Published in final edited form as:

Pharmacol Biochem Behav. 2012 December ; 103(2): 157–167. doi:10.1016/j.pbb.2012.07.017.

Sulpiride, but not SCH23390, Modifies Cocaine-Induced Conditioned Place Preference and Expression of Tyrosine Hydroxylase and Elongation Factor 1 α in Zebrafish

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Abstract

Finding genetic polymorphisms and mutations linked to addictive behavior can provide important targets for pharmaceutical and therapeutic intervention. Forward genetic approaches in model organisms such as zebrafish provide a potentially powerful avenue for finding new target genes. In order to validate this use of zebrafish, the molecular nature of its reward system must be characterized. We have previously reported the use of cocaine-induced conditioned place preference (CPP) as a reliable method for screening mutagenized fish for defects in the reward pathway. Here we test if CPP in zebrafish involves the dopaminergic system by co-treating fish with cocaine and dopaminergic antagonists. Sulpiride, a potent D2 receptor (DR2) antagonist, blocked cocaine-induced CPP, while the D1 receptor (DR1) antagonist SCH23390 had no effect. Acute cocaine exposure also induced a rise in the expression of tyrosine hydroxylase (TH), an important enzyme in dopamine synthesis, and a significant decrease in the expression of elongation factor 1 α (EF1 α), a housekeeping gene that regulates protein synthesis. Cocaine selectively increased the ratio of TH/EF1 α in the telencephalon, but not in other brain regions. The cocaine-induced change in TH/EF1 α was blocked by co-treatment with sulpiride, but not SCH23390, correlating closely with the action of these drugs on the CPP behavioral response. Immunohistochemical analysis revealed that the drop in EF1 α was selective for the dorsal nucleus of the ventral telencephalic area (Vd), a region believed to be the teleost equivalent of the striatum. Examination of TH mRNA and EF1 α transcripts suggests regulation of expression is post-transcriptional, but this requires further examination. These results highlight important similarities and differences between zebrafish and more traditional mammalian model organisms.

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Keywords

zebrafish; addiction; dopamine; behavior; sulpiride; SCH23390; tyrosine hydroxylase; elongation factor 1 α

1. Introduction

Future strategies for medical treatment of psychological disorders with a strong genetic component, including drug abuse, are likely to include personalized pharmaceutical therapy based on polymorphisms in one or more pathologically related genes. Several heritability studies conducted on monozygotic twins have indicated a strong genetic component for drug abuse consistent with a relatively small number of relevant genes (Kendler & Prescott 1998; Kendler et al 2003). However, it is likely that several such polymorphisms lead to the same endpoint, given the complexity by which the brain is affected by addictive drugs (Goldman et al 2005). Several such polymorphisms have been identified, but finding new targets remains a major goal of addiction research today. Forward genetics in model organisms is a powerful, unbiased approach to finding such novel targets. While at present the use of forward genetics is becoming more popular in rodents (Adams & van der Weyden 2008), the technique is used much more frequently and extensively in invertebrates and zebrafish. Forward genetics in these organisms has been used to identify novel genes affecting physiology and behavioral sensitivity to addictive drugs (Darland & Dowling 2001; Ninkovic & Bally-Cuif 2006; Wolf & Heberlein 2003).

Addictive drugs have distinct molecular targets and thereby have different physiological effects; yet, by definition, all share the common ability to create a state by which the individual (human or model organism) compulsively seeks the drug in spite of adverse consequences and to the exclusion of other normally pleasurable stimuli (DSM-IV-TR 2000). All addictive drugs and naturally pleasurable stimuli also elevate dopamine concentration in the nucleus accumbens (NAc) of the ventral striatum (DiChiara & Imperato 1988). The NAc and the afferent dopaminergic neurons of the ventral tegmental area (VTA) have long been considered the central pathway governing reward, or reinforcement of behavior (Pierce & Kumaresan 2006; Wise 2004). This central pathway also includes distinct but parallel circuits containing other clusters of midbrain dopaminergic neurons and projections to the frontal cortex in mammals (Ikemoto 2007). Numerous types of experimental paradigms have been developed to measure the rewarding properties of addictive drugs in model organisms. Conditioned place preference (CPP) is a classic experimental paradigm in which the model organism is exposed to a primary stimulus in the context of certain environmental cues (Tzschentke 1998). The degree of behavioral reinforcement is measured by how frequently the animal approaches the environmental cues in the absence of the primary stimulus in subsequent trials. CPP has proven amenable to the study of reward in most vertebrate model organisms, including zebrafish.

The zebrafish is a popular model organism because it can be readily manipulated genetically, it can be raised in large numbers similar to flies and worms, and it shares many common features of development and the basic neurological layout with mammals. For example, there have been a number of studies indicating that zebrafish have a central dopaminergic pathway analogous to the midbrain-forebrain pathways described in mammals (Rink & Wullimann 2002; 2004). Furthermore, several studies have documented drug-induced CPP in zebrafish, as well as, abnormal CPP in certain mutagenized families (Darland & Dowling 2001; Lau et al 2006; Ninkovic & Bally-Cuif 2006; Ninkovic et al 2006; Swain et al 2004; Webb et al 2009). In our previous work, we reported three families with abnormal cocaine-induced CPP (Darland & Dowling 2001). These types of studies

validate the potentially powerful approach of applying forward genetic approaches in zebrafish to find novel genes affecting addiction in humans. What remains to be seen is whether the molecular substrates that underlie reward in the zebrafish are analogous to those in mammals.

Cocaine is believed to raise the level of dopamine in the NAc by blocking monoaminergic transporters, principally the dopamine transporter (DAT) (Hall et al 2004). The rewarding effect is therefore likely mediated through dopamine receptors in the NAc (Holmes et al 2004). It is not yet known if the same is true for cocaine-induced CPP in zebrafish. Similarly, several studies have examined gene expression changes in the mesolimbic reward pathway after acute and chronic drug exposure to determine the molecular mechanisms underlying the shift in behavior from casual use to compulsion, as well as, genetic vulnerability to drug abuse (Goldman et al 2005). There has thus been a great deal published about the expression of certain genes after exposure in mammalian systems (Nestler 2004). While some studies have shown similar drug-induced gene expression changes in zebrafish, these studies did not involve cocaine and often made use of microarrays rather than detailed examination of specific candidate genes (Kily et al 2008; Webb et al 2009). Regional changes, statistical stringency and the absence of protein verification may limit the comparison of zebrafish with similar studies in mammals. Examination of genes regulated by addictive drugs in mammals needs to be performed in zebrafish in order to assess similarities and thus validate the model.

The list of genes regulated by addictive drugs is extensive (Nestler 2004; Nestler 2005). Among the most studied is tyrosine hydroxylase (TH), an important enzyme in dopamine synthesis. Several studies have reported changes in the expression of this gene after acute and chronic drug exposure, revealing an extensive array of regulatory mechanisms (Beitner-Johnson D 1991; Jedynek et al 2002; Kumar & Vrana 1996). The expression changes of genes important in dopaminergic function have to be compared to that of genes normally considered unaffected by the same stimuli. Among these so-called house-keeping genes, elongation factor 1 α (EF1 α) a protein involved in translational elongation (Negrutskii & El'skaya 1998), has emerged as one of the more consistent normalizing controls, at least in terms of transcription analysis (Tang R 2007). Frequently, however, regulation of the control genes turns out to be more complex and profound than that of genes considered directly relevant to the process in question. In the current study, the regulation of TH and EF1 α to a single cocaine exposure with and without dopaminergic antagonists was examined in zebrafish for comparison with what has been reported in mammalian species.

2. Materials and Methods

2.1 Fish Maintenance and husbandry

Fish were maintained at the University of North Dakota zebrafish facility in accordance with well-established procedures (Westerfield 2007). The fish were kept at 28.5 C on an Aquatic Habitat freestanding system with a 14–10 light-dark cycle. Water conditions were kept at a pH of approximately 7.8 and conductivity typically between 800 and 1000 μ S. Fish were fed twice daily with artemia and pelleted food. AB strain zebrafish (Harvard Biological Laboratories) hatched and raised through four generations in North Dakota were used for this study (IACUC number 0606-1). All studies involved male sibling fish from single clutches 6–8 months old. In previous experiments males were found to be more consistent in their behavioral responses. However, since the completion of this study, we have found similar results with both sexes (data not shown).

2.2 Conditioned Place Preference

CPP was performed similarly to what has been previously described (Darland & Dowling 2001), with some notable modifications. The CPP chamber was adapted from that used previously to include three compartments with removable barriers (Figure 1A). Figure 1A shows artificial plants in the front section that were not used in the CPP experiments, but provide contrast in the picture. Visual cues included duct tape that was wrapped around the rear of the apparatus, while the front remained clear. Fish also had the rear laboratory wall and differential light shading to use as visual cues. The apparatus had walls dividing it into three equal runways containing 1 liter of water each. The runways were completely isolated from one another: there was absolutely no chance for leakage between them. Removable barriers compartmentalized each runway into three sections. The middle section contained twice the volume of the end compartments, which were equal in volume. While the barriers between chambers within a single runway allowed some exchange, studies with phenol red showed that with a solid barrier in place, this exchange was extremely slow, noticeably slower than the 45-minute time course of the drug exposures described below. Three fish were tested simultaneously in the same apparatus, each in its own runway.

On the first day, fish were introduced to the central compartment of the apparatus and habituated during a one-hour long session in which barriers with holes allowed free access to all three chambers. On days 2 and 3 fish were conditioned without drug by isolation first in the front compartment for 45 minutes and then in the rear compartment for 45 minutes. Isolation was accomplished by replacing the passable barrier connecting the end compartment to the middle compartment with a solid barrier. Baseline preference for the rear compartment was determined during a ten-minute test swim the following morning on days 3 and 4. Treatment groups were assembled such that they shared the same average baseline preference for the rear compartment. Since observations were made visually, we do not have data on overall activity. However, we were able to assess the number of times fish changed compartments during baseline trials to provide a comparison of activity between the different treatment groups before drug exposure. Importantly, no fish were excluded from analysis based on excessive stress-related behaviors, like freezing. These factors complicated our previous studies (Darland & Dowling 2001). On day 4, fish were isolated in the front compartment for 45 minutes, then in the rear compartment for 45 minutes. It was during confinement in the rear compartment that fish received drug treatment. The next morning preference for the rear compartment was assessed again (CPP1 in Figure 1B). A second conditioning trial was performed that afternoon and CPP was assessed the following morning (CPP2 in Figure 1B).

Treatment groups included untreated controls, fish treated with 10 mg/L lidocaine, 10 mg/L cocaine, and cocaine with either 10 μ M sulpiride or 10 μ M SCH23390. Lidocaine served as control since this compound shares the local anesthetic properties of cocaine, but lacks the rewarding effects caused by blocking monoaminergic transporters. These doses proved maximally effective in unpublished experiments using the old CPP assay of previous investigations. Final treatment concentrations were calculated using 250 ml as the volume for the rear compartment. Drugs were dissolved as a stock solution and dispensed directly into the rear compartment with the fish. We assume based on behavioral and molecular changes seen that the drugs were readily absorbed across the gills of the fish. Results were tabulated as time spent in the rear compartment (seconds) and repeated measure ANOVA followed by Bonferroni posttests were used to analyze differences among and between the experimental groups.

2.3 Drug treatments and Dissections

For experiments in which brain tissue was collected for total RNA or protein, fish were equilibrated in the behavioral apparatus for mock CPP assays and were treated with water (untreated), 10 mg/L lidocaine (data not shown), 10 μ g/L cocaine, and 10 μ g/L cocaine with 10 μ M sulpiride or 10 μ M SCH23390. After one hour of exposure in the rear compartment of the apparatus, the fish were anesthetized in tricaine, sacrificed and dissected. Our animal protocol required tricaine treatment to eliminate any possibility of suffering to the animal. For western analysis tissue from three fish was combined per treatment condition in any given experiment. In the initial experiments, we collected tissue from brainstem and cerebellum. We combined tissue from multiple animals because of the low amount of tissue and TH expression in the cerebellum. We combined tissue from the telencephalon and diencephalon for experimental consistency. Brains from single fish were examined for mRNA using quantitative real time polymerase chain reaction (QPCR).

The brains were removed and dissected as diagrammed in Figure 2A (Wullimann 1996). The telencephalon (light gray) was separated from the diencephalon (charcoal gray) at the telencephalic flexure, just rostral to the optic chiasm and the olfactory bulbs were trimmed away. The cerebellum (black) was separated from the optic tectum and a cut was made straight through the midbrain flexure separating the diencephalon from the brain stem (stippled gray). The corpus cerebelli was then separated from the rest of the brainstem by cutting through the cerebellar tracts. Immunohistochemical surveys of the dissected regions in separate experiments showed that the diencephalic region included the dopaminergic neurons of the ventral thalamus, the posterior tuberculum (PT), pre-tectal, posterior tuberal nucleus, and the various groups of the hypothalamus (Yamamoto et al 2010; 2011). The region we call the telencephalon includes all the TH positive cells and fibers of the pallidum and subpallidum, and cells of the anterior preoptic areas (PPA) (Yamamoto et al 2010; 2011). The current thought is that the connections from the PT in the diencephalon to the ventral and dorsal telencephalon comprise the teleost equivalent to the midbrain-striatal connection in mammals (Rink & Wullimann 2002; 2004). We therefore confined our molecular analysis in this study to the regions we refer to as telencephalon and diencephalon.

2.4 Quantitative real time reverse transcription polymerase chain reaction (QPCR)

Total RNA was collected from individual brain regions using an RNeasy kit (Qiagen). 200 ng of RNA was used to synthesize cDNA with a GeneAMP kit (ABI). QPCR reactions were carried out in an ABI 3000 thermocycler, using 2 ml of cDNA, representing 2.5 ng worth of total RNA, and SYBR-green supermix (ABI). DNA fragments amplified from plasmid constructs of each gene tested were used to construct standard curves relating detection threshold values to starting amounts of DNA. The standard curves ranged from one to 1×10^{-10} picograms of fragment in 10-fold dilution steps. Unknown levels were determined using the slope of the standard curve. Primer amplification efficiencies were calculated with the standard formula: $E = -1 + 10^{(-1/\text{slope st. curve})}$. The primers used for cloning and QPCR analysis were taken directly from references in the literature or chosen by analysis of GenBank sequences using OligoAnalyzer software (Qiagen). Table 1 has a list of the primer sequences used, amplicon size and either a literature reference or Genebank annotation. All amplicons quantified in this study were cloned and verified by sequencing (ABI Big Dye kit and 3100 Genetic Analyzer). Results were recorded as attograms (ag) transcript/2.5 nanogram (ng) RNA. Comparisons were made between untreated and cocaine treated fish using an unpaired t-test of natural log transformed values.

2.5 Western Blotting

Protein lysates were made by passing tissue dissected as described above and suspended in RIPA buffer with DTT and protease inhibitors through an 18-gauge needle and tuberculin syringe. Total protein in these lysates was then quantified using a standard colorimetric assay (BioRad). 20 mg of lysate was loaded per lane onto 2–15% gradient gels and proteins separated using a standard SDS-PAGE apparatus (Mini Protean II, BioRad). As mentioned above, cerebellar extracts had very low TH expression and so the maximal amount of protein recommended by the manufacturer of the nylon membrane was used to assess level of expression in all brain regions. After charge-based transfer to PVDF membrane, the blots were incubated with primary antibodies to TH (Chemicon IHCR1005-6) and EF1 α (Chemicon-Millipore 05-235, since discontinued), followed by treatment with an alkaline phosphatase linked secondary antibody (BioRad). Specific protein bands were visualized using CDP-STAR substrate (Fisher) and a UVP Bio Imaging gel dock with LabWorks software. Pixel density was quantified using Image J software (NIH). Each band represents pooled tissue from three fish and a minimum of three experiments were done for each drug treatment. TH and EF1 α were graphed separately and as a ratio depicting a profile of “gene expression” in Figures 3 and 4. Differences between treatment groups were analyzed using one-way ANOVA with Bonferroni’s multiple comparison post-test.

2.6 Immunohistochemistry

Fish were treated as described for the other experiments, except that after tranquilization they were decapitated and the heads were placed immediately in 4% paraformaldehyde. The next day brains were removed, saturated with 30% sucrose and embedded in OCT. The entire telencephalon of 6 untreated and 6 cocaine-treated fish were cryosectioned, with each 10 μ M section collected. Slides from each brain were immunostained with the same TH antibody used in the western analysis and visualized and photographed under fluorescence using a FITC-conjugated secondary antibody (Chemicon-Millipore). EF1 α was detected with a rabbit polyclonal antibody (Santa Cruz Biotechnology, SC68481) and visualized using an anti-rabbit cy3-conjugated secondary (Chemicon-Millipore). The sections with TH and EF were imaged using a Flowview 100 multi-photon microscope (Olympus). Sections shown in Figure 5 D–I from the two testing conditions were compared based on overall morphology, the presence and morphology of the telencephalic blood vessels on either side, and the dopaminergic cells of the adjacent subpallidum.

2.7 Statistical Analyses

The statistical analyses were conducted with GraphPad Prism 4.0c for Macintosh (GraphPad Software, Inc., LaJolla, CA) with the specific tests and p values reported in the legends. For the CPP analysis repeated measures two-way ANOVA was used to calculate the effects of time, treatment, and the interaction between time and treatment. The post test following two-way ANOVA calculated significance by comparing the t ratio with the t distribution based on the degrees of freedom for each and with the Bonferroni correction applied for multiple comparison as recommended by GraphPad Prism. For one-way ANOVA analysis of the western blot data, the populations showed a Gaussian distribution; therefore, a parametric analysis was conducted followed by pair-wise comparisons of the data using the Bonferroni post-test. For QPCR analysis an unpaired t test was used in combination with an F test to determine if the variances differed significantly. When appropriate, the data were transformed to the natural log prior to analysis and the specific approaches are indicated in the text or legends.

3. Results

3.1 The effect of dopaminergic antagonists on cocaine-induced CPP

As detailed in the methods, we employed a revised method of measuring cocaine-induced CPP. The new apparatus had three chambers instead of two, trials were longer and fish could actually see one another although solid barriers between the runways prevented any other kind of contact (Figure 1A). We did not assess overall activity of the fish at all stages in the experiments as scoring was visual, but we did record the number of times the fish transitioned from one compartment to another in baseline trial. Treatment groups were matched for baseline preference. While we did not measure overall activity, we were able to record the number of transitions of fish from one compartment to another. This was very consistent between treatment groups before drug exposure. The mean number of transitions for all fish was 20.5 ± 7.1 during the 10-minute final baseline assessment. ANOVA analysis showed no significant differences in transitions between treatment groups during final baseline assessment (dfn = 3, dfd = 25, $F = 0.68$, $p < 0.57$). This shows that the fish in each group had comparable activity and exploratory behavior before drug exposure. Also significant was that in all these experiments, not one fish had to be excluded from analysis because of freezing or erratic behavior.

The treatment groups were assembled such that they all had the same average baseline preference. Thus, fish receiving different treatments were grouped in adjacent runways. The responses reported in Figure 1B, then, are not augmented by effects of fish following adjacent fish. Any potential effects from fish following one another did not eliminate differences in CPP between treatment groups. We determined optimal drug dosages through preliminary unpublished experiments (data not shown) and by extrapolating from previous results using the older method of measuring CPP (Darland & Dowling 2001). After assessing baseline preference, fish were treated in the rear compartment of the apparatus with water (untreated), lidocaine, cocaine, cocaine with the DR2 antagonist sulpiride, or cocaine with the DR1 antagonist SCH23390. Preference for the rear compartment was assessed the following day during a ten-minute swim. A second round of conditioning was used to assess any further changes in behavior for untreated and cocaine-treated fish.

Results from three experiments are summarized in Figure 1B. Untreated fish did not display a significant change in preference for the rear compartment ($t = 0.63$, $p > 0.05$). In contrast, 10mg/L cocaine induced a robust CPP response after a single exposure ($t = 3.6$, $p < 0.01$, when compared against baseline), with fish displaying an increased preference for the rear compartment by 60% (Figure 1B). This is comparable to results using the older system, though the CPP is expressed differently, and similar to what has been reported in mice (Darland & Dowling 2001; Sora et al 1998). Multiple trials did produce higher CPP than a single exposure, but the changes were not statistically significant from the first trial (repeated measures ANOVA of CPP1 and CPP2 gave the following statistical results, untreated: $t = 0.92$, $p > 0.05$, cocaine: $t = 0.54$, $p > 0.05$, cocaine and sulpiride: $t = 1.7$, $p > 0.05$, cocaine and SCH23390: $t = 0.042$, $p > 0.05$). All data involving antagonists are reported after one and two conditioning trials to obtain maximal CPP.

Lidocaine, a local anesthetic without rewarding properties, was used in additional behavioral experiments to control against cocaine effects not related to monoamine transporter activity. The time spent after 2 conditioning trials for 6 fish with 10 mg/L lidocaine was 88 ± 13.5 (SEM) seconds, as compared to baseline which was 76 ± 14.4 (SEM) seconds (data not shown in Figure 1). Six baseline matched untreated controls spent an average of 98 ± 25 (SEM) seconds in the rear compartment after two conditioning trials (data not shown). When examined by repeated measures ANOVA, untreated fish and those treated with lidocaine did not induce significant CPP (df 25, $F = 0.88$, $P < 0.51$). Similar results came

from 3 other lidocaine treated-fish baseline matched with the antagonist groups and from those obtained using the older assay (Darland & Dowling 2001).

To test the dopaminergic component of cocaine-induced CPP, a series of experiments were performed using DR1 and DR2 antagonists together with cocaine. Co-treating with 10 μ M sulpiride, a DR2-specific antagonist, completely blocked CPP induced by 10mg/L cocaine (Figure 1B: $t = 0.084$, $p > 0.05$ when compared to baseline). In contrast the D1 antagonist SCH 23390 had no effect on cocaine induced CPP at any dose tested ($t = 5.1$, $p < 0.001$ when compared to baseline). The experiments in Figure 1B do not include dose response curves for the antagonists. This was performed in experiments using the CPP assay reported previously in the older apparatus and the doses used here were maximally effective in the earlier experiments (data not shown). We include the result with 10 μ M SCH23390 for comparison with sulpiride. SCH23390, though ineffective at preventing CPP, clearly had pharmacological effects on the fish at the dosage reported here. These fish displayed obvious ataxia that persisted 5–10 minutes after returning them to their home tanks. The fact that there was an observable behavioral effect suggests that the drug entered the bloodstream of the fish, but failed to affect CPP.

3.3 Cocaine induces changes in telencephalic TH2 transcript levels

We used QPCR to test if there were cocaine-induced changes in telencephalic gene expression that might correlate with CPP. The genes chosen for examination were zebrafish homologs of cocaine-regulated genes in mammals. We focused on the forebrain for this analysis since all dopaminergic cell groups are localized to the telencephalon and diencephalon (Ma 1997), (Yamamoto et al 2011). The ventral and dorsal telencephalon is innervated by diencephalic dopaminergic projections and includes structures analogous to the mammalian striatum. While we do not report the other genes here, the most convincing and consistent change after treatment with cocaine was TH2 in the telencephalon. Figure 2C and 2D shows the results from experiments quantifying transcript levels of the two TH genes in zebrafish (TH1 and TH2). In addition, we used a number of housekeeping genes as controls (Tang R 2007). One of these control genes, EF1 α , is shown in Figure 2B for comparison. TH2 mRNA was significantly reduced by cocaine treatment ($t = 2$, $df = 9$, $p < 0.0087$), while TH1 and EF1 α were not significantly changed ($t = 0.52$, $df = 10$, $p < 0.61$ and $t = 1$, $df = 10$, $p < 0.2$ respectively). Experiments looking at the diencephalon showed no cocaine-induced differences in any of the three transcripts (data not shown in Figure 2, TH2: $t = -1.9$, $df = 10$, $p < 0.09$, TH1: $t = -0.02$, $df = 10$, $p < 0.69$, EF1 α : $t = 0.63$, $df = 10$, $p < 0.55$).

3.4 Cocaine and dopaminergic antagonists affect regional protein levels in the zebrafish brain

The change in TH2 mRNA and the availability of antibodies prompted studies to validate the results by looking for changes in TH protein, using EF1 α as a control. We therefore used western analysis to quantify TH levels in the brain after treatment with cocaine and cocaine with dopaminergic antagonists. Fish were dissected and brains were collected after mock conditioning trials. Brains were dissected as described in methods and summarized in Figure 2A. As for the mRNA quantification we focused on the telencephalon and diencephalon for analysis (Figures 3 and 4 respectively). Probing with the TH antibody resulted in a single band for all lysates tested, suggesting that in western analysis, the antibody recognized only one gene product, or that the two isoforms could not be distinguished on our gradient gels. The antibody we used is different than that used in other studies looking at TH isoforms in zebrafish (Yamamoto et al 2010). Initial investigations into possible TH antibodies suggested that the monoclonal we used would be sufficient to detect both TH isoforms. A more detailed examination indicated that the antibody we used most likely detected TH1,

but not TH2. After probing for TH the blots were re-probed with the EF1 α antibody (Figure 3A and 4A).

In contrast to what we detected using QPCR analysis, cocaine treatment resulted in a modest increase in TH1 protein in both the telencephalon ($t = 3.3$, $p > 0.05$) and diencephalon ($t = 3.3$, $p < 0.05$) though the former was not statistically significant (Figure 3C and 4C). In the telencephalon, both dopamine antagonists offset the modest cocaine-induced increase in TH protein (Figure 3C), but the effect of sulpiride was more robust and statistically significant ($t = 6.3$, $p < 0.001$, when compared to cocaine). The control EF1 α was not significantly affected by any drug treatment (Figure 3D, $t = 1.7$, 0.37 and 3.0 for cocaine, cocaine with sulpiride, and cocaine with SCH23390 respectively, $p > 0.05$ for all conditions), although the level of protein was consistently lower in cocaine treated fish and especially low in fish co-treated with the D1 antagonist. Initially we sought to use EF1 α as a normalizing control. When we normalized TH with EF1 α , we saw a significant increase in the ratio with cocaine treatment of 50% (Figure 3B, $t = 3.3$, $p < 0.05$). Sulpiride completely offset the effect of cocaine on the ratio ($t = 6.3$, $p < 0.001$ when compared to cocaine alone), while SCH23390 had no effect on the ratio ($t = 3.0$, $p > 0.05$ when compared to cocaine alone). The changes in the ratio of TH to EF1 α with cocaine and the antagonists in the telencephalon coincide well with the effects on CPP behavior (compare Figures 1B with 3B).

In the diencephalon levels of TH and EF1 α changed together (compare 4C and 4D), in contrast to differential regulation in the telencephalon. Cocaine induced a significant change in TH ($t = 3.3$, $p < 0.05$) and a similar change in EF1 α though the latter was not statistically significant ($t = 3.0$, $p > 0.05$). These changes were offset with co-treatment of SCH23390 ($t = 4.2$, $p < 0.01$ for TH and $t = 5.1$, $p < 0.01$ for EF1 α , when compared to cocaine alone), but not sulpiride ($t = 1.7$, $p > 0.05$ for TH and $t = 2.3$, $p > 0.05$ for EF1 α , when compared to cocaine alone). When normalized, none of the drug treatments resulted in any change in the ratio of TH to EF1 α (see Figure 4B $p > 0.05$ for all comparisons). Thus, changes in diencephalic gene expression induced by dopaminergic antagonists did not parallel changes in behavior in the same manner seen in the telencephalon. Lidocaine was used as a control in these studies and produced no changes in either telencephalic or diencephalic TH and EF1 α protein levels. Telencephalon EF1 α was 7958 ± 693 , TH was 4721 ± 540 ; Diencephalon EF1 α was 5003 ± 1739 , TH was 6477 ± 1916 . Comparisons of TH/EF1 α ratios between lidocaine and untreated controls showed no significant difference using least means squared analysis [Abs (Dif)-LSD with $\alpha = 0.05$; -316.5 , and -178.3 for telencephalon and diencephalon, respectively].

3.3 Cocaine induces regional changes in TH expression within the telencephalon

Given the results with western blotting we wanted to try localizing the changes in TH and EF1 α expression in the telencephalon, since these changes, when considered as a ratio, most closely correlated with CPP. We compared untreated brains with those from fish treated with cocaine. Dopaminergic cell groups in the region we refer to as the telencephalon include obvious neurons in and around the dorsal nucleus of the ventral telencephalon (Vd), as well as the anterior preoptic area (PPA) The Vd group is represented schematically in Figure 5A and extends for approximately 100 μm , including sections diagrammed in Figures 5B and C. Sections were matched using the cerebral blood vessels, morphology of the dorsal telencephalon, and the appearance of the midline as anatomical markers. The dopaminergic cell groups surrounding Vd also provided convenient anatomical markers for comparison between brains. After staining, we focused on dopaminergic cell groups for comparison, assuming that level of expression, rather than the number of positive neurons, would be different between conditions. Figure 5 (D–I) shows representative sections from three different pairs of untreated and cocaine treated brains. Figures 5D (untreated) and 5F (cocaine treated) show sections from one pair of brains that corresponds to the schematic

labeled 1. Figures 5E (untreated) and 5G (cocaine treated) correspond to the schematic labeled 2. Figures 5H and 5I show higher magnification of a third pair of brains and corresponding to the schematic 2.

TH expression changes in the western analysis might be due to changes in levels localized to terminals of diencephalic projection neurons, or local changes in expression by telencephalic neurons. While dopaminergic terminals were clearly visible in treated and control brains, it was impossible to assess any obvious difference at this level of investigation. All confocal images were shot at identical laser intensities, so differences in the brightness of telencephalic dopaminergic cell bodies (stained green in Figure 5) are reflective, at least in part, of expression differences, though this is hardly quantifiable. Along these lines, when examined at high power (Compare 5H with 5I), cocaine treated brains did have brighter TH positive cells, indicating that at least some of the modest increase seen in the western blots was localized to telencephalic dopaminergic neurons. While the significance of the TH results is certainly provocative, TH labeling in the telencephalon was perhaps most useful in providing the anatomical basis for comparison of EF1 α expression in the ventral telencephalon.

We had assumed the expression of EF1 α in the adult brain would be ubiquitous, but this was not the case with the antibody used. In untreated fish, expression was most prevalent in the Vd, Vm, and Dm. Many EF1 α cells (labeled red in Figure 5) were clustered around the TH-positive neurons, but the two markers only co-localized at punctate junctions, most likely synaptic contacts (thin arrows in Figure 5H). The origins of the TH-positive terminals cannot be reliably interpreted at this level of analysis, but the close proximity of the two cell types suggest that at least some of the Vd EF1 α -positive neurons could be regulated by local TH-positive neurons as well as by projection neurons from the diencephalon. EF1 α staining in all cocaine treated brains was profoundly lower relative to that of untreated controls and this was particularly obvious in the Vd. While EF expression in the Dm was comparable between treatment groups (compare 5D and 5F, for example), this difference was more variable than that seen in Vd. The effect of cocaine on EF1 α expression was seen in every brain examined and was much more pronounced than what our western blotting analysis would have led us to predict.

4. Discussion

4.1 Cocaine-induced CPP is blocked by sulpiride but not SCH23390

There is a growing interest in combining the powerful forward genetics approaches available with the zebrafish model system to find genes underlying complex behaviors, such as those associated with addictive drugs (Gerlai et al 2000; Ninkovic & Bally-Cuif 2006). In connection are a growing number of studies designed to validate this approach by assessing the neuroanatomical and neurochemical similarities of zebrafish and mammalian brains, including the neural pathways mediating reward (Ma 1997; Rink & Wullimann 2002). Extending this effort are recent studies reporting pharmacological bases (Lau et al 2006; Ninkovic et al 2006; Swain et al 2004) and molecular response (Kily et al 2008; Webb et al 2009) of these circuits to addictive drugs. We previously reported screening mutagenized zebrafish for abnormalities in cocaine-induced CPP (Darland & Dowling 2001). In the present study we attempted to determine if the dopaminergic system mediates the cocaine-induced CPP response and if select gene expression profiles are altered by acute cocaine exposure in wild type zebrafish.

A series of classic studies identified the ventral telencephalon as the recipient of dopaminergic projections from cell groups in the diencephalon (Rink & Wullimann 2002; 2004). These studies have been built upon using expression profiles of several dopaminergic

markers, including the two TH genes (Yamamoto et al 2010; 2011). The comparative picture that has emerged is that, in contrast to separate projections extending from the substantia nigra and the VTA to the dorsal and ventral striatum respectively, teleosts have a single pathway from a diencephalic region called posterior tuberculum (PT) to the ventral telencephalon, most notably the Vd, with a subset of fibers projecting to the dorsal telencephalon, in particular the Dm (Yamamoto & Vernier 2011). Both the Vd and Dm have been implicated in regulating zebrafish behavior; displaying changes in c-fos expression correlating with light-dark choice (Lau et al 2011).

Cocaine produces its psychostimulant effects by blocking monoamine transporters; but while elevated levels of serotonin and norepinephrine have well documented effects on behavior, it is the elevation in dopamine levels that have been most closely correlated with reward (Hall et al 2004). The effects of dopamine that underlie reward are presumably mediated by dopamine receptors (DR). The dopamine receptors are grouped as type I, including DR1 and DR5, which modulate neural activity via a G_s-coupled signaling pathway, and type II, including DR2, DR3 and DR4, which operate via a G_i-coupled signaling pathway. Most pharmacological and transgenic studies implicate DR1 and DR2 in mediating much of the rewarding effects of psychostimulants in the NAc; however, the interaction between these two receptor types is not entirely understood (Hall et al 2004). The classic model has DR1 expressed on the spiny GABAergic neurons projecting from the NAc shell to the ventral pallidum and mediating reward. The D2 receptor has been hypothesized to operate presynaptically on the dopaminergic neurons themselves, modulating dopamine release at the synapse. However, evidence also points to DR1 and DR2 being expressed on distinct populations of neurons in the NAc shell, with DR1 expressed by GABAergic projection neurons and DR2 expressed by cholinergic interneurons (Bertran-Gonzalez et al 2008; Holmes et al 2004).

The zebrafish DR1, DR2, DR3 and DR4 have all been cloned (Boehmler et al 2007; Boehmler et al 2004; Li et al 2007), but the detailed expression patterns, alternative splice variants, and pharmacology of all these receptors in zebrafish has not yet been fully described. In other teleosts, however, telencephalic expression of DR1 and DR2 overlaps and is particularly prevalent in structures equivalent to the Vd and Dm in zebrafish (Kapsimali et al 2000; Vacher et al 2003).

We present evidence for the importance of DR2, but not DR1 in mediating cocaine-induced CPP in zebrafish. In behavioral studies using co- treatment with sulpiride, the DR2 antagonist abolished cocaine-induced CPP, while SCH23390, the DR1 antagonist, had little effect (Figure 1B). Since DR2 is most highly expressed in the Vd and Dm, these areas are excellent candidates for the teleost equivalent of the NAc. Studies investigating CPP induced by other addictive drugs in zebrafish have antagonized the behavioral response with dopaminergic compounds. SCH23390, for example blocked morphine-induced CPP (Lau et al 2006). Sulpiride had the same effect, but only at much higher doses than that used in the present study. While methodology differences might lead to disparate effects, it is intriguing to speculate that CPP induced by cocaine and morphine are mediated by distinct, yet interrelated neural circuits.

4.2 Cocaine induces a decrease in TH2, but no change in TH1 mRNA levels

This study began as an investigation into transcript-level changes in addiction relevant genes. In a preliminary screen of candidate genes, TH2 was the one transcript that provided the most robust and consistent change, decreasing almost 50% with cocaine treatment. EF1 α was used as a control transcript. In contrast to the single gene found in mammals, two TH genes have been characterized in teleosts (Candy & Collet 2005). A comprehensive study on expression patterns of the two isoforms has shown the expression patterns to be largely

distinct, with some notable areas of overlap (Yamamoto et al 2010). One area in which both isoforms are expressed is the anterior preoptic area (PPa), a region included in our dissection of the telencephalon. In our efforts to validate the QPCR with western analysis, we were limited by our choice of reagents. In our western blot experiments the antibody clearly recognized a single band approximately 60 kD, similar in size to that reported in other species. As it turns out, the antibody recognizes the N-terminal of TH and therefore probably recognizes TH1 in the zebrafish, since the TH2 protein differs considerably in this region (Candy & Collet 2005). We were therefore unable to validate the TH2 QPCR results at the protein level. Future analysis will make use of other antibodies that are more effective at distinguishing the isoforms (Yamamoto et al 2010). The dopaminergic neurons of the PPa are believed to project back to the hypothalamus in the diencephalon (Yamamoto et al 2011). The dopaminergic neurons originating in the PT and projecting to the ventral telencephalon are all TH1 positive, but TH2 negative. It is therefore difficult to speculate on how TH2 might impact reward. It was fortuitous that examination of the TH2 transcript initiated an examination of protein levels because we found that in contrast to mRNA levels, TH1 protein was noticeably changed by cocaine. Furthermore, the control we were using, EF1 α , turned out to be dramatically regulated by cocaine treatment independently of TH1.

The fact that TH protein level rises while mRNA decreases, or stays the same, is not necessarily surprising, given that regulation of the TH gene involves almost every known type of transcriptional, post-transcriptional, translational, post-translational and allosteric mechanism (Kumar & Vrana 1996). Post-translational regulation by mRNA stabilization could be induced by cocaine in zebrafish, a possibility that has not been reported for other species. Our results showing change in TH expression in zebrafish differs somewhat from reports in rodents. Most studies examining chronic cocaine exposure report an increase in TH expression, while those looking at acute exposure report little or no change in expression (Beitner-Johnson D 1991; Sorg et al 1993). Presumably chronic exposure causes a depletion of dopamine stores, which stimulates TH gene expression. Perhaps, in zebrafish, the stores are depleted faster, or the signal that stimulates gene expression is more robust than in mammals. Regulation of DA neurons in the mesolimbic systems of mammals is likely far more complex because of extensive cortical input, which is undoubtedly less developed in the teleost brain. There are also the local dopaminergic circuits in the zebrafish telencephalon that are not present in mammals. Mutant zebrafish having lower TH expression also display lower food and opiate induced CPP (Bretaud et al 2007). On the other hand, experiments examining microarrays after cocaine treatment, changes in TH mRNA were not mentioned (Kily et al 2008). However, microarray analysis typically regards changes lower than 1.5 to 2-fold insignificant. The changes in TH2 that we saw would likely not have been detected.

4.3 Cocaine-induced changes in gene expression are also blocked by sulpiride but not SCH23390

The effect of the dopamine antagonists on behavior closely paralleled effects on protein expression in the telencephalon (compare Figure 1B and 3B). We saw a 60% increase in the telencephalic ratio of TH to EF1 α protein after cocaine treatment above untreated and lidocaine treated controls. The profound changes in the telencephalic TH/EF1 α ratio with cocaine treatment were driven by modestly increased TH and decreased EF1 α levels that, when examined independently, were not statistically significant. Co-treatment of the D2 antagonist sulpiride completely eliminated cocaine-induced elevation of the TH/EF1 α ratio. In contrast, the D1 antagonist SCH23390 had no effect on the cocaine-induced increase in TH/EF1 α ratio. The close parallel between gene expression changes and behavioral effects mediated by the antagonists suggests that the increase in TH and the decrease in EF1 α *together* are important for reward. In contrast, the diencephalic changes in gene expression

did not correlate with behavioral responses. Furthermore, in the diencephalon TH and EF1 α expression were co-regulated, while in the telencephalon the two genes were differentially regulated. This is certainly not to say that TH expression in the diencephalon is irrelevant in reward, indeed the increased TH in the telencephalon may well be driven in large part by increased synthesis in the projection neurons of the diencephalon. Cocaine may simultaneously drive EF1 α and TH expression in projection neurons, a possibility that will require a more extensive immunohistochemical analysis of the diencephalon, which is beyond the scope of this study.

Immunohistochemical analysis of the telencephalon supported and augmented the western analysis on the effect of cocaine on the ventral telencephalon. Western analysis indicated that TH and EF1 α are differentially regulated by cocaine and dopamine antagonists, suggesting that expression might be segregated into different cell types. Immunohistological analysis supports this. Wherever we looked in the telencephalon, TH and EF1 α we expressed in different cells. Co-localization was limited to punctate staining at the surface of EF1 α -positive cells, indicating that a subset of these EF1 α -positive cells may be regulated by dopamine. Increased TH protein by itself was not significant in western analysis, but was generally higher than in untreated brains. We explored this further using immunohistochemistry. While we could not assess the state of terminals from projection neurons we did notice that cell bodies of dopaminergic neurons in the ventral telencephalon, a region referred to as the subpallidum, were consistently brighter than those in untreated cells. These cells project to other regions of the telencephalon and to diencephalic targets (Rink & Wullimann 2004). Since these cells were in close proximity to the EF1 α cells of the Vd, we cannot discount the possibility that part of the increased TH protein detected originates in these cells and that they may even play some role in reward. One study looking at MPTP treatment of goldfish correlated the loss of dopaminergic neurons in the ventral telencephalon with Parkinsonian-like syndrome in goldfish (Goping et al 1995). It may be that the equivalent population in zebrafish residing in the subpallidum, near the Vd could regulate some aspect of locomotion or reward.

Our immunohistochemical data show that expression of EF1 α is profoundly decreased in the Vd with cocaine treatment. Given the differential effects of dopamine antagonists on cocaine-induced behavioral and gene expression changes, it may be possible to use EF1 α as a marker in a more extensive immunohistochemical analysis of fish co-treated with cocaine and dopaminergic antagonists to localize the teleost equivalent of the NAc. EF1 α is a translational elongation factor, important in regulating protein synthesis. Beyond its role as a so-called housekeeping gene, there has not been much published with regard to a specific role in complex behavior. EF1 α mRNA and protein are upregulated in hippocampal dendrites in response to stimuli that normally induce long-term depression (Huang et al 2005). In one study examining the impact of cocaine in regulating HIV infection of human astrocytes, the drug was shown to significantly decrease EF1 α protein (Reynolds et al 2006). EF1 α has not been specifically mentioned in the many studies looking at gene expression in the NAc after drug treatment, though most of these reports involve transcriptional analysis, rather than analysis at the protein level. The potential role for EF1 α may not be reward specific, but rather a general neural mechanism for strengthening or diminishing signal conduction across dopaminergic synapses.

5. Conclusions

The question remains how good a model organism is zebrafish for the study of addiction? Clearly zebrafish have a dopaminergic pathway that drives reward. The change in TH expression with cocaine that we observed is somewhat different than that which has been described for rodents; but this may point to an advantage, the relative simplicity of the

zebrafish nervous system. What may prove most telling is whether cocaine regulates genes like EF1 α in higher vertebrates. It will also be interesting to expand the examination in zebrafish to include other candidate genes such as the calcium response element binding protein (CREB), FosB, DA related protein phosphatase (DARPP-32) and the dopamine transporter, among others shown to be rapidly regulated by cocaine in mammals, to see how conserved the reward pathway is in teleosts (Nestler 2004; Nestler 2005). Such comparisons will be critical in assessing the use of zebrafish in finding novel “addiction genes”.

Acknowledgments

This investigation was supported through (TD) NIH-NIDA (KO1 DA016291-02). This work was also supported in part by National Science Foundation (NSF) Research Experience for Undergraduates (REU) Site Grants 0639227 and 0851869 (PIs Drs. Van Doze and Peter Meberg). It was also supported by the NIH National Center for Research Resources Grant P20RR016471 (PI Dr. Don Sens). Additional resources came from (DCD) NIH-NINSS R15 NS057807-01. We would also like to thank Sarah Abrahamson of the UND Medical School Imaging Core for her stellar technical assistance with the multi-photon microscope. The imaging core is supported by an NIH Center of Biomedical Research Excellence.

Abbreviations

CPP	conditioned place preference
DR1	dopamine receptor type 1
DR2	dopamine receptor type 2
Dm	medial zone of the dorsal tegmental area
EF1α	elongation factor 1 alpha
NAc	nucleus accumbens
PPa	anterior preoptic area
PT	posterior tuberculum
TH	tyrosine hydroxylase
Vd	dorsal nucleus of the ventral telencephalic area
VTa	ventral tegmental area
Vv	ventral nucleus of the ventral telencephalic area

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Highlights

- We describe a new conditioned place preference (CPP) assay used for zebrafish.
- We describe the impact of dopamine antagonists on CPP.
- We show gene expression changes in the telencephalon that correspond with CPP.
- EF1 α is lowered in the ventral telencephalon of cocaine-treated fish.

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Figure 1. Cocaine-induced CPP is abrogated by a D2 antagonist, but not a D1 antagonist

(A) The three-partition CPP chamber is shown with the rear compartment indicated. The fish were allowed to habituate to the apparatus and were tested for baseline preference. After confinement to the rear compartment, they were untreated, cocaine-treated (10 $\mu\text{g/L}$), or cocaine-treated (10 $\mu\text{g/L}$) with either sulpiride (10 μM) or SCH23390 (10 μM). Subsequent conditioning trials one day (CPP1) and two days (CPP2) after initial exposure were conducted. Scale bar is 9 cm. (B) The values for time spent in the rear compartment relative to initial treatment condition are graphed as the mean and standard deviation (SD) for six animals comparing baseline, CPP1, and CPP2. Repeated measures ANOVA indicated a significant percentage of variation from treatment condition [F (DFn = 3, DFd = 40) = 8.05, $p < 0.001$; 17.32%], baseline versus CPP1 and CPP2 [F (DFn = 2, DFd = 40) = 22.62, $p < 0.0001$; 29.89%], and the interaction between these variables [F (DFn = 6, DFd = 40) = 3.04,

$p < 0.0153$; 12.03%]. Bonferroni posttests to compare baseline to either CPP1 or CPP2 for each treatment showed significant differences for the cocaine-treated or cocaine plus SCH23390-treated groups. Asterisks reflect level of significance for pairs with “**” indicating $p < 0.01$, “***” indicating $p < 0.001$ and “n.s.” indicating paired comparisons were not significant. This experiment has been repeated three times with similar results and a single representative experiment is shown.

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Figure 2. The tyrosine hydroxylase 2 (TH2) mRNA but not the TH1 mRNA is reduced by cocaine treatment

(A) Schematic [traced and adapted from (Wullmann 1996)] showing brain dissections used in this study. The light gray indicates the telencephalon, dark charcoal gray indicates the region we call the diencephalon, stippled gray indicates the brainstem, and the cerebellum is black. QPCR analysis for EF1 α (B), TH1 (C), and TH2 (D) is graphed as the mean $\pm$ SD of 6 samples expressed as the number of attograms (ag) relative to the amount of RNA in the original cDNA reaction and compared to a gene-specific amplicon standard curve. Zebrafish were untreated or treated with cocaine (10 μ g/L) for one hour and the brains microdissected in RNALater^{TM}. Total RNA (200 ng) was reverse transcribed to cDNA and amplified with gene-specific primers. The efficiencies for the three transcripts were as follows: EF1 α (99.9%), TH1 (99.9%), and TH2 (99.6%). An unpaired, two-tailed t test was used to compare the natural log transformed values for untreated and cocaine-treated populations with “**” indicating $p < 0.01$.

Figure 3. TH and EF1α protein levels in the telencephalon change significantly in response to cocaine

(A) A representative western blot of TH and EF1α protein bands in lysates from telencephalon is shown with the treatment conditions indicated below. Adult animals were untreated, cocaine-treated (10 μg/L), or cocaine-treated (10 μg/L) with either sulpiride (10 μM) or SCH23390 (10 μM) for 1 hour. The brains were microdissected into lysate buffer and 20 μg of total protein were loaded per lane. The relative density of the TH and EF1α bands is graphed as a ratio for (B) with the mean ± SD of 3–6 samples pooled from three independent experiments. The relative density values for TH (C) and EF1α (D) are shown for comparison to highlight the changes in individual protein levels that occur with treatment condition. The ratios for TH/EF1α were transformed to the natural log and the population means compared by one-way ANOVA. The ANOVA p values for the transformed ratios [F (DFn = 3, DFd = 12) = 12, p < 0.0006], the TH alone [F (DFn = 3, DFd = 12) = 14, p < 0.0004], and the EF1α alone [F (DFn = 3, DFd = 12) = 4, p < 0.0355] indicated that the population means were significantly different. The post hoc analysis was completed with Bonferroni's Multiple Comparison Test for pair-wise comparison. Significant differences between paired means are indicated with asterisks; "*" indicating a p < 0.05, "***" indicating p < 0.01, and "****" indicating p < 0.001.

Figure 4. TH and EF1α protein levels in the diencephalon are changed in response to cocaine
 (A) A representative western blot of TH and EF1α protein bands in lysates from diencephalon is shown with the treatment conditions indicated below. Adult animals were untreated, cocaine-treated (10 μg/L), or cocaine-treated (10 μg/L) with either sulpiride (10 μM) or SCH23390 (10 μM) for 1 hour. The brains were microdissected into lysate buffer and 20μg of total protein was loaded per lane. The relative density of the TH and EF1α bands is graphed as a ratio for (B) with the mean ± SD of 3–6 samples pooled from three independent experiments. The ratios for TH/EF1α were transformed to the natural log and the population means compared by one-way ANOVA. The ANOVA p values for the transformed ratios [F (DFn = 3, DFd = 12) = 1.3, $p < 0.3054$], the TH alone [F (DFn = 3, DFd = 12) = 7.3, $p < 0.0049$], and the EF1α alone [F (DFn = 3, DFd = 12) = 9.4, $p < 0.0018$] indicated that the population means were significantly different only when the proteins were considered alone rather than as a ratio. The post hoc analysis was completed with Bonferroni's Multiple Comparison Test for pair-wise comparison. Significant differences between paired means are indicated with asterisks; "*" indicating a $p < 0.05$, "**" indicating $p < 0.01$, and "***" indicating $p < 0.001$.

Figure 5. The distribution of TH and EF1 α positive cells in the telencephalon and changes seen with cocaine treatment

Sections were cut and stained from the telencephalon of 5 untreated and 5 cocaine-treated fish. Sections were compared for immunoreactivity by aligning morphology of the brain, presence and shape of associated blood vessels and the presence, morphology, and number of TH-positive cells. The sections from three untreated and three cocaine-treated brains compared in this figure correspond to lines numbered 1 and 2 on the brain schematic (A). Schematics for the cross sections are shown in (B) and (C). The sections in (D) and (F) correspond to schematic 1 and the sections in (E), (G), (H) and (I) correspond to schematic 2. TH positive cells and processes are green and EF1 α positive cells are red. Comparing (D) and (F), or (E) with (G), at 20X magnification, it is clear that the number of EF1 α positive cells are markedly reduced in the cocaine-treated brains. This is particularly evident in the dorsal nucleus of the ventral telencephalic area (Vd), but not in the medial nucleus of the dorsal telencephalic area (Dm). The scale bar in panels D-G represents 100 μ m. Higher magnification shots (60X) of a third pair of brains is shown in H and I. Again there is a marked reduction of EF1 α expression in cells of the Vd immediately adjacent to the TH-positive neurons of the subpallidum of the cocaine treated brain. The arrowheads point to EF1 α positive neurons in the Vd of the untreated brain. Thin arrows indicate apparent synaptic contacts between TH-positive terminals and EF1 α -positive neurons. The scale bar in H and I represents 30 μ m.

Table I

Gene-specific primer sequences for qPCR, amplicon size, and reference source.

Gene	Sequence (5'-3')	Size	Reference
EF1 α	5'-CTGGAGGCCAGCTCAAACAT-3' (F) 5'-ATCAAGAAGAGTAGTACCGCTAGCATTAC-3' (R)	87	(Tang R 2007)
TH1	5'-TGAAGAGAAGTGCAGAGGATGTCA-3' (F) 5'-ATTTCGGCGATCATCTCCGTCGT-3' (R)	184	AL954320
TH2	5'-AGACAGCTTCGTGTTTGAGGAGGA-3' (F) 5'-TCTTTCTGCTCGCTCGACTTTCCA-3' (R)	161	AL845537