sigma	P3029	0.36 - 3.6 (0.1 - 1)	water	(O'Connor et al., 2011; Kyzar et al., 2012)
Procaine HCl	sigma	P9879	92 -733 (25 - 200)	water	(Woolverton and Balster, 1979; Johanesson and Aigner, 1981)
Retigabine HCl	Sequoia	SRP01080r	5.3 - 40 (2 -15)	DMSO	(Chege et al., 2012)
Rimonibant	Sequoia	SRP01287r	0.68 - 13.5 (0.3 - 6)	DMSO	(Braida et al., 2007; Justinova et al., 2011)
Tetracaine HCl	Sigma	T7508	0.83 - 6.3 (0.25 - 1.9)	water	(Woolverton and Balster, 1979; Wilcox et al., 2000; Wilcox et al., 2005)
9 delta THC	sigma	T2386	0.16 - 0.64 (0.05 - 0.2)	ethanol	(O'Connor et al., 2011)
WIN 55 212 - 2 mesylate	Sequoia	SRP00600w	0.06 - 1.15 (0.03 - 0.6)	DMSO	(Wiley et al., 1995; Lecca et al., 2006)

Table 2. Descriptions of and calculations for diagnostic tests conducted.

Endpoint	Description	Calculation
Concordance	Proportion of compounds where the model accurately predicted clinical measure	(TP + TN)/ Total # of compounds
Sensitivity	Proportion of +HSE or scheduled drugs accurately identified by the model	TP/(TP+FN)
Specificity	Proportion of drugs that do not induce +HSE or are unscheduled accurately identified by the model	TN/(TN+FP)
Positive Predictive Value (PPV)	Proportion of drugs that are TPs relative to all drugs with a positive result	TP/(TP+FP)
Negative Predictive Value (NPV)	Proportion of drugs that are TNs relative to all drugs with a negative result	TN/(TN+FN)
Adjusted PPV	PPV adjusted for a pretest probability (prevalence) of 0.3	(sensitivity x prevalence)/ [sensitivity x prevalence + (1 – specificity) x (1 – prevalence)]
Adjusted NPV	NPV adjusted for a pretest probability (prevalence) of 0.7	specificity x (1 – prevalence)/ [specificity x (1 – prevalence) + (1 –

		sensitivity) x prevalence)]
Value added PPV (VaPPV)	Quantitative measurement of value added for positive results relative to pretest probability	Adjusted PPV – prevalence
Value added NPV (VaNPV)	Quantitative measurement of value added for negative results relative to pretest probability	Adjusted NPV – (1 – prevalence)
Proportionate reduction in uncertainty (PRU) positive (+)	Proportion by which model reduces uncertainty in predicting drugs with +HSE or are scheduled	VaPPV/(1 – prevalence)
Proportionate reduction in uncertainty (PRU) negative (–)	Proportion by which model reduces uncertainty in predicting drugs without +HSE or are unscheduled	VaNPV/prevalence

Table 3. Summary of binary classification for zebrafish and rat to human subjective effects and scheduling status.

Abbreviations: 1 = positive finding; 2 = aversive in CPP; 0 = no effect; X = no data available

Compound	Class	HSE	Scheduling	ZF CPP	Rat CPP	Rat Self-Administration	References
Ketamine	Anesthetic	1	1	0	1	1	(Horton et al., 2013; Guo et al., 2016)
Phencyclidine	Anesthetic	1	1	1	1	1	(Marglin et al., 1989; Horton et al., 2013)
Procaine	Anesthetic	0	0	0	1	1	(Spyraki et al., 1982; Fischman et al., 1983; Kiyatkin and Stein, 1994)
Tetracaine	Anesthetic	X	0	1	X	X	X
Atomoxetine	Anti-depressant	0	0	0	0	0	(dela Pena et al., 2011; Horton et al., 2013)
Bupropion	Anti-depressant	0	0	0	1	1	(Horton et al., 2013; Mori et al., 2013)
Citalopram	Anti-depressant	X	0	0	X	0	(Horton et al., 2013)
Fluoxetine	Anti-depressant	X	0	0	1	X	(Horton et al., 2013; Faillace et al., 2015)
Chlorpheniramine	Anti-histamine	X	0	1	1	X	(Suzuki et al., 1999; Horton et al., 2013)
Diphenhydramine	Anti-histamine	1	0	0	1	1	(Halpert et al., 2003; Horton et al., 2013)
Methohexital	Barbiturate	X	1	2	0	1	(Pain et al., 1996; Horton et al., 2013)
Pentobarbital	Barbiturate	1	1	2	1	1	(Bossert and Franklin, 2003; Horton et al., 2013)
Diazepam	Benzodiazepine	1	1	0	0	1	(Leri and Franklin, 2000; Horton et al., 2013)
Rimonabant	Cannabinoid 1 receptor antagonist	0	X	0	0	X	(Huestis et al., 2007; Li et al., 2008)
Δ9-THC	Cannabinoid receptor agonist	1	1	0	1	1	(Braida et al., 2004; Horton et al., 2013)

WIN-55 212	Cannabinoid receptor agonist	X	X	0	2	1	(Chaperon et al., 1998; Fattore et al., 2001)
Retigabine (Ezogabine)	K^+ channel opener	1	1	0	X	X	(DEA, 2011)
MPEP	mGluR5 receptor antagonist	X	X	0	1	1	(van der Kam et al., 2009)
Fentanyl	μ Opioid receptor agonist	1	1	1	1	1	(Miller and Nation, 1997; Vitale et al., 2003; Horton et al., 2013)
Morphine	μ Opioid receptor agonist	1	1	1	1	1	(Zhang et al., 2012; Horton et al., 2013)
Oxycodone	μ Opioid receptor agonist	1	1	1	1	1	(Rutten et al., 2011; Horton et al., 2013)
Naloxone	Opioid receptor antagonist	0	0	0	0	0	(Bardo and Neisewander, 1986; Braida et al., 2005; Horton et al., 2013)
Amphetamine	Stimulant	1	1	1	1	1	(Spyraki et al., 1982; Horton et al., 2013)
Caffeine	Stimulant	1	0	0	1	0	(Bedingfield et al., 1998; Horton et al., 2013)
Cocaine	Stimulant	1	1	1	1	1	(Bedingfield et al., 1998; Horton et al., 2013)
Ethanol	Stimulant	1	0	1	1	1	(Morales et al., 2012; Horton et al., 2013)
Nicotine	Stimulant	1	0	1	1	1	(Pascual et al., 2009; Horton et al., 2013)

Table 4. Statistical summary of diagnostic tests for the prediction of human subjective effects. Summary for prediction of human subjective effects, demonstrating the number of false positives, true positives, true negatives, false negatives, and outcome ratios for various statistical outputs with regards to diagnostic value. Values in parentheses indicate 95% confidence intervals. Values in bold indicate a significant difference from pretest probability [concordance, PPV, NPV, PPV (30% prev), NHP (30% prev)] or from zero predictive value (VaPPV, VaNPV). PPV and NPV represent observed predictive values. PPV (30%) and NPV (30% prev) represent predictive values corrected for an estimated prevalence of 0.3. Abbreviations: PPV = positive predictive value, NPV = negative predictive value, PRU positive = proportionate reduction in uncertainty for positive findings; PRU negative = proportionate reduction in uncertainty for negative findings.

Model	False positives	True positives	True negatives	False negatives	Concordance	Sensitivity	Specificity	PPV	Adjusted PPV (30% prev)	Value added PPV	NPV	Adjusted NPV (30% prev)	Value added NPV	PRU positive	PRU negative
Zebrafish CPP	0	8	5	7	0.65 (0.43, 0.82)	0.53 (0.30, 0.75)	**1.0 (0.57, 1.0)**	1.0 (0.68, 1.0)	1.0 (0.29, 1.0)	0.7 (-0.01, 0.70)	0.42 (0.19, 0.68)	**0.83 (0.70, 0.92)**	**0.13 (0, 0.22)**	1.0 (-0.01, 1.0)	**0.44 (0, 0.72)**
Rat CPP	2	13	3	1	**0.84 (0.62, 0.94)**	**0.93 (0.69, 0.99)**	0.6 (0.23, 0.88)	0.87 (0.62, 0.96)	**0.5 (0.3, 0.87)**	**0.2 (0, 0.57)**	0.75 (0.3, 0.95)	**0.95 (0.7, 1.0)**	**0.25 (0, 0.3)**	**0.28 (0, 0.82)**	**0.84 (0.01, 0.99)**
Rat self-administra	2	13	2	1	**0.83**	**0.93**	0.5	0.87	0.44	0.14	0.67	0.94	0.24	0.20	0.81

tion	**(0.61, 0.94)**	**(0.69, 0.99)**	(0.15, 0.85)	(0.62, 0.96)	(0.29, 0.84)	(-0.01, 0.54)	(0.21, 0.94)	(0.62, 1.0)	(-0.08, 0.3)	(-0.02, 0.77)	(-0.25, 0.99)

Table 5. Statistical summary of diagnostic tests for the prediction of scheduling status. Summary for prediction of scheduling status, demonstrating the number of false positives, true positives, true negatives, false negatives, and outcome ratios for various statistical outputs with regards to diagnostic value. Values in parentheses indicate 95% confidence intervals. Values in bold indicate a significant difference from pretest probability [concordance, PPV, NPV, PPV (30% prev), NHP (30% prev)] or from zero predictive value (VaPPV, VaNPV). PPV and NPV represent observed predictive values. PPV (30%) and NPV (30% prev) represent predictive values corrected for an estimated prevalence of 0.3. Abbreviations: PPV = positive predictive value; NPV = negative predictive value, PRU positive = proportionate reduction in uncertainty for positive findings; PRU negative = proportionate reduction in uncertainty for negative findings.

Model	False positives	True positives	True negatives	False negatives	Concordance	Sensitivity	Specificity	PPV	Adjusted PPV (30% prev)	Value added PPV	NPV	Adjusted NPV (30% prev)	Value added NPV	PRU positive	PRU negative
Zebrafish CPP	4	6	8	6	0.58 (0.39, 0.76)	0.5 (0.25, 0.75)	0.67 (0.39, 0.86)	0.6 (0.31, 0.83)	0.39 (0.19, 0.7)	0.09 (-0.11, 0.4)	0.57 (0.33, 0.79)	0.76 (0.59, 0.88)	0.06 (-0.11, 0.18)	0.13 (-0.16, 0.57)	0.19 (-0.37, 0.59)
Rat CPP	8	9	2	2	0.52 (0.32, 0.72)	**0.82 (0.52, 0.95)**	0.20 (0.06, 0.51)	0.53 (0.31, 0.74)	0.30 (0.20, 0.44)	0 (-0.10, 0.14)	0.50 (0.15, 0.85)	0.72 (0.15, 0.97)	0.02 (-0.55, 0.27)	0.01 (-0.14, 0.20)	0.07 (-1.83, 0.91)
Rat self-administra	5	11	4	0	**0.75**	**1.0**	0.44	0.69	**0.44**	**0.14**	1.0	**1.0**	**0.3**	**0.19**	**1.0**

tion	**(0.53, 0.89)**	**(0.74, 1.0)**	(0.19, 0.73)	(0.44, 0.86)	**(0.32, 0.67)**	**(0.02, 0.37)**	(0.51, 1.0)	**(0.77, 1.0)**	**(0.07, 0.3)**	**(0.03, 0.53)**	**(0.22, 1.0)**

Table 6. Summary of false positives and false negatives for each model with respect to positive human subjective effects.

Zebrafish CPP		Rat CPP		Rat Self-Administration		NHP Self-Administration	
False positive	**False negative**	**False positive**	**False negative**	**False positive**	**False negative**	**False positive**	**False negative**
	Ketamine Diphenhydramine Pentobarbital Diazepam Δ9-THC Retigabine Caffeine	Procaine Bupropion	Diazepam	Procaine Bupropion	Caffeine	Procaine Bupropion	

Table 7. Summary of false positives and false negatives for each model with respect to scheduling status

Zebrafish CPP		Rat CPP		Rat Self-Administration		NHP Self-Administration	
False positive	**False negative**	**False positive**	**False negative**	**False positive**	**False negative**	**False Positive**	**False negative**
Tetracaine Chlorpheniramine Ethanol Nicotine	Ketamine Methohexital Pentobarbital Diazepam Δ9-THC Retigabine	Procaine Bupropion Fluoxetine Chlorpheniramine Diphenhydramine Caffeine Ethanol Nicotine	Methohexital Diazepam	Procaine Bupropion Diphenhydramine Ethanol Nicotine		Procaine Tetracaine Bupropion Chlorpheniramine Diphenhydramine Caffeine Ethanol Nicotine	

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Figures

Figure 1

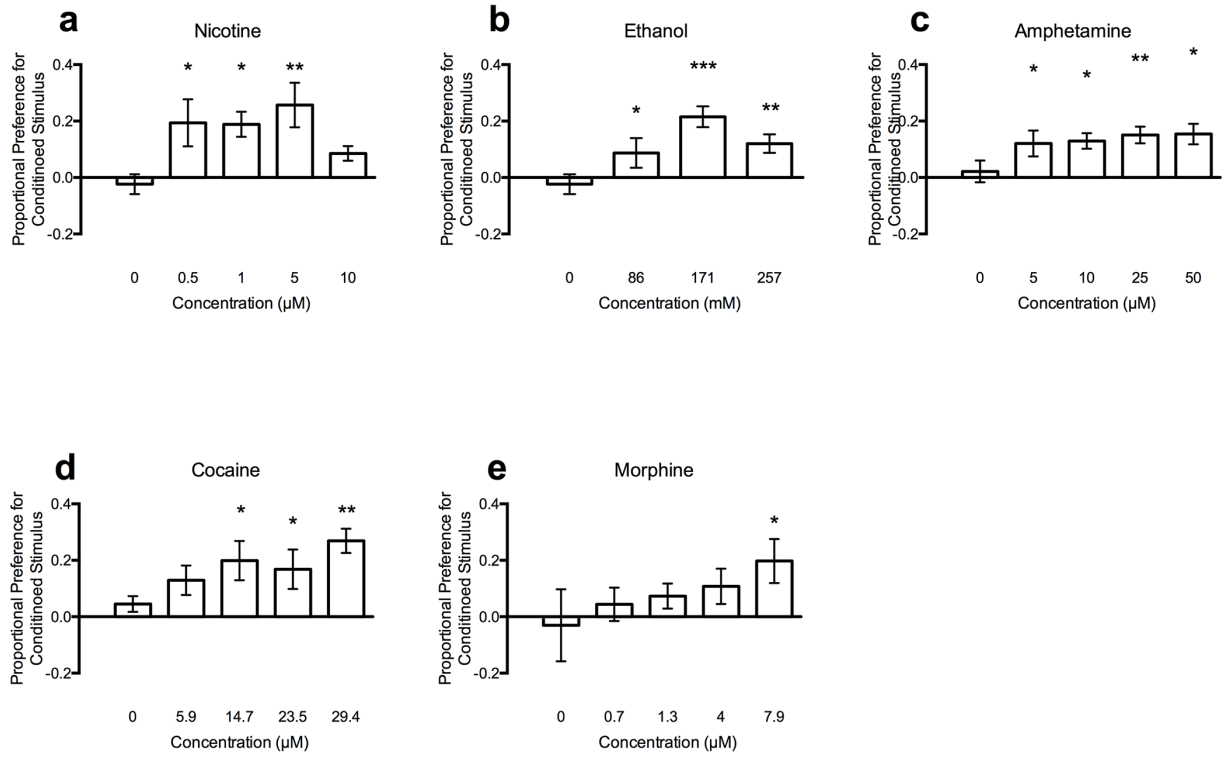

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Figure 2

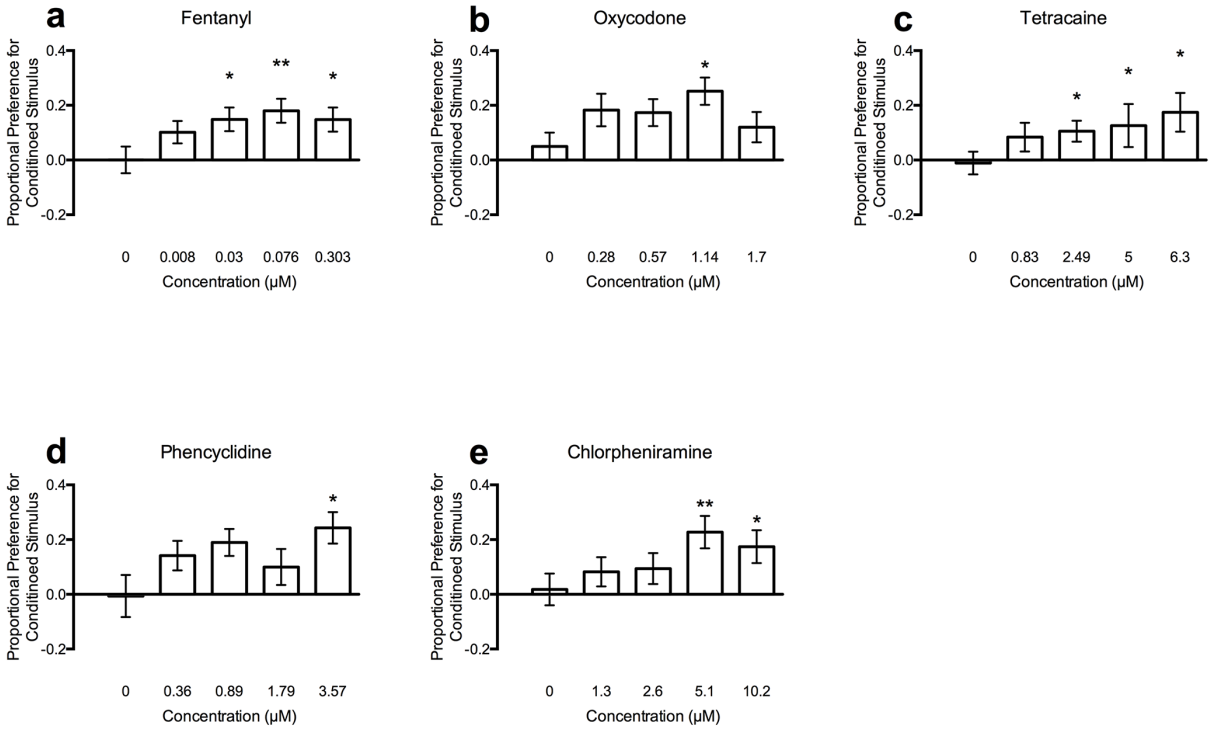

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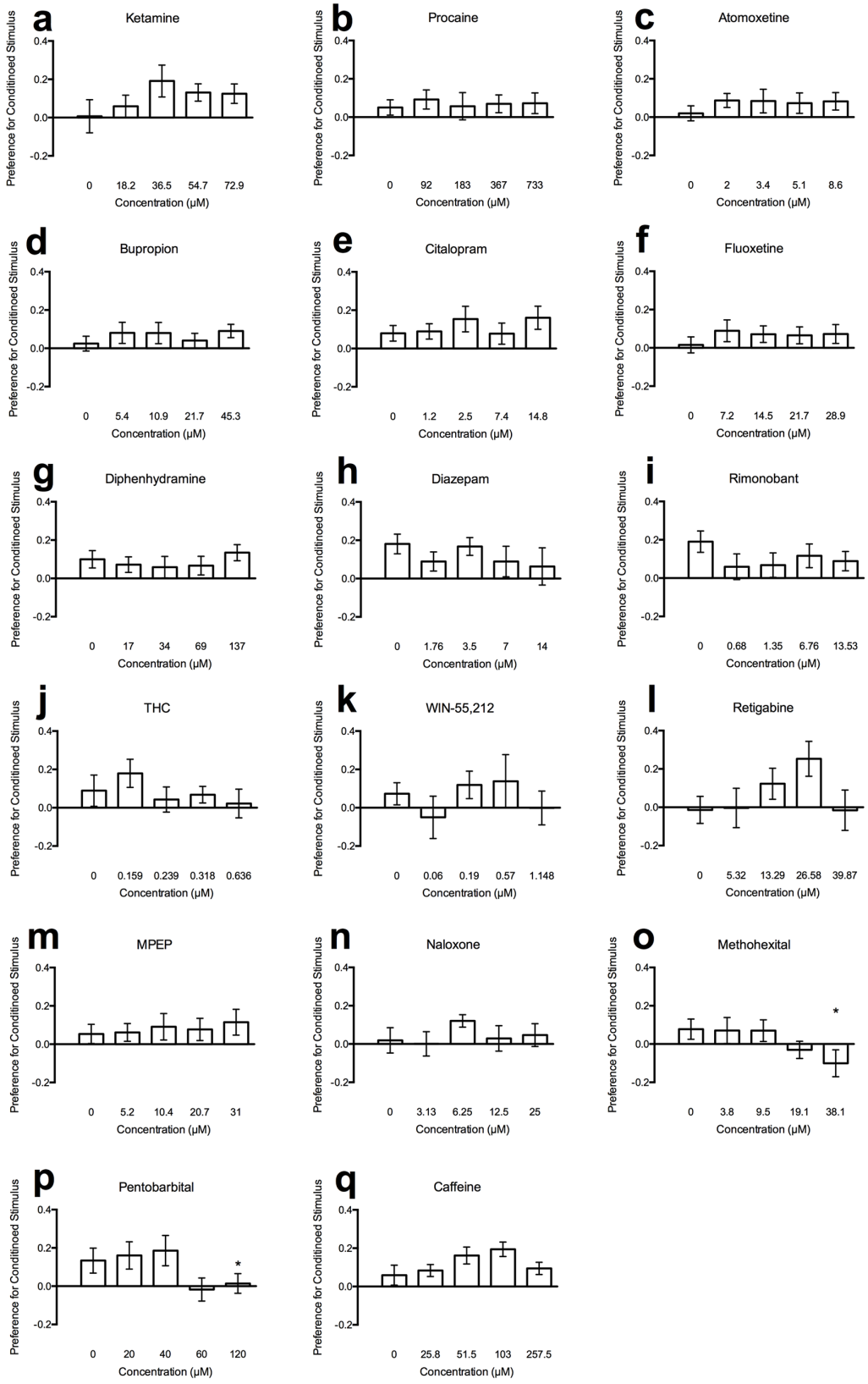
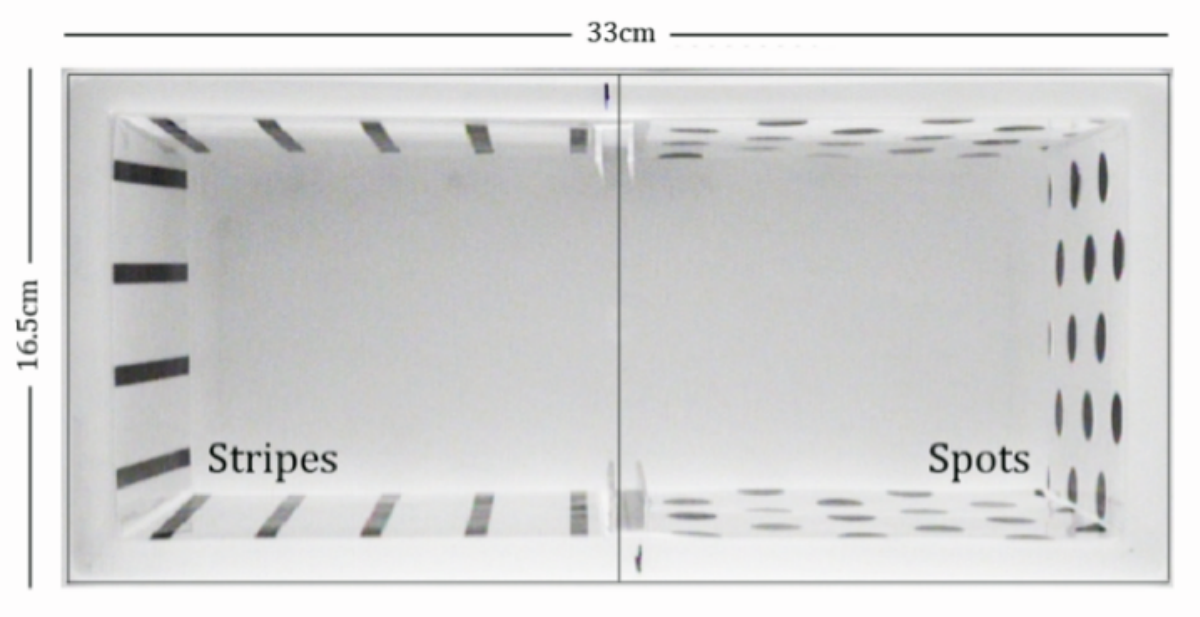
Figure 3

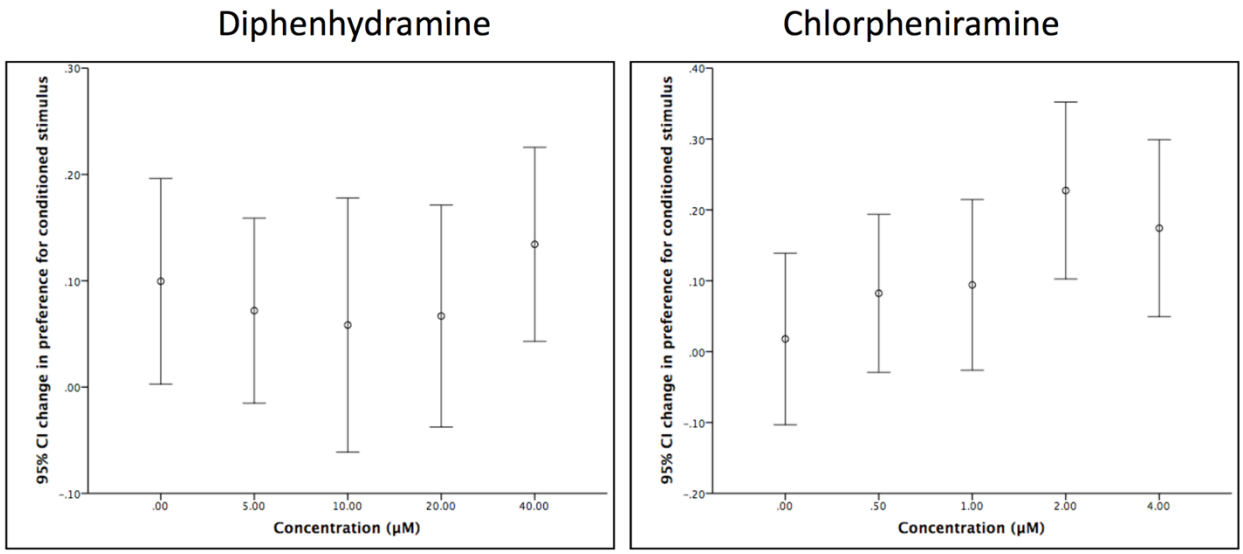


Supplementary Figures and Tables

Supplementary Figure 1. CPP was carried out in an opaque rectangular tank (20 cm (W) x 15cm (H) x 30cm (L)) containing 2.5L of aquarium water. All testing was carried out on a single fish at a time. Baseline preference was assessed by allowing the fish free access to both sides of the tank. During conditioning, the fish was incubated in its least preferred side in drug solution, and in its preferred side with no drug. Conditioning was assessed following training by repeating the baseline preference assessment.



Supplementary Figure 2. Mean change in preference according to dose of all drugs tested ±95% confidence intervals according to drug class.



Assessing the value of the zebrafish conditioned place preference model for predicting human abuse potential

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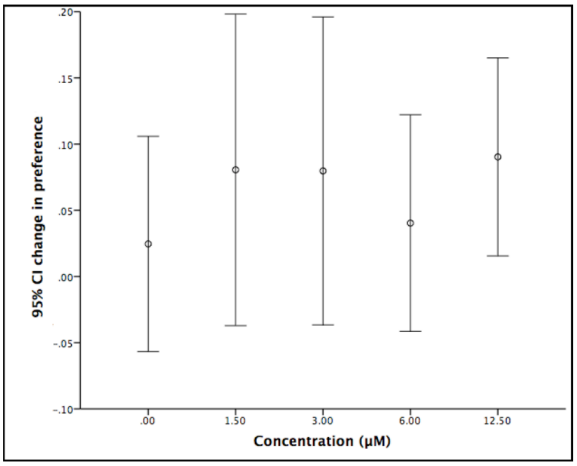
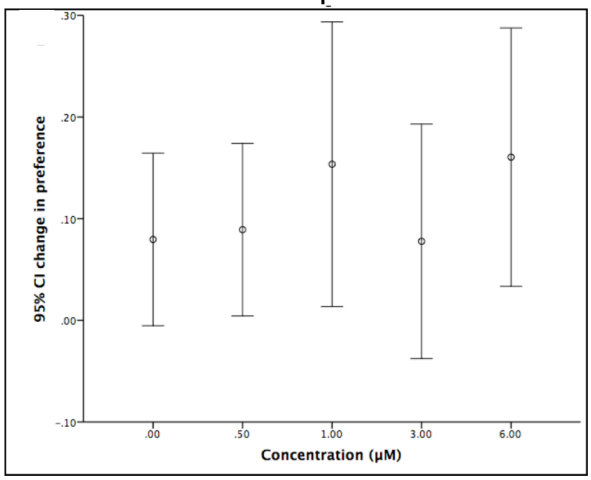
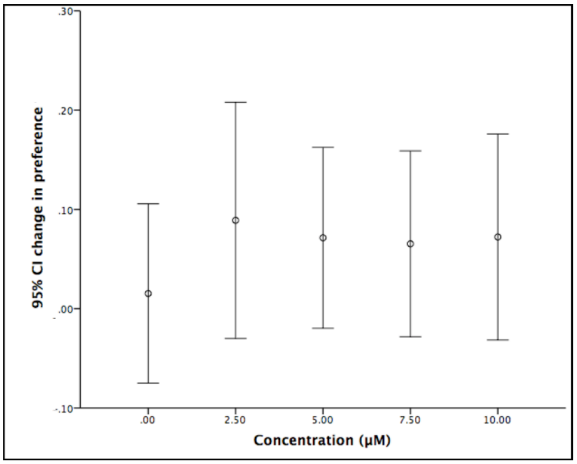
Bupropion



Citalopram



Fluoxetine



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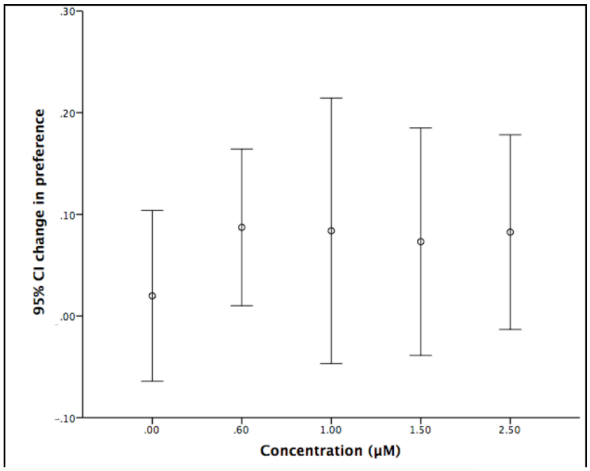

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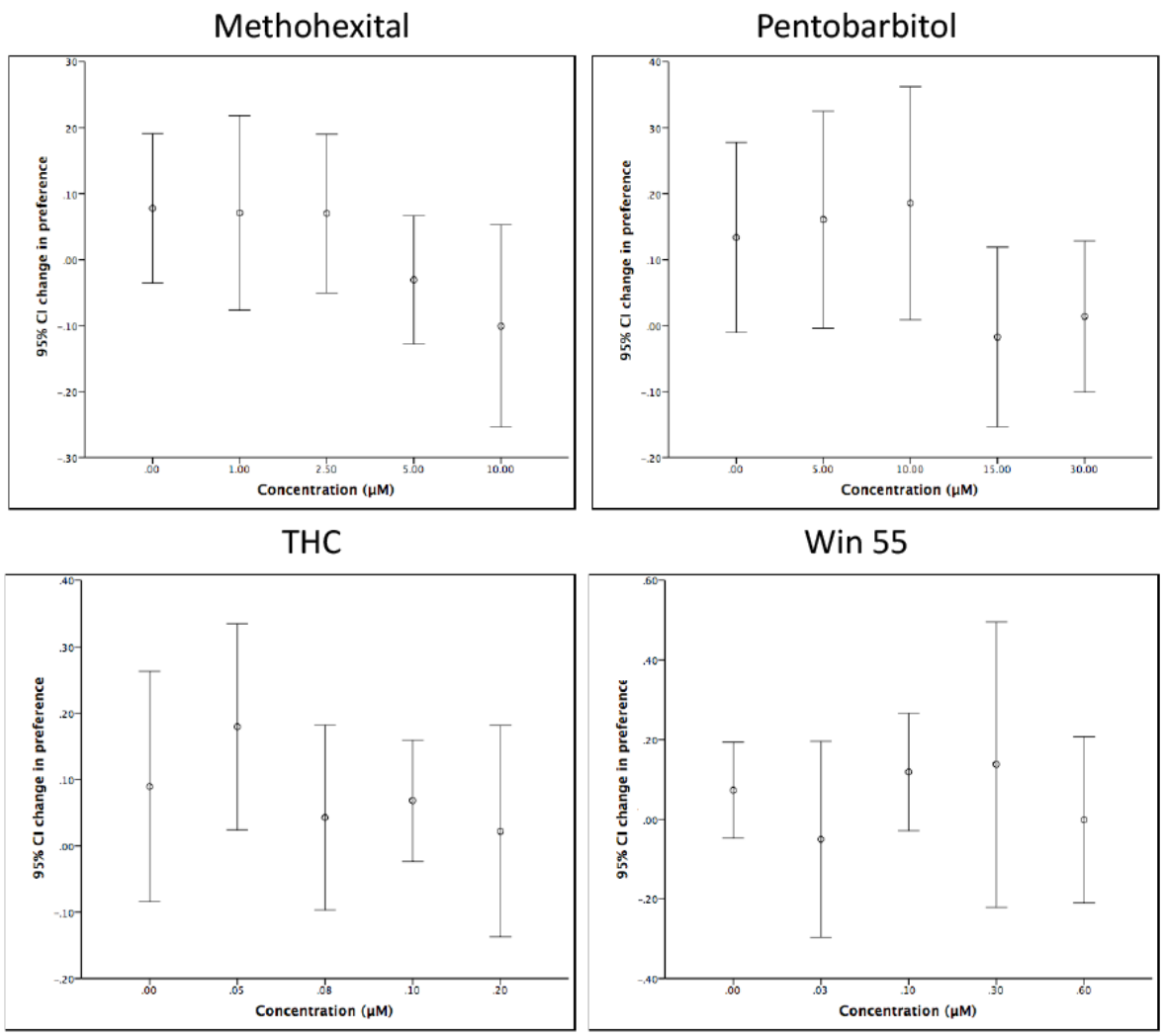

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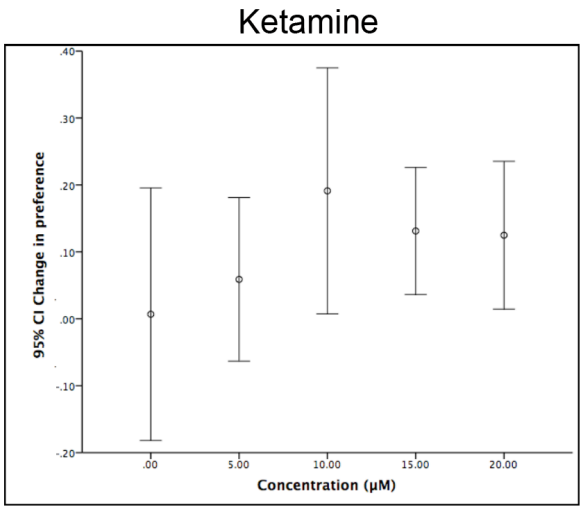

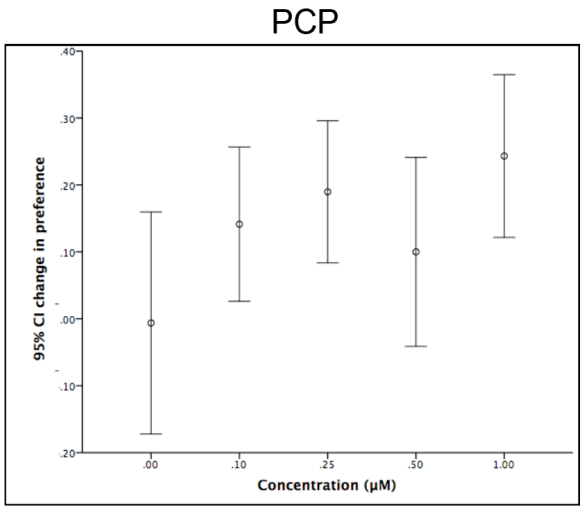

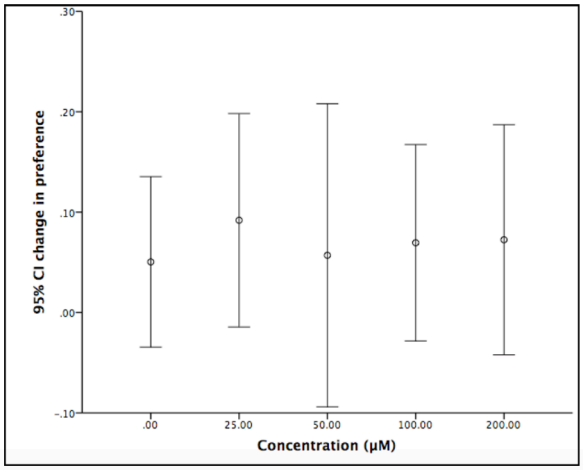

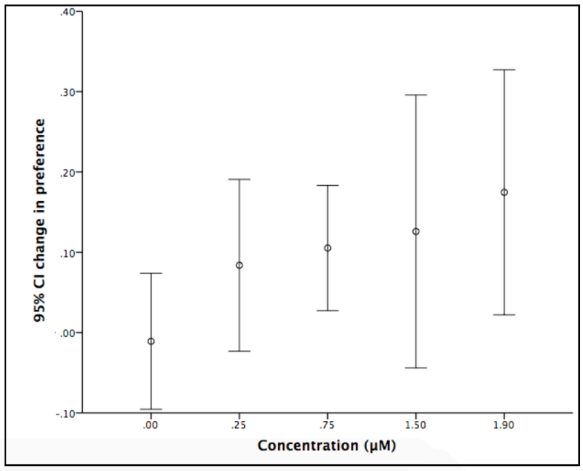

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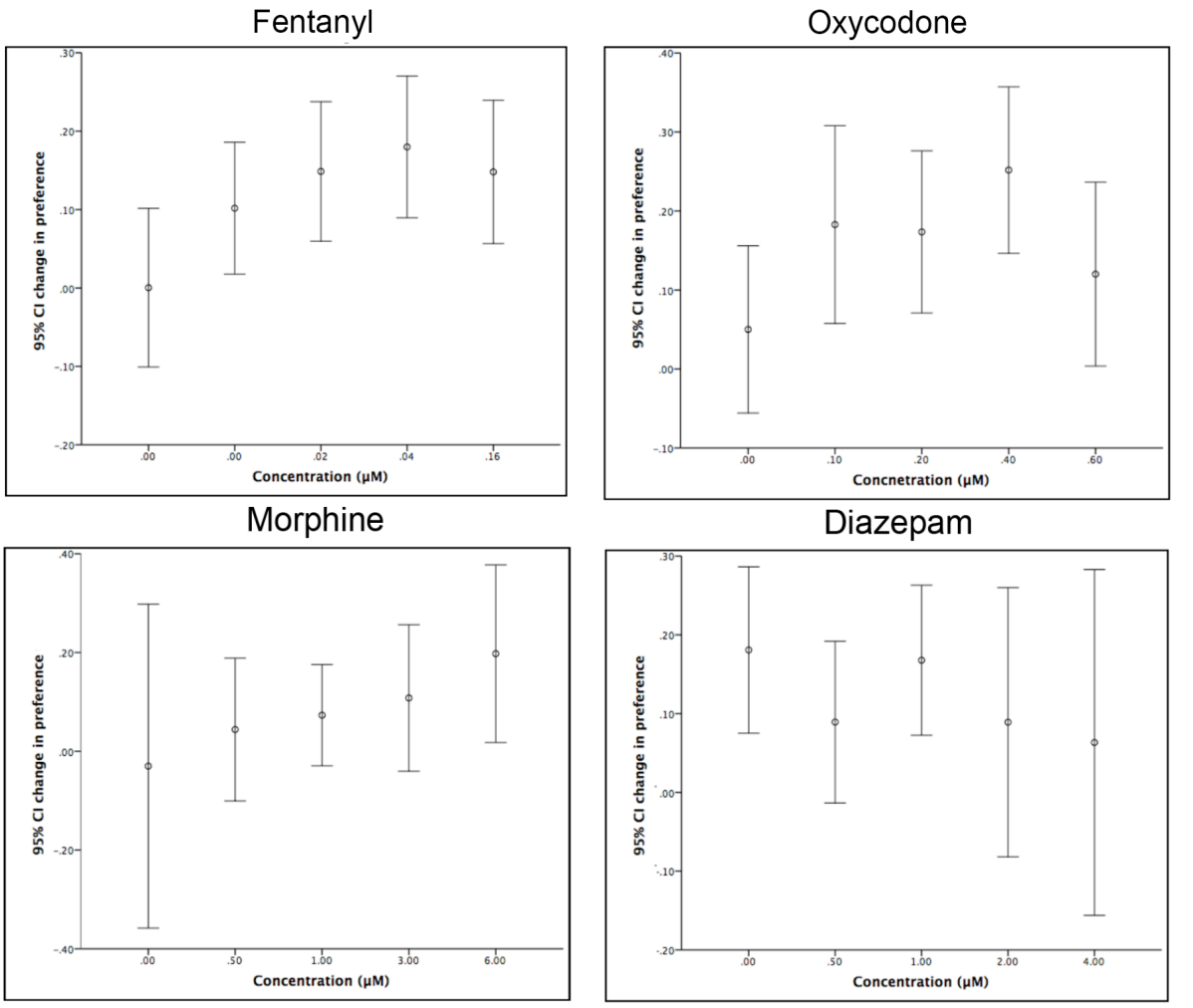

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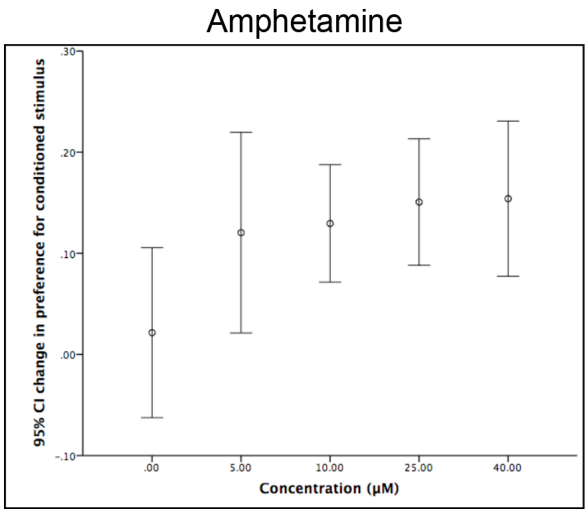

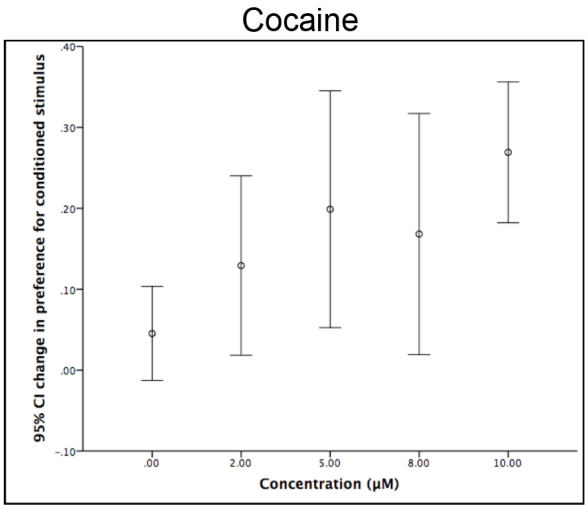

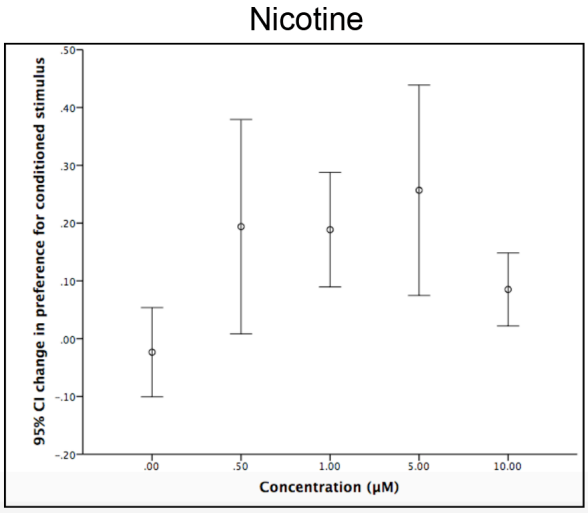

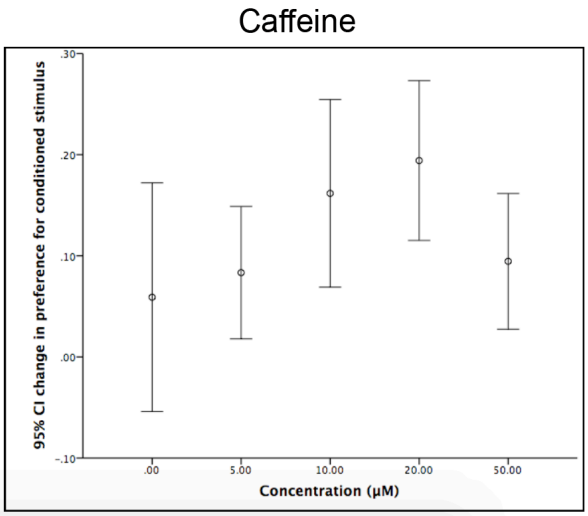

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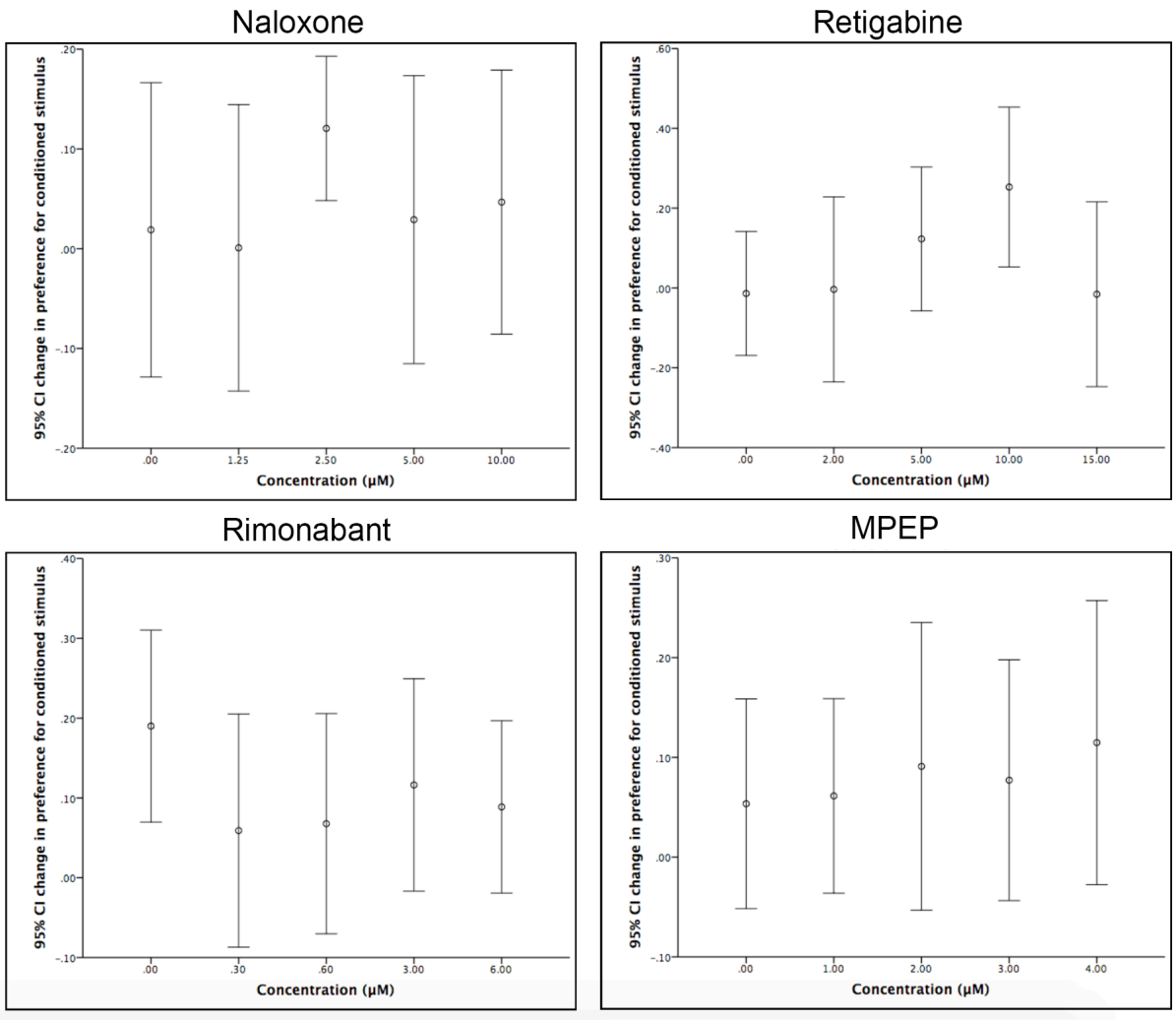

Supplementary Figure 3. Concentration-dependent locomotor effects following pretreatment of fish with drugs used in CPP studies. All drugs are displayed according to class of drug. Significant effects of drug are illustrated on the graphs as compared *post hoc* to concentration = 0. *Note:* *P < 0.05; **P < 0.01.

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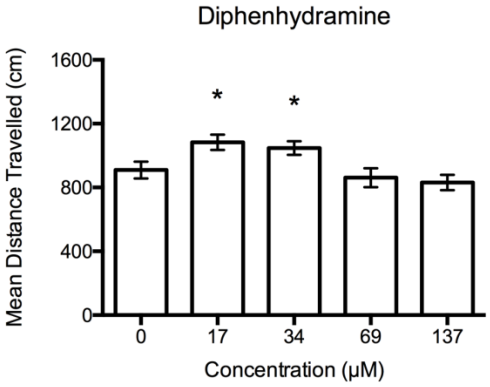

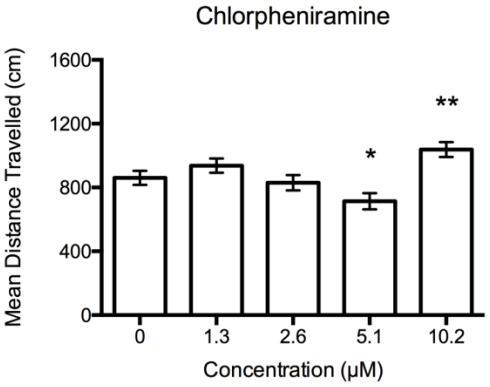

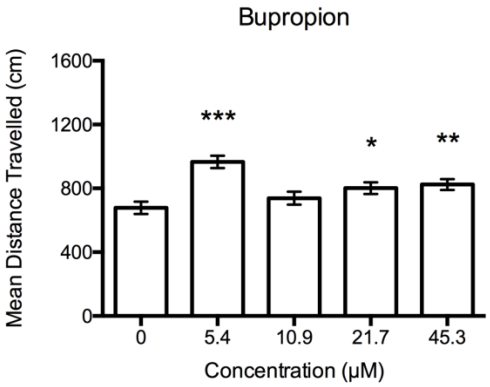

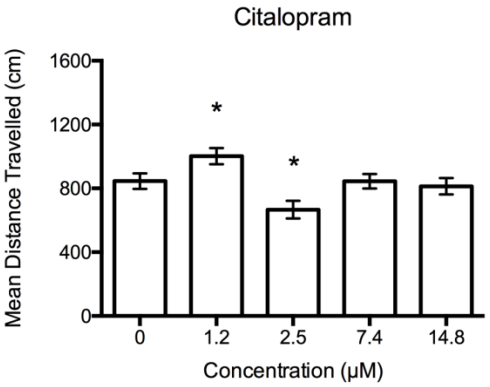

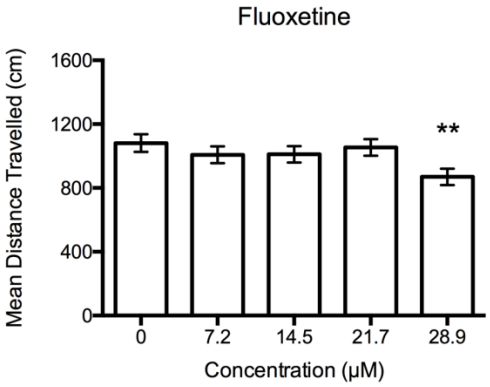

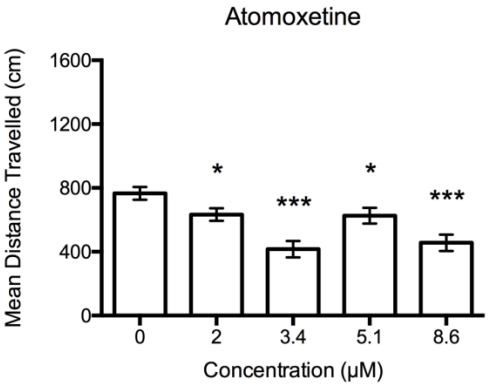

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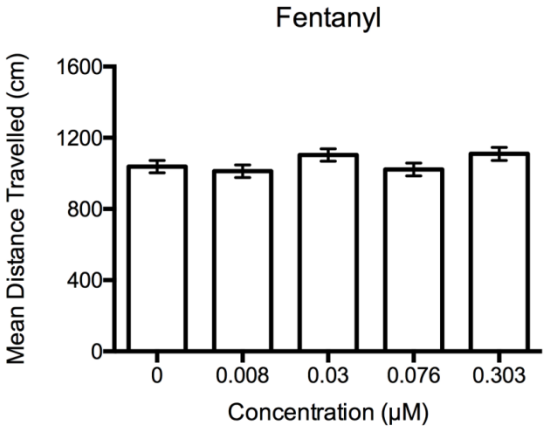

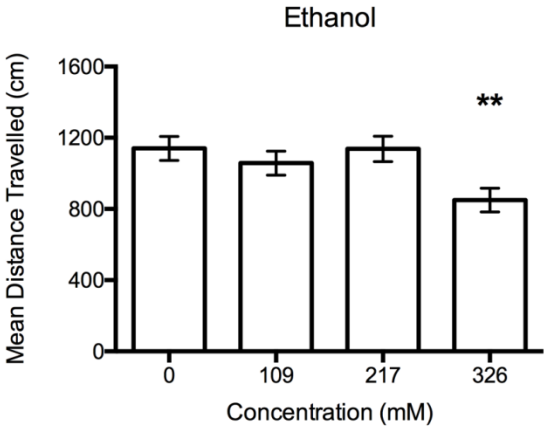

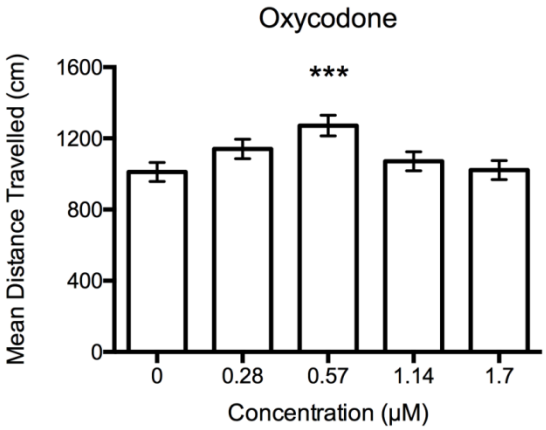

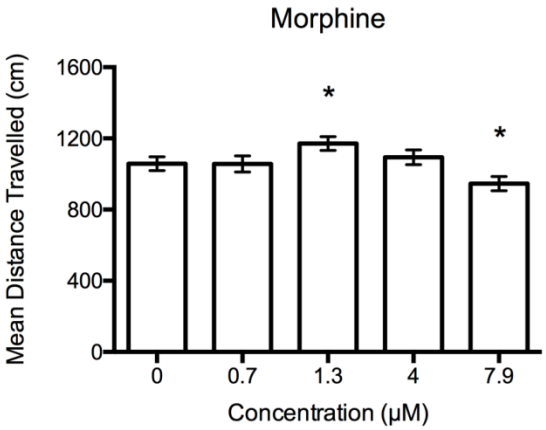

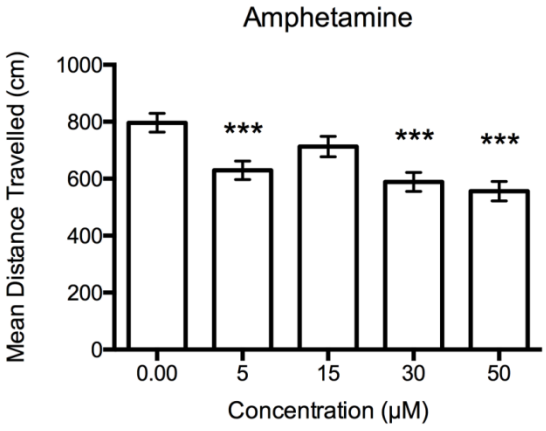

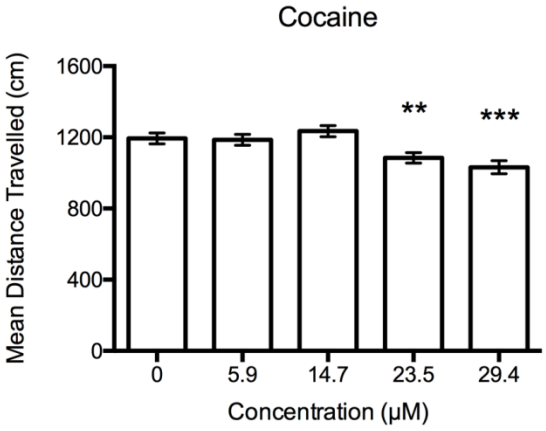

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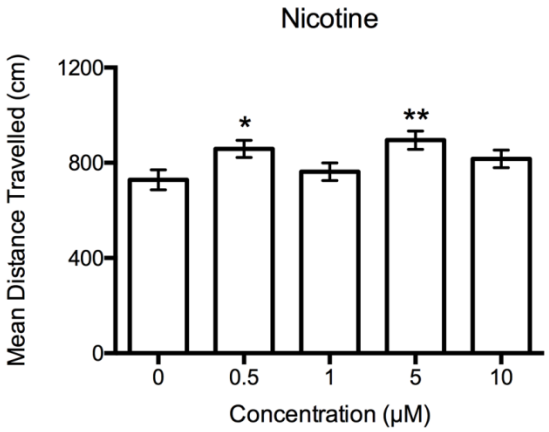

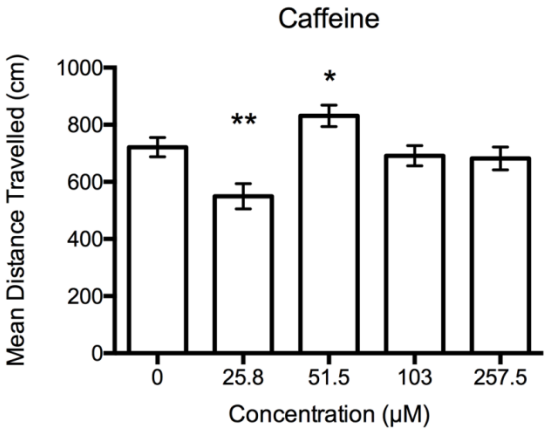

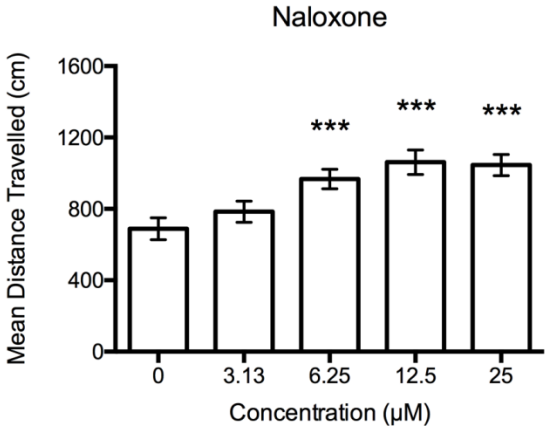

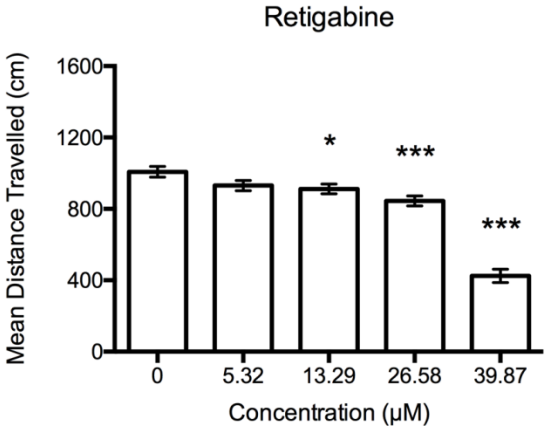

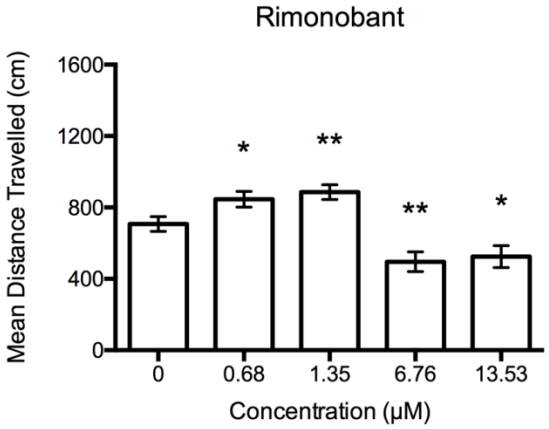

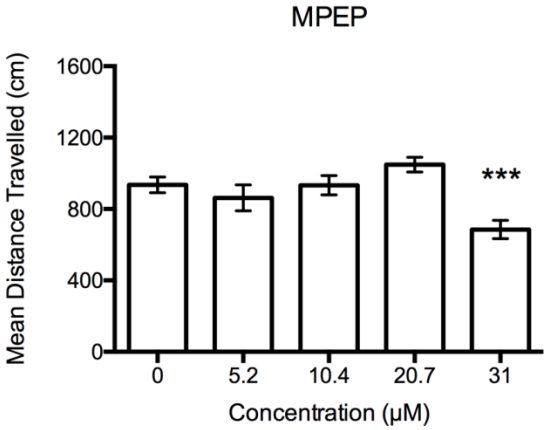

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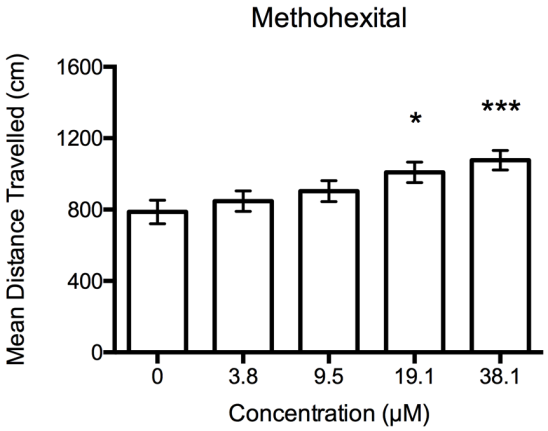

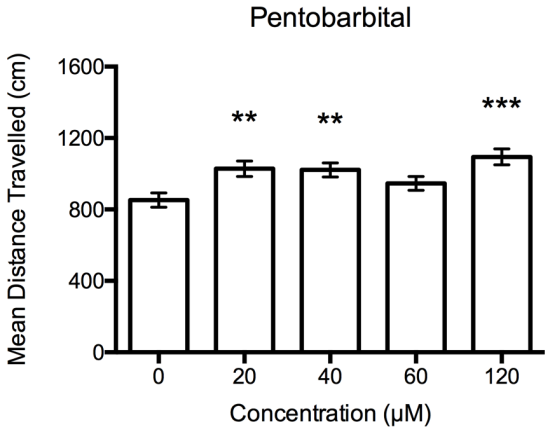

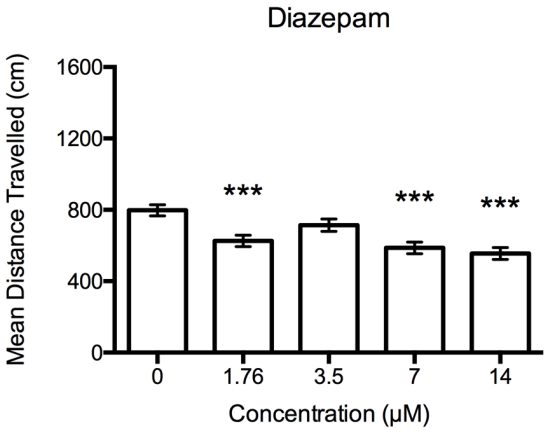

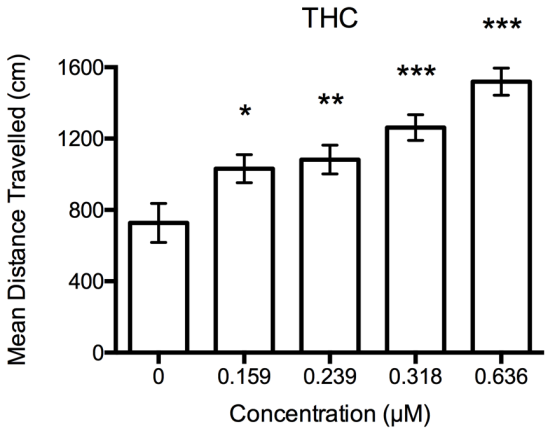

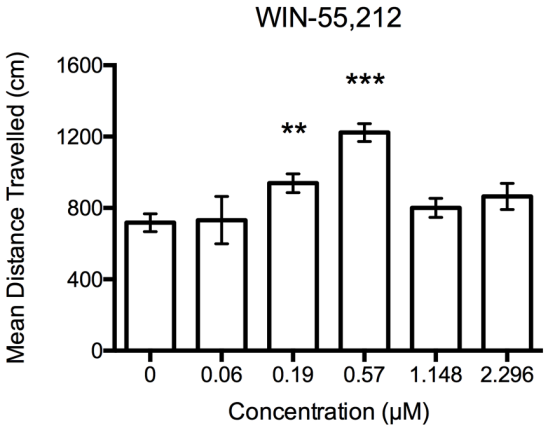

Supplementary Table 1. Summary of zebrafish CPP means and statistics. Data were first analyzed using an overall test for evidence of non-monotonicity. If there was no evidence of non-monotonicity (ie, there was a monotonic response, or in other words, followed an increasing or decreasing trend with increasing concentration of drug), a sequential trend test was used to determine statistical significance. If there was evidence of non-monotonicity (ie, the response did not follow an increasing or decreasing trend with increasing concentration of drug), a Dunnett's post hoc test was performed to determine statistical significance. Abbreviations and symbols: CB = cannabinoid; n.d. = no data (only 3 concentrations tested); n/a = not applicable (Dunnett Test not performed due to monotonic response); -- = analysis not performed because next higher concentration was not statistically significant from vehicle; *p<0.05 compared with vehicle.

				Means								Monotonic Response Sequential Trend Test p-value vs. vehicle				Non-Monotonic Response Dunnett Test p-value vs. vehicle			
Drug	Class	CPP Direction	N	Vehicle	Conc 1	Conc 2	Conc 3	Conc 4	Mono-tonic response?	Mono-tonicity F1	p value for non-mono-tonicity	Conc 1	Conc 2	Conc 3	Conc 4	Conc 1	Conc 2	Conc 3	Conc 4
Ketamine	Anesthetic	no change	78	0.007	0.059	0.191	0.131	0.125	Yes	0.562	0.456	--	--	--	0.146	n/a	n/a	n/a	n/a
Phencyclidine	Anesthetic	positive	78	-0.006	0.141	0.190	0.100	0.243	Yes	1.045	0.310	--	--	0.098	0.020*	n/a	n/a	n/a	n/a
Procaine	Anesthetic	no change	82	0.050	0.092	0.057	0.069	0.072	No	0.216	0.643	--	--	--	0.900	0.953	1.000	0.997	0.995
Tetracaine	Anesthetic	positive	175	-0.011	0.084	0.105	0.126	0.175	Yes	0.000	1.000	0.077	0.038*	0.049*	0.029*	n/a	n/a	n/a	n/a
Atomoxetine	Anti-depressant	no change	88	0.020	0.087	0.084	0.073	0.083	Yes	0.048	0.828	--	--	--	0.486	n/a	n/a	n/a	n/a
Bupropion	Anti-depressant	no change	116	0.025	0.081	0.080	0.040	0.090	Yes	0.466	0.497	--	--	--	0.536	n/a	n/a	n/a	n/a
Citalopram	Anti-depressant	no change	95	0.080	0.089	0.154	0.078	0.161	Yes	1.011	0.317	--	--	--	0.376	n/a	n/a	n/a	n/a
Fluoxetine	Anti-depressant	no change	89	0.015	0.089	0.071	0.065	0.072	Yes	0.139	0.710	--	--	--	0.575	n/a	n/a	n/a	n/a
Chlorpheniramine	Anti-histamine	positive	98	0.018	0.082	0.094	0.227	0.174	Yes	0.421	0.518	--	0.183	0.008*	0.011*	n/a	n/a	n/a	n/a
Diphenhydramine	Anti-histamine	no change	78	0.100	0.072	0.058	0.067	0.134	No	0.437	0.511	--	--	--	0.676	0.982	0.924	0.969	0.964
Methohexital	Barbiturate	negative	72	0.078	0.071	0.070	-0.031	-0.101	Yes	7.093	0.010	--	--	0.105	0.018*	n/a	n/a	n/a	n/a
Pentobarbital	Barbiturate	negative	52	0.134	0.161	0.186	-0.017	0.014	Yes	7.423	0.009	--	--	0.076	0.048*	n/a	n/a	n/a	n/a
Diazepam	Benzodiaze pine	no change	59	0.181	0.089	0.168	0.089	0.063	Yes	3.081	0.082	--	--	--	0.281	n/a	n/a	n/a	n/a
Rimonabant	CB 1 receptor antagonist	no change	73	0.190	0.059	0.068	0.116	0.089	Yes	3.032	0.086	--	--	--	0.442	n/a	n/a	n/a	n/a
Δ9-THC	CB receptor agonist	no change	82	0.089	0.179	0.043	0.068	0.022	Yes	3.207	0.077	--	--	--	0.266	n/a	n/a	n/a	n/a
WIN-55 212	CB receptor agonist	no change	72	0.073	-0.050	0.119	0.138	-0.001	No	1.974	0.165	--	--	--	0.901	0.739	0.975	0.984	0.961
Retigabine (Ezogabine)	K^+ channel opener	no change	58	-0.014	-0.004	0.123	0.253	-0.016	No	4.615	0.036	--	--	--	0.374	1.000	0.669	0.120	1.000
MPEP	mGluR5 receptor antagonist	no change	99	0.054	0.061	0.091	0.077	0.115	Yes	0.029	0.864	--	--	--	0.473	n/a	n/a	n/a	n/a

Fentanyl	μ Opioid receptor agonist	positive	129	0.000	0.102	0.149	0.180	0.148	Yes	0.251	0.617	0.052*	0.011*	0.002*	0.011*	n/a	n/a	n/a	n/a
Morphine	μ Opioid receptor agonist	positive	39	-0.030	0.044	0.073	0.108	0.198	Yes	0.000	1.000	--	--	0.106	0.039*	n/a	n/a	n/a	n/a
Oxycodone	μ Opioid receptor agonist	positive	92	0.050	0.183	0.174	0.252	0.120	No	2.950	0.089	--	--	--	0.222	0.241	0.280	0.038*	0.759
Naloxone	Opioid receptor antagonist	no change	55	0.019	0.001	0.121	0.029	0.047	No	1.405	0.241	--	--	--	0.660	0.999	0.575	1.000	0.992
Amphetamine	Stimulant	positive	78	0.021	0.120	0.130	0.151	0.154	Yes	0.000	1.000	0.035*	0.018*	0.008*	0.012*	n/a	n/a	n/a	n/a
Caffeine	Stimulant	no change	87	0.059	0.083	0.162	0.194	0.095	No	3.533	0.064	--	--	--	0.159	0.976	0.228	0.068	0.921
Cocaine	Stimulant	positive	107	0.045	0.129	0.199	0.168	0.269	Yes	0.138	0.711	0.146	0.024*	0.044*	0.003*	n/a	n/a	n/a	n/a
Ethanol	Stimulant	positive	49	-0.023	0.087	0.215	0.120	n.d.	Yes	2.534	0.119	0.027*	0.0001*	0.004*	--	n/a	n/a	n/a	--
Nicotine	Stimulant	positive	44	-0.023	0.194	0.189	0.257	0.085	No	3.252	0.078	--	--	--	0.167	0.031*	0.037*	0.006*	0.588

Supplementary Table 2. Summary of zebrafish locomotor activity statistics. Data were fitted to linear mixed effects models, with distance travelled as the dependent variable and time and concentration as fixed effects (fish ID as a random effect). Abbreviations: CB = cannabinoid; Y = yes; N = no.

Drug	Class	Main effect of Concentration			Main effect of time			Concentration by time interaction		
		Effect of Concentration?	F	*p* value	Effect of time?	F	*p* value	Concentration * time?	F	*p* value
Ketamine	Anesthetic	Y	F(4,305)=3.023	0.018	N	F(7,305)=1.489	0.17	N	F(28,305)=0.458	0.992
Phencyclidine	Anesthetic	N	F(4,306)=1.168	0.325	N	F(7,306)=0.743	0.636	N	F(28,306)=0.684	0.887
Procaine	Anesthetic	N	F(4,285)=1.497	0.203	N	F(7,285)=0.296	0.955	N	F(28,285)=0.446	0.994
Tetracaine	Anesthetic	Y	F(4,322)=9.979	<0.0005	N	F(7,322)=0.483	0.847	N	F(28,322)=0.263	1
Atomoxetine	Anti-depressant	Y	F(4,221)=9.600	<0.0005	N	F(7,221)=0.513	0.824	N	F(28,221)=0.467	0.991
Bupropion	Anti-depressant	Y	F(4,277)=7.727	<0.0005	N	F(7,277)=0.806	0.583	N	F(28,277)=0.720	0.851
Citalopram	Anti-depressant	Y	F(4,277)=5.131	0.001	N	F(7,277)=0.977	0.448	N	F(28,277)=0.342	0.999
Fluoxetine	Anti-depressant	Y	F(4,300)=2.401	0.05	N	F(7,300)=1.364	0.22	N	F(28,300)=0.357	0.999
Chlorpheniramine	Anti-histamine	Y	F(4,292)=6.345	<0.0005	Y	F(7,292)=2.807	0.008	N	F(28,292)=0.740	0.83
Diphenhydramine	Anti-histamine	Y	F(4,301)=5.360	<0.0005	N	F(7,301)=731	0.646	N	F(28,301)=0.454	0.993
Methohexital	Barbiturate	Y	F(4,275)=4.045	0.003	N	F(7,275)=1.768	0.094	N	F(28,275)=0.756	0.811
Pentobarbital	Barbiturate	Y	F(4,279)=5.005	0.001	N	F(7,279)=1.488	0.171	N	F(28,279)=0.640	0.922
Diazepam	Benzodiazepine	Y	F(4,288)=8.548	<0.0005	N	F(7,288)=0.533	0.809	N	F(28,288)=0.421	0.996
Rimonabant	CB 1 receptor antagonist	Y	F(4,238)=12.721	<0.0005	N	F(7,238)=0.825	0.567	N	F(28,238)=0.633	0.926
THC	CB receptor agonist	Y	F(4,235)=10.854	<0.0005	N	F(7,235)=0.869	0.531	N	F(28,235)=0.339	0.999
WIN 55,121-2	CB receptor agonist	Y	F(5,206)=12.153	<0.0005	N	F(7,206)=0.576	0.775	N	F(28,206)=0.335	1
Retigabine	K^+ channel opener	Y	F(4,307)=42.26	<0.0005	N	F(7,307)=0.733	0.645	N	F(28,307)=0.230	1
MPEP	mGluR5 receptor antagonist	Y	F(4,226)=7.689	<0.0005	N	F(7,226)=0.384	0.911	N	F(28,226)=0.820	0.728
Fentanyl	μ Opioid receptor agonist	N	F(4,332)=1.667	0.157	N	F(7,332)=0.883	0.52	N	F(28,332)=0.453	0.993
Morphine	μ Opioid receptor agonist	Y	F(4,290)=4.291	0.002	N	F(7,290)=1.136	0.34	N	F(28,290)=0.515	0.981
Oxycodone	μ Opioid receptor agonist	Y	F(4,299)=3.651	0.006	Y	F(7,299)=3.931	<0.0005	N	F(28,299)=0.496	0.986

Naloxone	Opioid receptor antagonist	Y	F(4,243)=7.168	<0.0005	Y	F(7,243)=2.035	0.052	N	F(28,243)=0.658	0.907
Amphetamine	Stimulant	Y	F(4,288)=8.548	<0.0005	N	F(7,288)=0.533	0.809	N	F(28,288)=0.421	0.996
Caffeine	Stimulant	Y	F(4,284)=6.170	<0.0005	N	F(7,284)=0.367	0.921	N	F(28,284)=0.394	0.998
Cocaine	Stimulant	Y	F(4,314)=6.622	<0.0005	N	F(7,314)=0.957	0.463	N	F(28,314)=0.503	0.985
Ethanol	Stimulant	Y	F(3,285)=14.224	<0.0005	N	F(7,285)=0.545	0.8		F(21,285)=0.443	0.985
Nicotine	Stimulant	Y	F(4,229)=3.003	0.019	N	F(7,299)=1.568	0.144	N	F(28,299)=0.509	0.983

Supplemental Table 3. Summary of binary classification for non-human primate (NHP) self-administration. Classification was conducted as described in (Horton et al., 2013). In brief, PubMed was the primary tool for locating peer-reviewed source documents. Google Scholar search engine was used as a follow-up to obtain additional resources, but only peer reviewed data or government documents were used for classifications. A positive in NHP self-administration was defined as a drug maintaining a higher level of responding under a fixed ratio (FR) schedule than the drug's vehicle. In cases where there were differences between data published in the literature, a drug was considered positive if any studies revealed a positive result. Abbreviations: 1 = positive finding; 0 = no effect; X = no data available.

Compound	Class	NHP Self-Administration	Citations
Ketamine	Anesthetic	1	(Broadbear et al., 2004)
Phencyclidine	Anesthetic	1	(Balster and Woolverton, 1980)
Procaine	Anesthetic	1	(Johanson and Aigner, 1981)
Tetracaine	Anesthetic	1	(Woolverton and Balster, 1979)
Atomoxetine	Anti-depressant	0	(Wee and Woolverton, 2004)
Bupropion	Anti-depressant	1	(Lamb and Griffiths, 1990)
Citalopram	Anti-depressant	X	X
Fluoxetine	Anti-depressant	X	X
Chlorpheniramine	Anti-histamine	1	(Beardsley and Balster, 1992)
Diphenhydramine	Anti-histamine	1	(Banks et al., 2009)
Methohexital	Barbiturate	1	(Broadbear et al., 2005)
Pentobarbital	Barbiturate	1	(Meisch and Lemaire, 1988)
Diazepam	Benzodiazepine	1	(Grant and Johanson, 1987)
Rimonabant	Cannabinoid 1 receptor antagonist	0	(Beardsley et al., 2002)
Δ9-THC	Cannabinoid receptor agonist	1	(Justinova et al., 2003)
WIN-55 212	Cannabinoid receptor agonist	X	X
Retigabine	K^+ channel opener	X	X
MPEP	mGluR5 receptor antagonist	X	X
Fentanyl	μ Opioid receptor agonist	1	(Broadbear et al., 2004)
Morphine	μ Opioid receptor agonist	1	(Hoffmeister and Goldberg, 1973)
Oxycodone	μ Opioid receptor agonist	1	(Aigner and Balster, 1979)
Naloxone	μ Opioid receptor antagonist	0	(Aigner and Balster, 1979)
Amphetamine	Stimulant	1	(Hammerbeck and Mitchell, 1978)
Caffeine	Stimulant	1	(Deneau et al., 1969)
Cocaine	Stimulant	1	(Broadbear et al., 2004)
Ethanol	Stimulant	1	(Broadbear et al., 2005)
Nicotine	Stimulant	1	(Mello and Newman, 2011)

Supplemental Table 4. Statistical summary of diagnostic tests for the non-human primates (NHP) self-administration (SA) model and the prediction of human subjective effects (HSE) and scheduling status. Summary for NHP self-administration data published in the literature using the same list of compounds as zebrafish and rat models (see Figure 3) and the prediction of human subjective effects and scheduling status. The output demonstrates the number of false positives, true positives, true negatives, false negatives, and outcome ratios for various statistical outputs with regards to diagnostic value. Values in parentheses indicate 95% confidence intervals. Values in bold indicate a significant difference from pretest probability [concordance, PPV, NPV, PPV (30% prev), NHP (30% prev)] or from zero predictive value (VaPPV, VaNPV). PPV and NPV represent observed predictive values. PPV (30%) and NPV (30% prev) represent predictive values corrected for an estimated prevalence of 0.3. Abbreviations: PPV = positive predictive value; NPV = negative predictive value, PRU positive = proportionate reduction in uncertainty for positive findings; PRU negative = proportionate reduction in uncertainty for negative findings.

Model	False positives	True positives	True negatives	False negatives	Concordance	Sensitivity	Specificity	PPV	Adjusted PPV (30% prev)	Value added PPV	NPV	Adjusted NPV (30% prev)	Value added NPV	PRU positive	PRU negative
NHP SA to HSE	2	14	3	0	**0.89 (0.69. 0.97)**	**1.00 (0.78, 1.00)**	0.60 (0.23, 0.88)	0.88 (0.64, 0.97)	**0.52 (0.33, 0.89)**	**0.22 (0.03, 0.59)**	1.00 (0.44, 1.00)	**1.00 (0.84, 1.00)**	**0.30 (0.14, 0.30)**	**0.31 (0.05, 0.84)**	**1.00 (0.48, 1.00)**
NHP SA to scheduling	8	11	2	0	0.62 (0.41, 0.79)	**1.00 (0.74, 1.00)**	0.20 (0.06, 0.51)	0.58 (0.36, 0.77)	0.35 (0.27, 0.50)	0.05 (-0.03, 0.20)	1.00 (0.34, 1.00)	1.00 (0.51, 1.00)	0.30 (-0.19, 0.30)	0.07 (-0.04, 0.28)	1.00 (-0.64, 1.00)

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