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SCHOOL OF MEDICINE

Dissertation

EFFECTS OF COCAINE ON VISUAL PROCESSING IN ZEBRAFISH

by

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B.S., Massachusetts Institute of Technology, 2008

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requirements for the degree of

Doctor of Philosophy

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The truth may be puzzling. It may take some work to grapple with. It may be counterintuitive. It may contradict deeply held prejudices. It may not be consonant with what we desperately want to be true. But our preferences do not determine what's true. We have a method, and that method helps us to reach not absolute truth, only asymptotic approaches to the truth — never there, just closer and closer, always finding vast new oceans of undiscovered possibilities. Cleverly designed experiments are the key.

-Carl Sagan

DEDICATION

This work is dedicated to my son Silas, and to all the other children of his generation, in the hope that some very small piece of it may one day contribute to their health and happiness or relieve their suffering.

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First, my thanks to my zebrafish. Some of them were sacrificed for this work, and others may have suffered discomfort in my hands. Their lives were valued.

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EFFECTS OF COCAINE ON VISUAL PROCESSING IN ZEBRAFISH

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Boston University School of Medicine, 2015

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ABSTRACT

Psychostimulants are known to alter visual function acutely and on withdrawal, and can cause attention deficit following prenatal exposure. However, psychostimulants can also improve visual attention in patients with attention deficit. The mechanisms involved in these contrasting effects remain largely unknown.

To determine the role of specific brain regions and the dopamine system in the impact of cocaine exposure on visual processing, we employed two-photon microscopy and a transgenic larval zebrafish expressing the calcium indicator GCaMP-HS. We documented neuronal responses to contrasting visual stimuli, red light (LF) and dark (DF) flashes.

We found that in the optic tectum neuropil (TOn), both stimuli elicited similar responses, though after repeated stimulus presentation, habituation developed to dark flash only. The dorsal telencephalon (dTe) responded and habituated to LF only. Acute cocaine (0.5 μM) reduced neuronal responses to LF in both brain regions and prevented habituation of dTe neurons to LF, but did not modify responses or habituation to dark flash.

Prenatal cocaine exposure (PCE) did not modify baseline responses, but it prevented the acute effects of cocaine on LF responses in both regions and habituation in

dTe, with no impact on dark flash responses. PCE also significantly reduced D1 dopamine receptor expression in TOn and cerebellum, but not dTe or the eye.

Fish lacking the dopamine transporter (DATKO) retained normal D1 expression throughout the brain, baseline responses to LF in both TOn and dTe, and response reduction following cocaine in TOn. However, they demonstrated abnormal swimming behavior, and neither their swimming behavior nor dTe responses to LF were modified by cocaine.

We discovered that in zebrafish, a diurnal vertebrate, responses to light not only require the primary visual processing center TO (superior colliculus in mammals), but also higher level processing by dTe. Responses to light but not darkness are modified by cocaine, unless the fish lack DAT or were exposed to PCE. Together, our results demonstrate specific effects of cocaine on visual processing mediated by the dopamine system, and provide a novel animal model for further investigation of these phenomena and development of new therapeutic approaches.

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LIST OF ABBREVIATIONS

ADHD .... attention deficit hyperactivity disorder

DAT .... dopamine transporter

DATKO .... dopamine transporter knockout

DF .... dark flash

dpf .... days post fertilization

dTe .... dorsal telencephalon

GBC .... genetic background control

GECI .... genetically encoded calcium indicator

hpf .... hours post fertilization

LED .... light emitting diode

LF .... light flash

NET .... norepinephrine transporter

PCE .... prenatal cocaine exposure

SERT .... serotonin transporter

SPV .... stratum periventriculare

Te .... telencephalon

TO .... optic tectum

TOn .... optic tectum neuropil

UAS .... upstream activating sequence

ZT .... Zeitgeber time

GLOSSARY

Genetically encoded calcium indicators (GECIs) and GCaMP: engineered proteins produced by fusing a fluorescent protein with a calcium-sensitive protein such that the resulting product fluoresces more brightly when calcium is bound. The gene encoding a GECI can then be inserted into the genome of an animal (transgenesis) at a precise location such that it is expressed only in certain areas of interest. In these areas of interest, the GECI will fluoresce more brightly when calcium levels increase, which occurs whenever neurons are active. GCaMP, of which there are now many improved versions, is a fusion between green fluorescent protein (GFP) and calmodulin and was originally produced in 2001 (Nakai, Ohkura, & Imoto, 2001).

Optic tectum: a major component of the mesencephalon (midbrain) in vertebrates. The optic tectum is referred to as the superior colliculus in mammals. It processes sensory information and projects to motor areas. A principal function in mammals is controlling eye movement. In zebrafish it is the main visual processing center.

Telencephalon: the most rostral and most complex part of the forebrain. It is responsible for higher-order processing and voluntary behavior. In mammals it contains the cerebral cortex and basal ganglia. Homology between specific cortical structures in mammals and regions of the telencephalon in zebrafish is just beginning to be understood.

Zebrafish (*Danio rerio*): a small (1-inch) freshwater teleost fish native to the Indian subcontinent. They are popular as an animal model for the study of vertebrate development, neuroscience, genetics, and other areas of biology and medicine.

CHAPTER ONE

Introduction

The goal of this project was to investigate the effects of both acute and prenatal cocaine exposure on visual processes. It was motivated both by numerous reports in the literature of harmful changes to visual processing and visual attention following exposure to this psychostimulant (Church, Crossland, Holmes, Overbeck, & Tilak, 1998; Good, Ferriero, Golabi, & Kobori, 1992; A. Heffelfinfger, Craft, & Shyken, 1997; Siegel, 1978), and by the well-known benefits of psychostimulant drugs on attention. The mechanisms behind these contrasting effects are still unknown. We wanted to understand how, and where within the brain, cocaine modulates responses to simple visual stimuli during acute exposure. Next we addressed whether prenatal cocaine exposure influenced either baseline responses to visual stimuli, or the subsequent actions of cocaine later in life. After characterizing several effects of cocaine on visual processing, we then investigated the role of specific molecular targets in mediating those effects.

We accomplished these goals by recording changes in neural activity in response to contrasting visual stimuli in two relevant brain areas simultaneously, the telencephalon (Te) and optic tectum (TO). We studied these responses during acute exposure to cocaine in drug-naïve animals, and also following cocaine exposure during early embryonic development. To further investigate the mechanisms responsible for the effects of cocaine on visual processing, we also studied neuronal responses, behavior and gene expression in animals lacking cocaine's canonical molecular target, the dopamine transporter (DAT).

In studying visual behavior and its neuronal correlates, the choice of model organism is critical. In most vertebrate models, it is difficult to observe neural activity in several brain areas simultaneously with microscopy, especially at single cell resolution. It typically requires cranial surgery to implant viewing a viewing port and partial restraint of the animal during imaging, as well as injection of an indicator or virus carrying an indicator gene into the regions of interest (Leinweber et al., 2014). Furthermore, due to the optical properties of brain tissue, data can only be gathered from a depth of 1-2 mm (Kobat, Horton, & Xu, 2011), limiting the amount of information that can be collected. Considering that behavioral responses to visual stimuli depend in part on their adaptive value, it is also important to consider whether an animal is diurnal, like humans, or nocturnal.

For our studies we chose zebrafish (*Danio rerio*), a diurnal vertebrate, as our model. The special characteristics of young zebrafish, referred to as larvae, allow for a set of effective techniques to be employed. Larval zebrafish are transparent and small, around 4 mm in length at 5 days post fertilization (dpf) when they become active (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Their optical transparency means that direct, live recording of neuronal activity in their 0.5 mm brains is possible without the need for surgery or invasive procedures of any kind. Larvae are capable of transcutaneous respiration, allowing them to be lightly restrained in soft agar for imaging, without the need for sedation or anesthesia, for several hours with no ill effects. It is even possible to restrain just the head, leaving the rest of the body to move freely and provide a behavioral correlate of the neuronal activity being recorded (Aizenberg &

Schuman, 2011). At 7 dpf, these diurnal vertebrates are independent swimmers capable of hunting live prey by sight (Nevin, Robles, Baier, & Scott, 2010) and classical conditioning (Aizenberg & Schuman, 2011), and display a wide variety of instinctive behaviors (Nevin et al., 2010).

Zebrafish are genetically tractable, and many transgenic lines have been developed which allow targeted expression of genetically encoded calcium indicators (GECIs). GECIs are indicators that fluoresce more brightly in the presence of calcium. Because calcium concentration increases significantly when a neuron is active, it is an excellent proxy for neural activity (Muto et al., 2011; Rose, Goltstein, Portugues, & Griesbeck, 2014). Thus GECIs allow visualization of neural activity wherever they are expressed in the brain. The use of two-photon microscopy allows excitation of GECIs with infrared light invisible to the zebrafish, and better tissue penetration than with visible lasers. In darkness, test visual stimuli can be presented to the larvae without distraction. The fortuitous combination of imaging suitability and rapid neurological development makes zebrafish very valuable as a model organism for the study of visual and behavioral neuroscience.

To address our goal of understanding the impact of acute and prenatal cocaine exposure on visual processing in a diurnal vertebrate, and the mechanisms involved, we first developed several transgenic zebrafish lines expressing GECIs in areas of the brain related to visual processing, the optic tectum and telencephalon. This chapter describes the details of the development of these transgenic lines.

s1013t-GCaMP-HS

To make the s1013t-GCaMP-HS line, which expresses the GECI GCaMP-HS and was used for the majority of experiments in this project, we started with two transgenic lines developed by other labs. The first was UAS: GCaMP-HS (Muto et al., 2011), produced by Dr. Akira Muto in the laboratory of Dr. Koichi Kawakami at the National Institute of Genetics in Japan, and given to us as a generous gift. In this line, GCaMP-HS is not expressed because UAS ("upstream activating sequence") is not bound by the protein Gal4. GCaMP-HS expression can be induced by crossing the line to one that expresses Gal4. GCaMP-HS was first developed in 2011 and has better fluorescent and kinetic properties than previous GCaMP fluorophores. It can detect as few as 3 action potentials (Muto et al., 2011). The second transgenic line, s1013t, derived from an enhancer trap screen, drives expression of the Gal4:VP16 protein primarily in the optic tectum and dorsal telencephalon, under control of an unknown promoter. It was produced by the laboratory of Dr. Herwig Baier ("s1013t") (Scott & Baier, 2009) and obtained from the Zebrafish International Resource Center. When the two lines are bred together, in the offspring, Gal4 is produced in the optic tectum and telencephalon, and drives GCaMP-HS expression in those structures via the UAS. Because the original s1013t line carries kaede, a green photoconvertible fluorophore, we screened for GCaMP-HS-positive, kaede-negative F1 offspring by illuminating 24 hours post fertilizaiton (hpf) embryos with full-spectrum light from a hydrogen lamp for 30 seconds, and then selecting only those embryos which did not show any color change. The embryos were grown up and interbred around 3-4 months of age, and their F2 offspring

were used for our initial experiments (Fig. 1). F3, F4 and F5 offspring were also used over the next 3 years. Because we were unable to determine, by visual inspection, which embryos were homozygous for GCaMP-HS, almost all of our adult s1013t-GCaMP-HS zebrafish are heterozygotes. 75% of their offspring carry GCaMP-HS, and GCaMP-HS-positive individuals were selected by hand at 24 hpf.

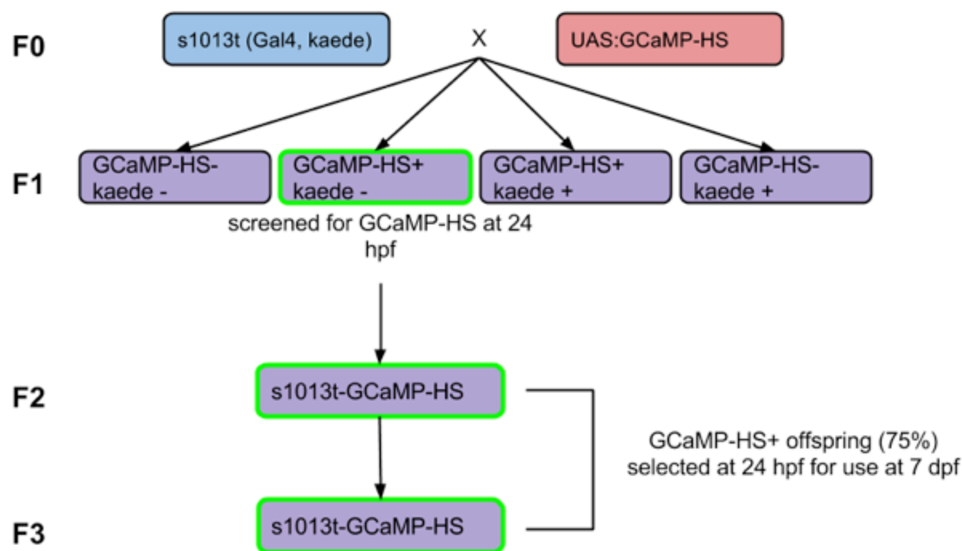

Figure 1. Breeding schematic for s1013t-GCaMP-HS transgenic line. F0-F3, consecutive generations.

s1013t-GCaMP-HS zebrafish express GCaMP-HS most prominently in the optic tectum (TO) (Fig. 2). Expression in this structure was characterized by the lab in which the s1013t line was made (Scott & Baier, 2009), showing that approximately 40% of labeled cells are periventricular neurons and 40% are radial glia, both of which have cell bodies in the stratum periventriculare (SPV). The other 20% of cells are superficial or neuropil neurons, or shallow stratum periventricular neurons. Overall, virtually all tectal cells, and their processes which comprise the neuropil portion of the TO, are labeled.

There is also strong expression in the pallium and the eye. Weaker expression is observed in the hindbrain, spinal cord, notochord, and muscles (Fig. 2)

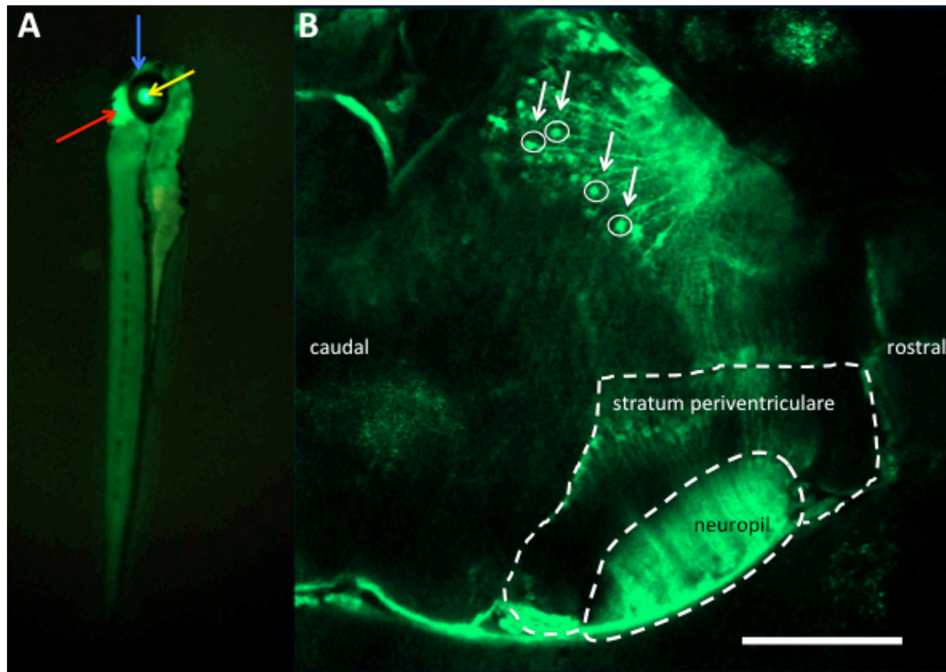

Figure 2. GCaMP-HS expression in a 7 dpf s1013t-GCaMP-HS transgenic larva. (A) Side view of whole fish using a fluorescent dissection microscope (40x). Red arrow - TO, blue arrow - Te, yellow arrow - lens of eye. (B) Optic tectum, two-photon image, dorsal view (scale bar 100 µm). Circles with arrows show individual periventricular neurons, with processes extending into the neuropil layer.

s1013t-GCaMP-7

A new GCaMP transgenic line, UAS:GCaMP-7a (Muto, Ohkura, Abe, Nakai, & Kawakami, 2013), with improved properties became available in 2012. This new version of GCaMP, also produced and gifted to us by Dr. Akira Muto, is capable of detecting single action potentials due to a 4-fold increase in calcium sensitivity. To see whether the improved fluorophore would allow us to image single-neuron activity in TO and Te, we developed the s1013t-GCaMP-7a line. Using the same procedure described above, we bred the UAS:GCaMP-7a line with the s1013t transgenic line. The resulting transgenic line was used in the calcium imaging experiment described in the Appendix, but could

not be substituted for the s1013t-GCaMP-HS line because it would have resulted in data sets which could not be compared with each other.

s1013t-GCaMP-HS-DATKO

After observing several effects of cocaine on visual processing (see Chapter 2), we wanted to determine whether any of the effects were mediated by the dopamine system. To do this, we needed a transgenic line lacking cocaine's dopamine system target, DAT. A DAT knockout (DATKO) line was generously gifted to us by Dr. Jing-Ruey J. Yeh (Foley et al., 2009). We found that DAT -/- individuals have a pronounced behavioral phenotype, swimming low in the water column (see Chapter 3), which we used to select DAT -/- individuals during the breeding process. To make the s1013t-GCaMP-HS-DATKO line, we crossed our s1013t-GCaMP-HS line with the DATKO line. F1 offspring, all of which were heterozygous for DAT, were screened for GCaMP-HS expression at 24 hpf. Expressers were grown for 3-4 months and then back-crossed with the F0 DATKO line. F2 offspring were screened for GCaMP-HS expression at 24 hpf, and then expressers were individually screened a second time at 3 months of age for the DAT -/- behavioral phenotype. These GCaMP-HS-positive, DAT-/- individuals were interbred to produce F3, and eventually F4 (Fig. 3) GCaMP-HS-positive F3 and F4 larvae were selected at 24 hpf, because virtually all adult fish were heterozygous for GCaMP-HS.

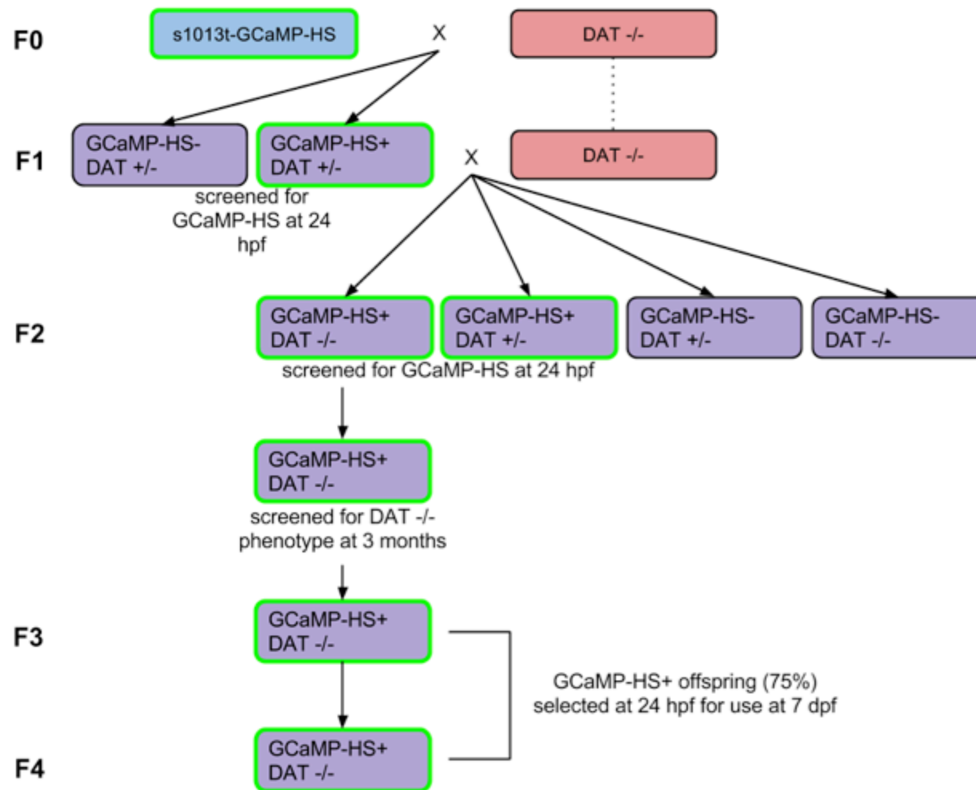

Figure 3. Breeding schematic for s1013t-GCaMP-HS-DATKO transgenic line. F0-F4, consecutive generations.

s1013t-GCaMP-HS-GBC

Because strain differences have the potential to affect many aspects of our experiments from baseline behavior to responses to drugs, we bred the s1013t-GCaMP-HS-GBC (“Genetic Background Control”) line to test in parallel with our s1013t-GCaMP-HS-DATKO line to ensure that we were measuring effects of DAT knockout, not strain differences. The procedure, using our s1013t-GCaMP-HS line and a DAT+/+ GBC provided to us by Dr. Jing-Ruey J. Yeh was the same as described above for the DATKO line, except that there was no behavioral phenotype to screen for.

CHAPTER TWO

Introduction

Cocaine, a psychostimulant and widely used drug of abuse, interferes with visual functions during intoxication and withdrawal, with up to 50% of chronic cocaine abusers experiencing simple non-formed visual hallucinations (Mitchell & Vierkant, 1991; Siegel, 1978; Vorspan et al., 2012). The consequences of prenatal cocaine exposure (PCE) also include a variety of deficits in visual functions, including nystagmus (Spiteri Cornish, Hrabovsky, Scott, Myerscough, & Reddy, 2013), strabismus (Block, Moore, & Scharre, 1997) and reduced visual attention (Accornero et al., 2007; Ackerman, Riggins, & Black, 2010; R L Hansen, Struthers, & Gospe, 1993; A. Heffelfinfger et al., 1997; A. K. Heffelfinfger, Craft, White, & Shyken, 2002; Struthers & Hansen, 1992), leading to increased risk of attention deficit and hyperactivity disorder (ADHD) (Bada et al., 2011; Bandstra, Morrow, Anthony, Accornero, & Fried, 2001; Leech, Richardson, Goldschmidt, & Day, 1999).

Visual perception is critical for processing conditional and unconditional stimuli, which are known to play a major role in drug addiction (Overton & Devonshire, 2008; Weiss, 2010). Thus, cocaine's effects on visual processing might also play an important role in the development of cocaine addiction and relapse, and could in part underlie the increased risk of drug abuse in humans exposed to cocaine prenatally (Delaney-Black et al., 2011; Richardson, Larkby, Goldschmidt, & Day, 2013). In studying PCE, a number of serious obstacles, including polysubstance abuse by most cocaine-using pregnant women, make it difficult to determine the specific effects of cocaine on human

development (Ackerman et al., 2010). Animal models therefore present a unique opportunity to document the effects of PCE under controlled conditions and to study the mechanisms involved. We employ a zebrafish model, which has recently become a popular organism for studying the effects of drugs on brain functions and behavior (Gerlai, Lahav, Guo, & Rosenthal, 2000; Guo, 2004; López-Patiño, Yu, Cabral, & Zhdanova, 2008; Thomas, Welsh, Galvez, & Svoboda, 2009). Adult zebrafish respond behaviorally to acute cocaine administration (Darland & Dowling, 2001) displaying anxiety-like behavior and showing changes in brain dopamine and dopamine transporter levels following cocaine withdrawal (López Patiño, Yu, Yamamoto, & Zhdanova, 2008; López-Patiño et al., 2008). Moreover, an earlier study in our laboratory in developing zebrafish demonstrated that PCE interferes with gene expression and brain morphology (Shang & Zhdanova, 2007), including in the optic tectum, known to be the principal area of visual processing in zebrafish (Nevin et al., 2010).

For this project we addressed whether, during early vertebrate development, acute or prenatal cocaine exposure, or a combination of the two, can interfere with processing of contrasting visual stimuli, whole-field red light flash (LF) and dark flash (DF). We assessed changes in calcium concentration in neurons and their projections, a well-established indicator of neuronal activity (Muto et al., 2011; Rose et al., 2014) using transgenic larvae expressing the calcium reporter GCaMP-HS (s1013t-GCaMP-HS, see Chapter 1), and focused on two brain structures, TO and Te.

The TO, the zebrafish homolog of the mammalian superior colliculus, is a highly conserved visual structure, responsible for eye and head movement control and sensory

integration, and contributing to visual attention. A series of studies support a view that hyper-responsiveness of the tectum is associated with increased distractibility and thus attention deficits, and that inhibition of tectal responses could underlie some of the therapeutic effects of psychostimulants in patients with ADHD (Clements, Devonshire, Reynolds, & Overton, 2014). Recent studies also suggest that the TO is involved in the pathways integrating visual information and its corresponding affective value, thus influencing affective biases in patients with mental disorders and addiction (Vuilleumier, 2014). Another principal component of these integration pathways is the cerebral cortex, critical for visual information processing and affected by psychostimulants, including cocaine (Muñoz-Cuevas, Athilingam, Piscopo, & Wilbrecht, 2013). In zebrafish, a homolog of the mammalian cortex is the dorsal Te (dTe) (Mueller, Dong, Berberoglu, & Guo, 2011).

In these experiments we have shown that the TO and dTe of a developing zebrafish have distinctly different patterns of response and habituation to contrasting visual stimuli. Cocaine can acutely modulate the responses to light but not darkness in this diurnal vertebrate. Importantly, we found that while prenatal exposure to cocaine alone does not modify neuronal responses to either visual stimulus tested, it induces tolerance to the effects of acute cocaine on visual signal processing later in life. Understanding the mechanisms through which acute or prenatal cocaine acts to interfere with visual functions, and which brain structures are affected, could help developing new treatment strategies for attention deficit and other consequences of early cocaine exposure.

Methods

Subjects

Transgenic s1013-t-GCAMP-HS larval zebrafish, as described in Chapter 1, were used in this study. (Wild type [AB strain] larvae were used for one behavioral experiment.) Sex determination is not possible during the larval stage, thus sex is not indicated.

Adult male and female zebrafish (6 fish/3-L tank) were housed in a 14h light/10h dark cycle, in a temperature (27°C) and pH (7.0–7.4) controlled multi-tank re-circulating water system (Aquaneering, San Diego, CA). Adult animals were fed three times a day with live brine shrimp (Brine Shrimp Direct, Ogden, UT), enriched with fish pellets (Lansy NRD, Salt Lake City, UT). Embryos and larvae were raised at 28°C in a 12 h light/12 h dark cycle, and fed paramecia three times a day starting at 5 days post fertilization (dpf). The experiments were conducted in 6-7 day old larvae, between ZT2 and ZT8 (Zeitgeber time, ZT0 is lights-on time). All animal procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Boston University School of Medicine.

Prenatal cocaine treatment

For PCE, embryos were dechorionated with pronase (Sigma-Aldrich, St. Louis, MO) at 24 hours post fertilization (hpf) and then repeatedly exposed to a solution of 0.5 μM cocaine in egg water (60 μg/mL Instant Ocean salts in deionized water) at 24, 48 and 72 hpf. Each exposure lasted 15 minutes and was followed by a thorough washout of the

drug, using a fine net to move larvae through 5 successive wells of fresh egg water, for 30 seconds each time. Controls were also dechorionated and treated with egg water.

Neuronal population imaging

At 7 dpf, s1013-t-GCaMP-HS larvae were immersed in 1.1 mM tubocurarine hydrochloride (Sigma-Aldrich, St. Louis, MO) for 6 min to induce paralysis and prevent movement during imaging. Larvae were then embedded in approximately 12 µL of 1% low-melting agar (40°C, IBI Scientific, Peosta, IA) and positioned dorsal side up in a glass petri dish. When the agar had set, the preparation was covered with egg water. Observation of a normal heartbeat and circulation in an embedded larva confirmed that it had not been injured by the procedure. A dish containing an individual larva was placed under the microscope and, depending on protocol, adapted to darkness or 630 nm red light whole-dish illumination for 3 min. Two-photon imaging of both optic tectum hemispheres and the telencephalon was conducted simultaneously using a Zeiss LSM 710 NLO microscope with a Chameleon Vision S short-pulse pre-chirped titanium sapphire laser for multiphoton excitation at 930 nm using a 20x NA 1.0 immersion objective. Zen software was used to operate the microscope. Care was taken to image at the same depth in the optic tectum and dorsal telencephalon each time, based on anatomical markers.

Dark flash and red light flash in neuronal imaging studies

The light flash was an automatically generated 1-sec 450-lux pulse of light produced by the 633 nM red laser triggered by the two-photon microscope used for imaging. Between light flash presentations larvae remained in total darkness. The dark

flash was an automatic 1-sec interruption in 450-lux 630 nM red light produced by 4 LEDs placed in a ring surrounding the embedded larva and controlled by the microscope trigger system (see Appendix). A similar setup was used in both calcium imaging studies and behavioral studies described below.

Experimental design of neuronal imaging studies

In our within-subject design, each experiment consisted of 3 consecutive trials per fish, with each trial including 1000 two-photon images taken every 750 ms over ~13 minutes (Fig. 4). During each trial, 9 stimuli were presented. Following a 3-min adaptation to darkness or 630 nm red light, the first trial (LF1 or DF1) documented neural responses at baseline. Dark-adapted animals received red light flash and light adapted animals received dark flash. Thereafter, the larva was exposed to either water (control) or cocaine (water in dish was carefully aspirated and replaced with 0.5 μM cocaine solution), followed by a 10-min incubation, to allow the drug to permeate the agar. Agar is porous (Schantz & Lauffer, 1962) and both previous experiments in our laboratory (Mabray, 2010) and the experiments described here, have shown that agar-embedded larvae display effects of cocaine within 10 minutes. Larval responses to dark flash or light flash following cocaine administration were documented in the second trial (LF2 or DF2). Then the larvae were exposed to a repeated stimulus presentation (RSP), with 60 light flash or dark flash stimuli over a 13-min period, with an inter-stimulus interval of 15 sec. Time-control larvae remained in constant conditions. The third trial (LF3 or DF3) then allowed us to determine whether larvae had habituated or sensitized to RSP, and whether the presence of cocaine and/or prior PCE affected the results.

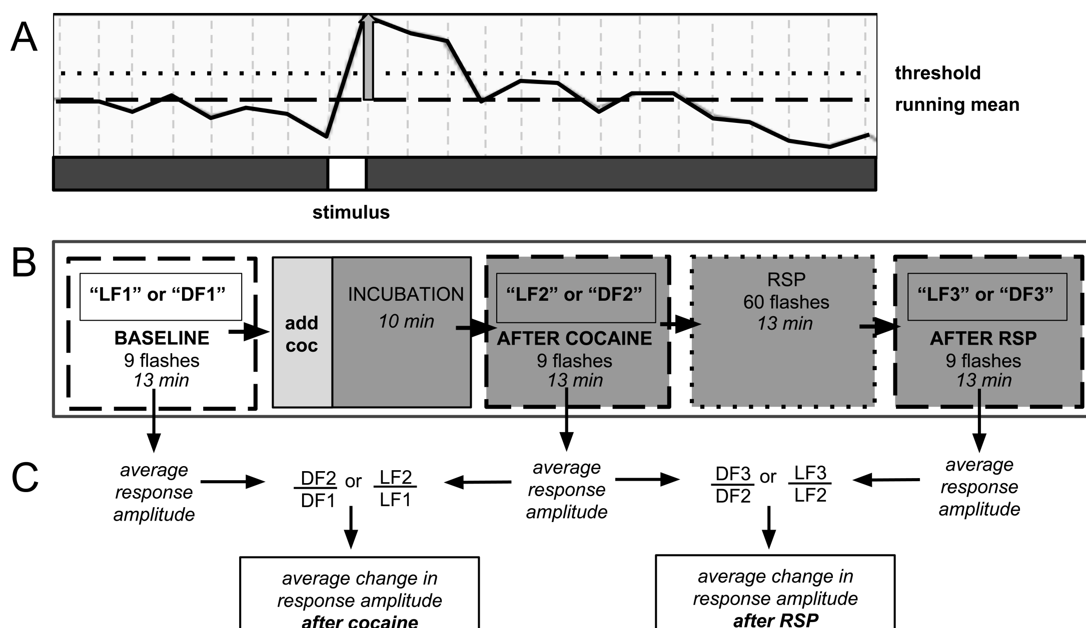

Figure 4. Neuronal population imaging protocol. (A) Detail of fluorescence signal from calcium reporter documented using two-photon microscopy. Y-axis - fluorescence intensity. Vertical gray bar -amplitude of individual stimulus response. Responses that did not reach the threshold line were not counted. (B) Schematic of 3-trial imaging protocol. Each trial consisted of 9 stimulus presentations over 13 minutes. (C) Schematic of data analysis. Change in signal amplitude was computed between trials. DF - dark flash, LF - light flash, LF1, DF1, etc. – light flash trial 1, dark flash trial 1, etc., RSP - repeated stimulus presentation.

Data analysis for neuronal imaging studies

Using custom software, regions of interest (ROIs) covering the optic tectum neuropil and dorsal telencephalon were manually defined for each fish, based on anatomical boundaries. The average brightness of each region was then tracked throughout each trial (1000 images) and normalized to a 100-frame running mean centered on each stimulus presentation (see Appendix). Individual responses were defined as being above threshold if the increase in fluorescence was at least 1.5 standard deviations greater than the running mean, for at least 1 frame (0.75 sec), immediately following a stimulus presentation. Response amplitude was reported as percent increase above running mean, and average RA for each stimulus train was computed. A 3% rise

in signal amplitude was equivalent to a 5-fold increase above background noise. Trials with movement artifacts due to incomplete restraint were not included in the analysis.

For each treatment group, the percent change in response amplitude between consecutive trials was reported (i.e., percent change between LF1 and LF2, and between LF2 and LF3). These percent change statistics were compared between treatment groups using linear mixed model analysis in SPSS (IBM, Armonk, NY, USA). Within groups, a one-sample t-test was used to determine whether response amplitude increased or decreased significantly between trials. Two-tailed t-tests were used to determine significance in the dose-response study. In all cases significance level was set at $p<0.05$.

Behavioral assessment

Note: This behavioral assessment was designed and carried out by Konstantin Kopotiyenko as part of our collaboration. The locomotor responses to visual stimuli in zebrafish larvae, 6-7 dpf, were assessed using high-speed video recordings at 1000 frames per second (NAC Image Technology, Simi Valley, CA, USA). All recordings were performed in a dark room, at 27 ± 1 ^{o}C using infrared light (IR045, Wisecomm, Cerritos, CA, USA). Activity was recorded in groups of 20 larvae. Light stimuli were delivered by a custom built circuit of four red (660 nm) LEDs (276-015, Radioshack, Fort Worth, TX, USA). The light intensity at dish level was 290 lux (red light) or 0 lux (dark).

Depending on the experiment, each group was adapted to either red light or darkness for 8 minutes prior to assessment. Each experiment included 13 trials, each 800 ms in duration, with 60 sec inter-trial interval. First, spontaneous activity in constant conditions (red light or dark) was evaluated (3 trials), followed by red light or dark

flashes (light flash or dark flash), accordingly (10 trials). Every 60 sec, the constant condition (dark or red light) was interrupted by a 1000-ms light flash or dark flash. The first 800 ms window of each 1000-ms light flash or dark flash was captured for further analysis.

All kinematic analyses were performed using Flote 2.0 high throughput automated analysis software (Burgess & Granato, 2007b). Individual movements were scored using the Fourier analysis method with the angle threshold value of 2. Tracked larvae with anomalies in curvature and movement were excluded from analysis. Larvae that were moving at the very beginning of the trial, and larvae situated on the dish periphery, were automatically excluded from the analysis by Flote. The kinematic analysis results are presented as means of 3 consecutive trials for corresponding constant lighting conditions, or 10 consecutive trials for light flash or dark flash presentation. Comparisons were performed using Student's t-test.

Results

Optic tectum neuropil responds differentially to light and dark flashes, while dorsal telencephalon responds only to light flash.

To investigate neuronal correlates of the light flash- and dark flash-induced behaviors (Burgess & Granato, 2007a; Burgess, Schoch, & Granato, 2010), we used calcium imaging in transgenic fish carrying the genetically encoded calcium indicator GCaMP-HS, with fluorescence intensity providing a correlate of neuronal activity (Muto et al., 2011; Rose et al., 2014). Calcium responses were simultaneously monitored in two brain areas using two-photon microscopy (Fig. 5). In constant red light or dark,

background fluorescence intensity in TO neuropil (TOn) and dorsal Te (dTe) generally did not deviate by more than 1%, consistent with prior characterization of the GCaMP-HS fluorophore (Muto et al., 2011). When the larva was exposed to light flash or dark flash stimuli, a pronounced peak in fluorescence intensity power at the stimulus presentation frequency (0.012 Hz) was documented. All individual responses peaked within the first image frame following the stimulus (Fig. 4A). Response amplitude was reported as percent change in fluorescence intensity between running mean and response peak. In TOn, an increase in response amplitude was documented following each stimulus, whether light flash (3.71 ± 0.38%) or dark flash (2.35 ± 0.19%). However, the duration of the response to dark flash (3.65 ± 0.34 sec) was significantly longer than to light flash (2.6 ± 0.23 sec, t test, df=18, p=0.026, Fig. 5).

The dTe responded to light flash with a response amplitude of 4.78 ± 0.55% and a duration of 1.62 ± 0.08, but no responses to dark flash could be documented in this region (Fig. 5). No significant responses were observed to either dark flash or light flash in the control region, TO stratum periventriculare.

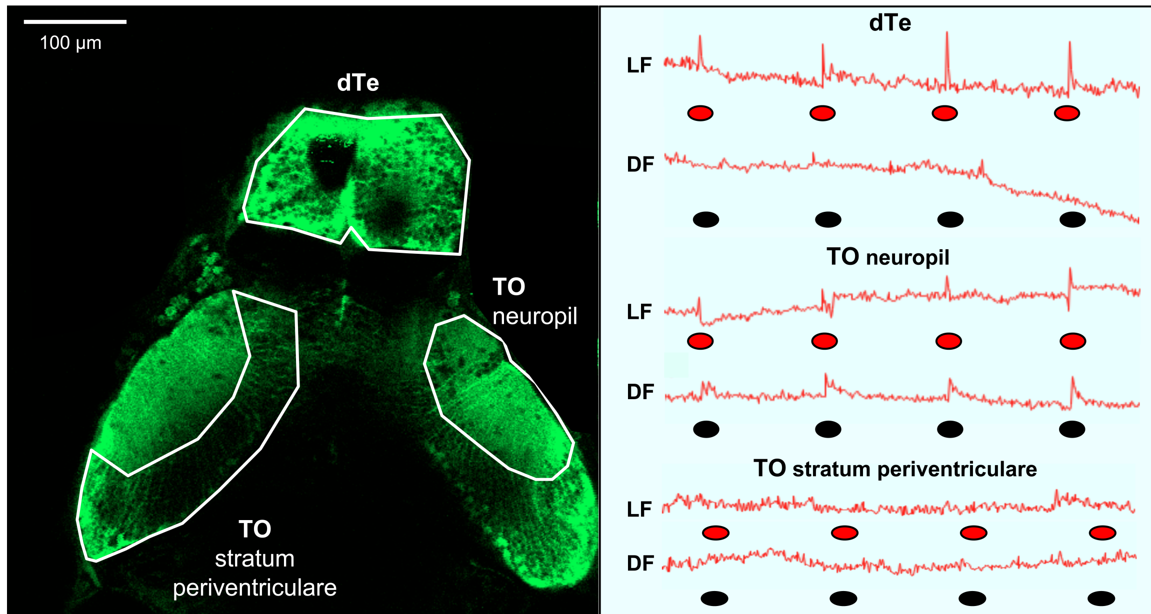

Figure 5. Summary of light flash and dark flash responses in the optic tectum and dorsal telencephalon. Left panel: Dorsal view of s1013t-GCaMP-HS larval brain (7 dpf), two-photon microscopy. Anatomical borders of the optic tectum (neuropil and stratum periventriculare) and the dorsal telencephalon are outlined. Right panel: Red line - traces of absolute fluorescence intensity during first trial and following exposure to red light (LF) or dark (DF) flashes, indicated by red and black ovals, respectively, for the optic tectum (neuropil and stratum periventriculare) and the dorsal telencephalon.

TOn habituates to repeated dark flash but not to light flash

To determine whether repeated stimulus presentation (RSP) can lead to habituation or sensitization of neural responses in either brain area studied, and whether these effects could be stimulus-dependent, we first conducted three 9-response "time control" trials (e.g., LF1, LF2, LF3), with a 13-min inter-trial interval (Fig. 4). We then compared the results obtained to the results following RSP, a train of 60 stimuli with an inter-stimulus interval of 15 seconds administered between LF2/LF3 or DF2/DF3.

In time control fish exposed to light flash, the response amplitude in TOn showed a tendency to increase between LF1 and LF2 (22 ± 16%, NS) and exhibited a significant increase between LF2 and LF3 (37 ± 11%, n=4, p=0.002) (Fig. 6A). In dTe, light flash response amplitude tended to increase between LF1 and LF2 (1 ± 5%, NS) and reached

significance between LF2 and LF3 (30 ± 7%, n=5, p=0.006, Fig. 6B). A similar trend was observed in TO in response to dark flash, though neither change reached significance (25 ± 16% between DF1 and DF2, and 16 ± 10% between DF2 and DF3, Fig. 6C).

On this time-control background, showing a tendency towards increasing response amplitude between subsequent trials, the introduction of RSP between the second and third trials led to further changes in response amplitude. In TOn, response amplitude to light flash increased between the second and third trials (15% ± 11%, NS, Fig. 6A but response amplitude to dark flash diminished between the second and third trials (-14% ± 13%, n=33, p=0.028 vs. time-control, Fig. 6c). In dTe, RSP led to a decrease in response amplitude to light flash between the second and third trials (-10% ± 7%, n=53, p=0.004 vs. time-control, Fig. 6B). There was no dTe response to dark flash over these trials. Together, these results suggested that TOn habituated to dark flash but not light flash, while dTe habituated to light flash. These groups, receiving RSP in the absence of any drug treatment, later served as “RSP control”.

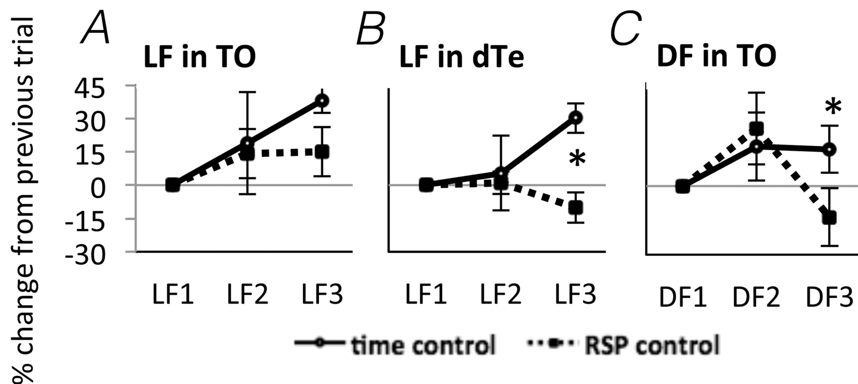

Figure 6. Response of TOn and dorsal Te neuronal activity to repeated stimulus presentation. (A) Light flash in TOn. Time-control (no RSP), n=4, RSP-control, n=17. (B) Light flash in dorsal Te. Time-control, n=5, RSP-control, n=15. (C) Dark flash in TOn. Time-control, n=5, RSP-control, n=6. LF1, LF2, LF3 – separate trials. Repeated Stimulus Presentation (RSP) exposure between LF2 and LF3 trials. All bars – group mean ± SEM. *p<0.05.

Acute cocaine exposure reduces light flash response amplitude in TOn and dTe, and prevents habituation to repeated light flash in dTe

To determine the effect of acute cocaine exposure on neuronal responses, we administered cocaine to the recording chamber of individual larvae between the first and second trials (Fig. 4). The light flash response was sensitive to acute cocaine. The dose dependence study showed that, in TOn, the response amplitude to light flash decreased significantly between the first and second trial during exposure to either 0.5 μM or 0.05 μM cocaine, but not 5 μM cocaine (n=31 and 25, p=0.001 and 0.003 respectively, Fig. 7). This is consistent with the previously observed bimodal dose effects of cocaine in larval zebrafish (Shang & Zhdanova, 2007). Based on this, the 0.5 μM cocaine dose, which is similar to those that a human fetus with PCE is typically exposed to (De Giovanni & Marchetti, 2012; A. W. Jones, Holmgren, & Kugelberg, 2008), and led to the least inter-individual variability in our assays, was used for all further experiments. This concentration evoked a 12 ± 9% decrease in TOn response amplitude (t test, df=23, p=0.049, vs. control, Fig. 8) and a 21 ± 5% decrease in dTe response amplitude (n=53, p=0.018, vs. control, Fig. 9).

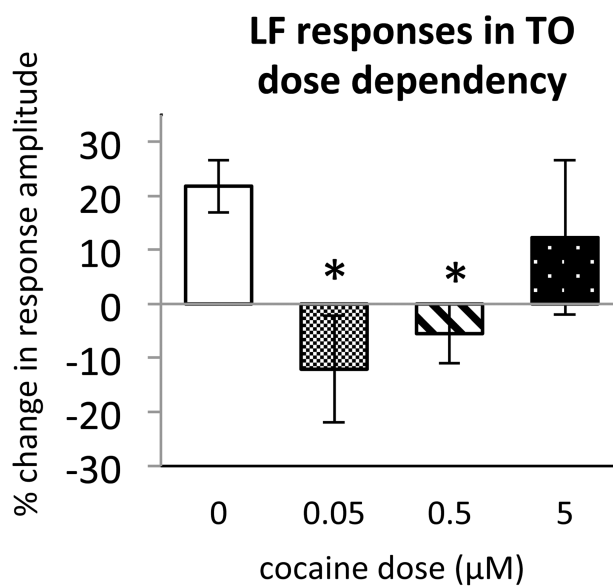

Figure 7. Dose dependence of cocaine effects on light flash response in TOn. Cocaine concentrations: 0 µM, n=17; 0.05 µM, n=8; 0.5 µM, n=16; 5 µM, n=10. All bars – group mean ± SEM. *p<0.05.

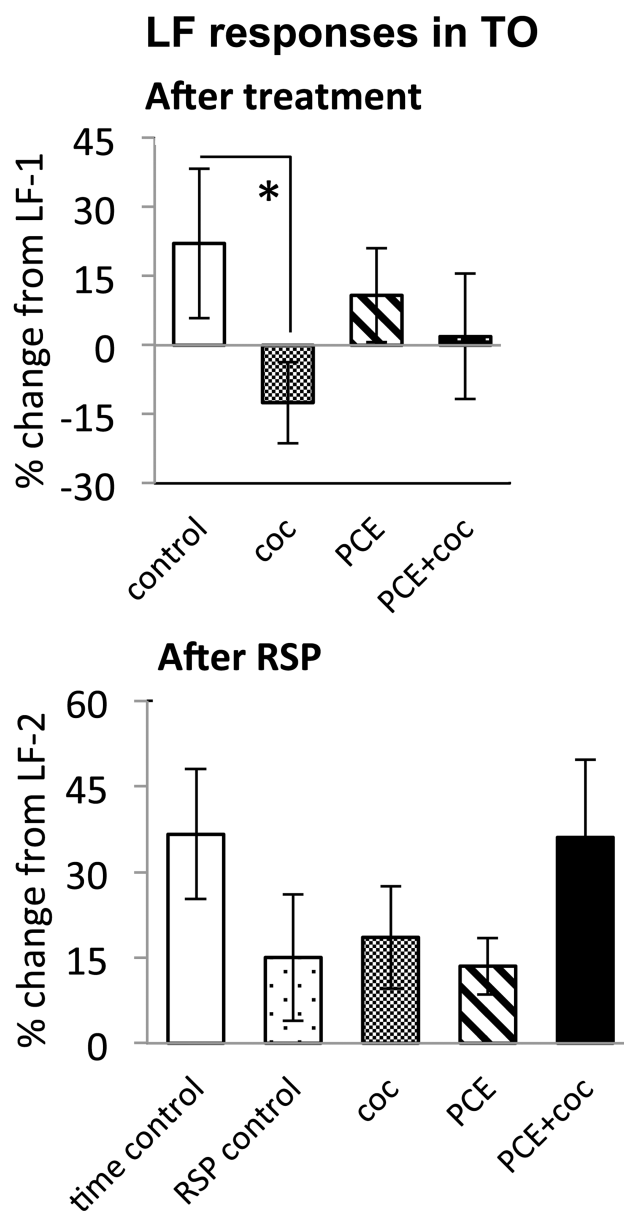

Figure 8. Effects of cocaine, PCE, and PCE + cocaine exposures on light flash responses in TOn, and modification of effects of repeated stimulus presentation by cocaine. (After treatment) Percent change in response amplitude between LF1 and LF2. (After RSP) Percent change in response amplitude between LF2 and LF3. RSP – repeated stimulus presentation. All bars – group mean ± SEM. Time-control, n=4; RSP-control, n=17; coc, n=12; PCE, n=12; PCE+coc, n=10. *p<0.05.

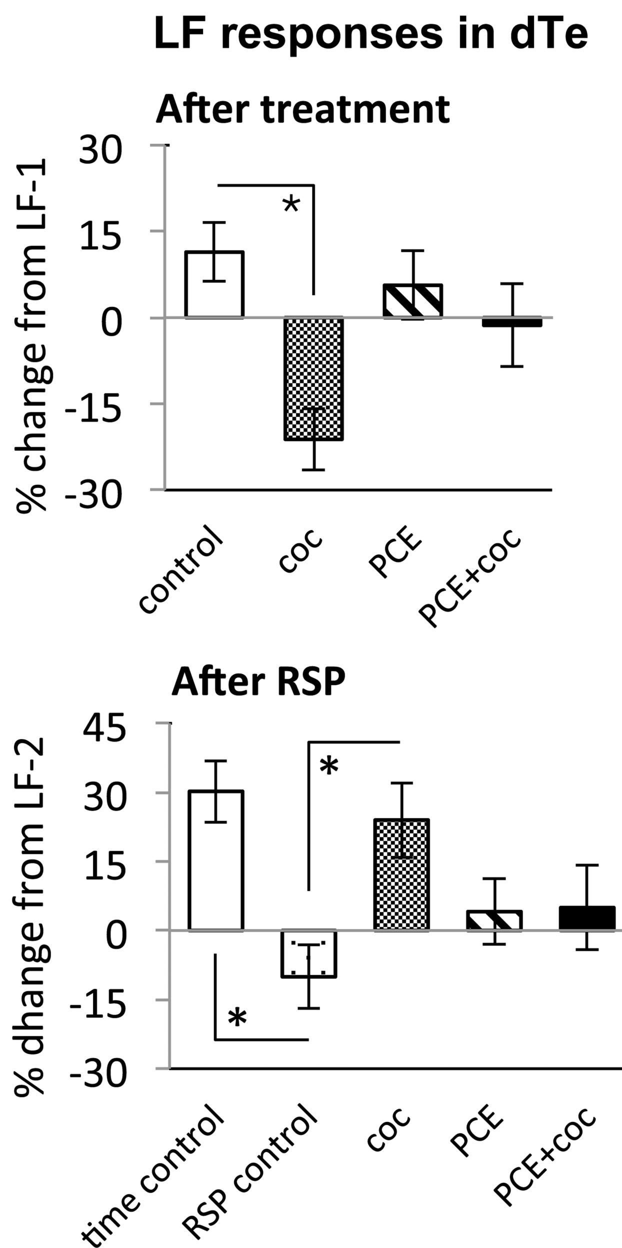

Figure 9. Effects of cocaine, PCE, and PCE + cocaine exposures on light flash responses in dorsal Te, and modification of the effects of repeated stimulus presentation by cocaine. (After treatment) Percent change in response amplitude between LF1 and LF2. (After RSP) Percent change in response amplitude between LF2 and LF3. RSP – repeated stimulus presentation. All bars – group mean ± SEM. Time-control, n=5; RSP-control, n=15; acute only, n=10; PCE-only, n=12; PCE and acute cocaine, n=11.*p<0.05.

In TOn, no habituation was observed following RSP in either control or cocaine conditions (Fig. 8). In dTe, however, following RSP the response amplitude to light flash increased by 24% ± 8% (n=53, p=0.002, vs. control), in contrast to the habituation that had been observed in control animals (Fig. 9).

Acute cocaine does not change responses to dark flash in TOn

The dark flash response in TOn was not sensitive to acute cocaine exposure. Similar to control fish, in which response amplitude to dark flash increased between the first and second trial, cocaine-treated fish showed a 25% ± 16% increase in response amplitude (NS vs. control, Fig. 10).

While time-control and RSP-control groups differed significantly from each other (n=33, p=0.028, Fig. 10), reflecting efficacy of RSP, animals treated acutely with cocaine did not differ significantly from either control group, suggesting a trend for cocaine to attenuate habituation to dark flash in TOn.

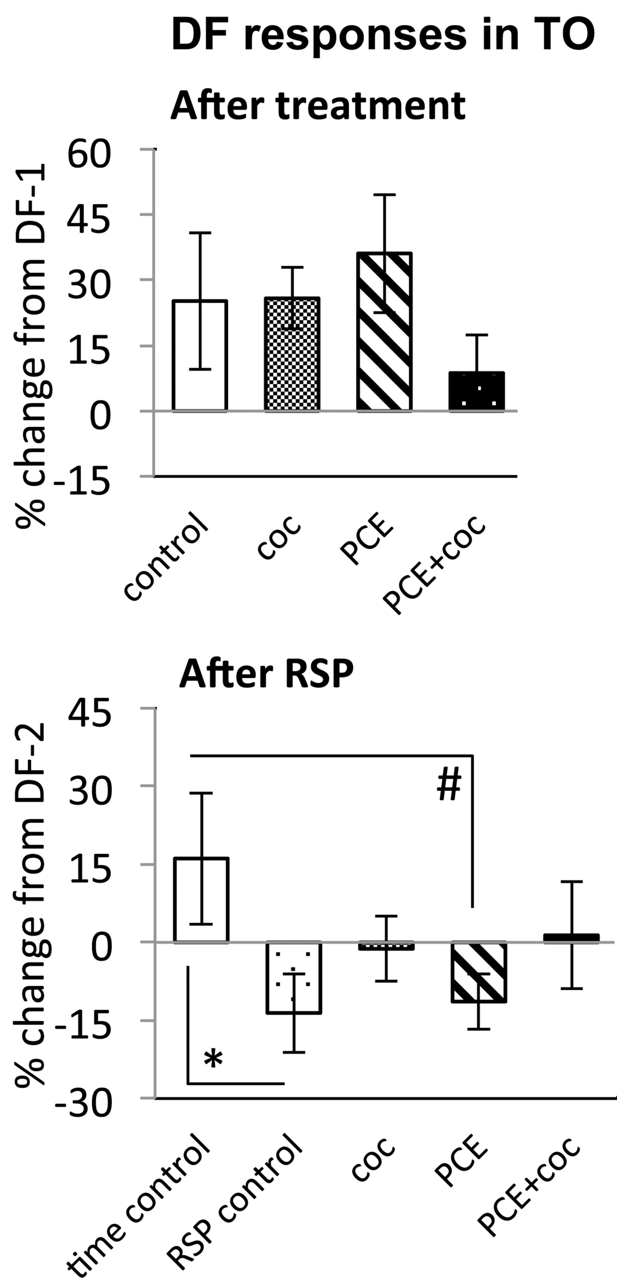

Figure 10. Effects of cocaine, PCE, and PCE + cocaine exposures on dark flash responses in TO, and modification of the effects of repeated stimulus presentation by cocaine. (After treatment) Percent change in response amplitude between DF1 and DF2. (After RSP) Percent change in response amplitude between DF2 and DF3. RSP – repeated stimulus presentation. All bars – group mean ± SEM. Time-control, n=5; RSP-control, n=6; acute only, n=8; prenatal only, n=8; acute and prenatal, n=6. *p<0.05.

Prenatal cocaine exposure makes larvae insensitive to the effects of acute cocaine

To determine whether PCE administered repeatedly at 24, 48 and 72 hpf would modify visual responses or the outcome of acute cocaine treatment in 7 dpf larvae, we documented the effects of PCE alone, and the effects of acute cocaine in PCE larvae (for all treatment groups, see Table 1). PCE alone did not significantly affect response to dark flash or light flash in either of the brain structures studied (Figs. 8, 9 and 10). However, in contrast to drug naive larvae, following acute cocaine administration PCE larvae did not show a reduction in light flash response amplitude in either TOn or dTe (Figs. 8 and 9). Moreover, acute cocaine treatment no longer prevented habituation to light flash in dTe of PCE larvae, in contrast to drug naïve animals (Fig. 9).

Treatment group	***Acute cocaine***	***Prenatal cocaine***	***Repeated stimulus presentation (RSP)***
time control	No	No	No
RSP control	No	No	Yes
coc	Yes	No	Yes
PCE	No	Yes	Yes
PCE+coc	Yes	Yes	Yes

Table 1. Cocaine treatment groups and controls

Response dynamics within individual trials

To ensure that we were not losing valuable information by averaging all 9 response amplitudes and reporting just one statistic per trial, we plotted average response amplitudes to each individual stimulus for all 3 trials in our experimental design. We generated 3 plots, each with RSP control and acute cocaine groups, showing light flash

responses in TO (Fig. 11), light flash responses in Te (Fig. 12), and dark flash responses in TO (Fig. 13). The only trends we observed in these plots were those that have already been reported above. There were no significant within-trial dynamics that had been masked by our use of a single average response amplitude for each trial.

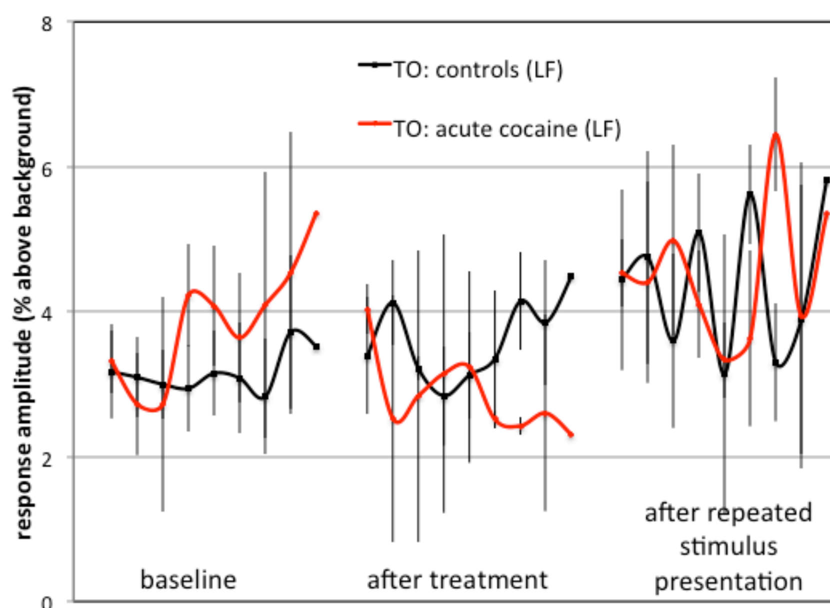

Figure 11. Average response amplitudes to individual light flash stimuli in TO for all 3 trials in RSP control and acute cocaine groups. RSP – repeated stimulus presentation. All points – n= 5, group mean ± SEM.

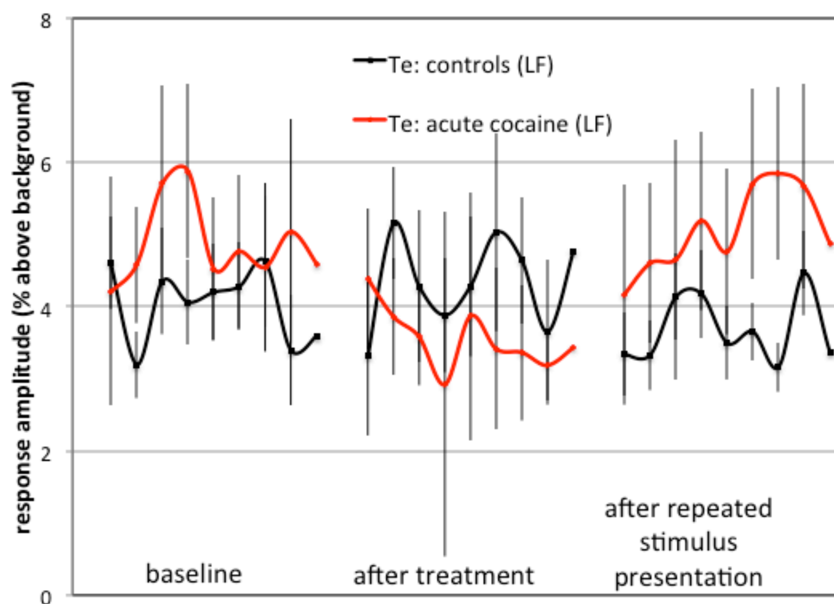

Figure 12. Average response amplitudes to individual light flash stimuli in Te for all 3 trials in RSP control and acute cocaine groups. RSP – repeated stimulus presentation. All points – n= 5, group mean ± SEM.

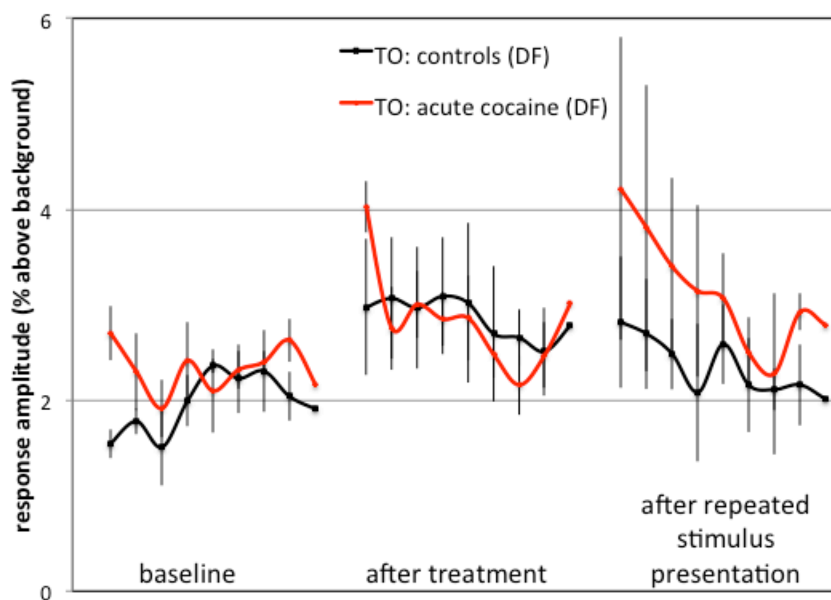

Figure 13. Average response amplitudes to individual dark flash stimuli in TO for all 3 trials in RSP control and acute cocaine groups. RSP – repeated stimulus presentation. All points – n= 5, group mean ± SEM.

Locomotor activity in darkness and response to dark flash differ from those in red light or in response to red light flash.

To image neuronal activity using infrared two-photon microscopy, we had to use red light flashes in order to prevent damage to the microscope's sensitive detector. To provide valid comparison to earlier studies that used white light flash vs. dark flash for studying larval behaviors, we documented locomotor activity over 13-min periods of constant darkness or red light, and then following dark flash or red light flash. (Note: these behavioral assessments were carried out by Konstantin Kopotiyenko as part of our collaboration.) We found that the s1013t-GCaMP-HS transgenic larvae, as well as wild-type fish, were both spontaneously more active in constant darkness than in red light (t test, df=310, p=0.0061, Fig. 14a). This was similar to results reported in other strains exposed to white light vs. darkness (Burgess & Granato, 2007a).

Following either red light flash or dark flash, latency to movement was significantly reduced when compared to spontaneous swimming in continuous red light or dark (t tests, light flash: df=655 groups, $p=6.97*10^{-5}$; dark flash: df=706, $p=4.8*10^{-6}$, Fig. 14b). Locomotor responses to light flash were significantly faster than to dark flash, with significant difference in latency to movement after light flash (183±20 ms) than after dark flash presentation (266± 6 ms, t test, df=1051, p=0.0011, Fig. 14b). Activity patterns were not significantly modified by light flash, in comparison to swimming in constant light. However, dark flash resulted in pronounced changes in behavior, e.g., increased bend angle (t test, df=706, $p=4.8*10^{-6}$) and significantly greater swim distance when compared to spontaneous swimming in dark (t test, df=706, $p=2.3*10^{-7}$, Fig. 14a)

or light (t test, df=700, $p=2.7*10^{-8}$, Fig. 14a). These results, similar to previously published work extensively characterizing larval zebrafish activity in white light and in darkness (Burgess & Granato, 2007a), suggest that larval locomotor responses to red light and white light are similar, and both differ from responses to darkness or dark flash. It is thus likely that the imaging results obtained in this study can be generalized to white light stimuli as well.

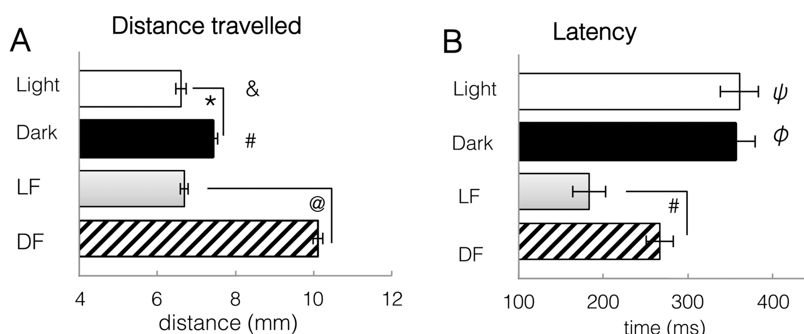

Figure 14. Spontaneous locomotor activity under constant conditions of light and dark, and kinematic responses to light flash and dark flash. Note: these experiments were carried out by Konstantin Kopotiyenko. (A) Distance travelled during a single trial, either spontaneously in constant conditions, or in response to light flash or dark flash. T tests: *: spontaneous light vs. spontaneous dark, df=310, p=0.0061. #: spontaneous dark vs. dark flash, df=706, $p=2.3*10^{-7}$. &: spontaneous light vs. dark flash, df=700, $p=2.7*10^{-8}$. @: light flash vs. dark flash, df=1051, $p=7.34*10^{-8}$. (B) Latency to first movement, either spontaneously in constant conditions, or in response to light flash or dark flash. T tests: *ψ:* spontaneous light vs light flash, df=655, $p=6.97*10^{-5}$. *Φ:* spontaneous dark vs dark flash, df=706, p=0.0035 . #: light flash vs. dark flash df=1051, p=0.0011. All bars – group mean ± SEM. DF – dark flash, LF – light flash.

Discussion

Our principal findings demonstrate region-specific responses to contrasting visual stimuli in the developing brain, stimulus-specific alteration of these responses by acute cocaine, and the ability of prenatal cocaine exposure to modify visual responses to cocaine later in life. We found that the optic tectum responds to both light flash and dark flash, while dorsal telencephalon responds only to light flash. Acutely, cocaine inhibits responses to light flash in both the optic tectum and dorsal telencephalon, but only

interferes with adaptation to repeated stimulus presentation in the dorsal telencephalon. Importantly, prenatal cocaine exposure produces no measurable effects on its own but prevents the inhibitory effects of cocaine on visual perception later in life.

These results are important in light of the major role that the optic tectum, in connection with the cerebral cortex, plays in sustaining visual attention, and the significant changes in attention induced by prenatal cocaine exposure in humans. Our findings further support the notion that the optic tectum mediates these prenatal cocaine-induced changes in attention (Overton, 2008) and establishes a new approach to studying the neurochemical circuits underlying both the adverse and therapeutic effects of psychostimulants on attention.

Effects of cocaine on the optic tectum neuropil are stimulus- and region-dependent, and relevant to attention.

Our choice of the optic tectum - the superior colliculus in mammals - as one of the brain structures of interest was based on its critical role in visual signal processing, control of eye and head movements and visual attention (Krauzlis et al., 2013; Nevin et al., 2010; Overton, 2008). It has been known for some time that superior collicular lesions attenuate distractibility in cats (Sprague & Meikle, 1965), rats (Goodale, Foreman, & Milner, 1978) and monkeys (Milner, Foreman, & Goodale, 1978). Consistent with this, in zebrafish, pharmacological augmentation of the tectal response to light flash, using bicuculline, is associated with decreased ability to catch prey, which requires a high level of visual attention and coordination (Del Bene et al., 2010). Tectal light flash responses are thought to represent active filtration of whole-field visual

information by the superficial layers of the tectum. The deeper layers of the tectum are then better able to track moving objects such as prey (Del Bene et al., 2010). Importantly, mammals also exhibit layer-specific filtration of visual information in the superior colliculus (Isa, 2002). These collicular effects are highly conserved and humans manifest increased distractibility after the loss of inhibitory control of the prefrontal cortex over the superior colliculus (Gaymard, François, Ploner, Condy, & Rivaud-Péchoux, 2003).

In our zebrafish model, cocaine suppresses responses to light flash in the superficial layer of the optic tectum. Similarly, another psychostimulant, amphetamine, suppresses responses to light flash in the superficial layer of the superior colliculus in mammals, and this is associated with increased visual attention (Gowan, Coizet, Devonshire, & Overton, 2008). These important observations led to a hypothesis that ADHD and perhaps other conditions of increased distractibility are associated with a hyperactive superior colliculus, and that consequently, reducing superior colliculus activity can attenuate distractibility, contributing to the therapeutic effects of psychostimulants in ADHD (Clements et al., 2014; Comoli et al., 2003; Overton, 2008).

In this context, we were especially interested to discover that prenatal cocaine exposure, in doses similar to those that human fetus can be exposed to in utero (De Giovanni & Marchetti, 2012; A. W. Jones et al., 2008), does not interfere with basal visual responses of the zebrafish optic tectum but prevents it from being modulated by acute cocaine later in life. This unexpected finding suggests that the optic tectum target of psychostimulants probably play a regulatory function, involved in modulating rather

than generating visual responses. Identifying these supposed regulatory targets could improve the design or discovery of treatments for prenatal cocaine exposure or other types of increased distractibility resistant to psychostimulant treatment.

Indeed, the superior colliculus contains all three monoaminergic targets for psychostimulants. Of the three, the superficial layer of the superior colliculus, corresponding to TOn, is most densely innervated by noradrenergic and serotonergic afferents (Parent, Descarries, & Beaudet, 1981; Wichmann & Starke, 1988), with fewer dopaminergic projections (Campbell, Takada, & Hattori, 1991; Parker, Brock, Walton, & Brennan, 2013; Weller, Rose, Jenner, & Marsden, 1987). However, the deep layers of the superior colliculus project directly to the dopaminergic neurons of the midbrain (Comoli et al., 2003), and can activate them in response to visual stimuli (Dommett et al., 2005). Thus, despite the relative lack of dopaminergic fibers in the superior colliculus itself, there is strong evidence that a hyper-responsive superior colliculus could contribute significantly to the dopaminergic dysregulation seen in increased distractibility.

Our findings also highlight the differences between the optic tectum responses to light and dark stimuli. While the optic tectum is activated by both light and dark flashes, repeated presentation of dark flash results in habituation, while no such effect is observed following repeated light flash. This disparity, and the fact that, in contrast to light flash, response to dark flash is not modulated by acute or prenatal cocaine exposure, suggest that distinct mechanisms are involved in processing light and dark stimuli in the optic tectum. This notion is strongly supported by an earlier study showing that dark flash stimuli are received and processed by the OFF retinal pathway, leading directly to turn

movements which cause the larva to navigate away from dark regions (Burgess et al., 2010). In contrast, light flash stimuli, as shown by the same study, are processed by the ON retinal pathway, and result in activation of serotonergic neurons which then allow the larva to navigate towards the stimulus.

Visual system alterations following prenatal cocaine exposure and tectal dysfunction

Initial reports on cognitive deficits in children of preschool age exposed to cocaine prenatally (Azuma & Chasnoff, 1993) have been further supported by work documenting cognitive and behavioral problems in their school-age and teenage counterparts (Ackerman et al., 2010; Buckingham-Howes, Berger, Scaletti, & Black, 2013). Extending from infancy through young adulthood, however, this population shows pronounced deficits in visual attention which may contribute significantly to their behavior problems (Accornero et al., 2007; Ackerman et al., 2010; R L Hansen et al., 1993; A. Heffelfinfger et al., 1997; A. K. Heffelfinfger et al., 2002; Struthers & Hansen, 1992). Moreover, given the effects of psychostimulants on the superior colliculus (Gowan et al., 2008), it is not surprising that several specific visual system problems noted in children exposed to cocaine prenatally are consistent with collicular dysfunction, including nystagmus (Spiteri Cornish et al., 2013), strabismus (Block et al., 1997), and poor visual memory (Robin L. Hansen, Struthers, & Gospe, 2008; Struthers & Hansen, 1992).

Based on earlier findings linking a hyper-activated optic tectum and increased distractibility (Overton, 2008), which is also observed following prenatal cocaine exposure (Bandstra et al., 2001; Delaney-Black et al., 2011), we therefore expected that

prenatal exposure to cocaine in zebrafish embryos would lead to altered activity of the superficial layers of the optic tectum. The results were different than expected. There were no significant changes in the baseline responses of the optic tectum to light flash or dark flash after prenatal cocaine exposure. However, when prenatally exposed larvae were challenged with a dose of acute cocaine later in development, they demonstrated a loss of the inhibitory effects of cocaine on tectal response to visual stimuli, suggesting that they had developed tolerance to the psychostimulant. This unexpected discrepancy may allow future studies to locate targets of prenatal cocaine exposure that do not affect the visual response itself but rather its modulation by psychostimulants. Studying this phenomenon further might also lead to an increased understanding of the mechanisms behind the therapeutic effects of psychostimulants on increased distractibility, or help to develop novel pharmacological treatments lacking potential for abuse.

Selective response to light flash in dorsal telencephalon and its modulation by cocaine

The dorsal telencephalon in zebrafish is homologous to the cerebral cortex in mammals (Mueller et al., 2011; Ng et al., 2012) and to our knowledge, its visual response properties have not been investigated until now. We find that dorsal telencephalon is responsive to light flash only, since it did not respond to dark flash under any of the conditions tested here, and that cocaine acutely inhibits this response, as it does in the optic tectum.. Importantly, cocaine prevents habituation of the dorsal telencephalon to repeated light flash. This is in striking contrast to the optic tectum, in which habituation to light flash does not develop, and acute cocaine has no impact upon adaptation to repeated stimulus presentation. Both inhibition of the light flash response and inhibition

of habituation to it by acute cocaine are prevented in the dorsal telencephalon by prenatal cocaine exposure.

Neither the specific functions of the zebrafish telencephalon, nor its homology to mammalian cortex have been explored well enough to precisely interpret the telencephalic response that we observed. However, our behavioral observations can provide some clues. We find that larval zebrafish respond more rapidly to light flash but with more pronounced locomotor behaviors following a dark flash, including increased bend angle and distance traveled. This has been observed in earlier studies and explained by the need to navigate away from dark towards preferred lighted environments (Burgess and Granato, 2007b; Burgess et al., 2010; Steenbergen et al., 2011). The diurnal lifestyle of zebrafish larvae, which are active during the day and sleep at night (Zhdanova, Wang, Leclair, & Danilova, 2001), necessarily means that bright light is associated with more complex visual processing related to a broad repertoire of daytime individual and social behaviors (Burgess & Granato, 2007a). Light is critical for prey capture, a behavior well developed in our 7 days post fertilization larvae, and one that requires exceptional attention and motor coordination (Borla, Palecek, Budick, & O'Malley, 2002; Gahtan, Tanger, & Baier, 2005; McElligott & O'Malley, 2005). Thus, the (Overton, 2008) fact that light flash but not dark flash activates dorsal telencephalon neurons might reflect the role of this structure in higher-level signal processing and/or learning and memory functions which are much less engaged when a diurnal animal is in dark environment.

Overall, as demonstrated here, the zebrafish model has the potential to significantly contribute to our understanding of the brain region-specific and stimulus-

specific effects of acute and prenatal cocaine exposure. Early development of sophisticated visual responses and the critical role of attention in prey capture activities make the zebrafish an excellent model to uncover the mechanisms of adverse and therapeutic effects of psychostimulants on vertebrate brain and behavior. The model could also potentially be used to aid in the development of therapeutics to prevent or treat the consequences of prenatal cocaine exposure.

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CHAPTER THREE

Introduction

Cocaine has several targets in the brain. It causes inhibition of the three main monoamine transporters, DAT, norepinephrine transporter (NET), and serotonin transporter (SERT) (Ritz, Cone, & Kuhar, 1990; Stolerman & Price, 2010). This leads to an increase in the concentration of the corresponding monoamines in the synaptic cleft, extending their action on both pre-and post-synaptic receptors. Cocaine can also modify gene expression (Shang & Zhdanova, 2007) and cause vasoconstriction and changes in heart rate and blood pressure (Schwartz, Rezkalla, & Kloner, 2010). All of these things can affect embryonic development, including that of the visual system (Block et al., 1997), and could contribute to the long-term consequences of prenatal cocaine exposure. Determining which targets of cocaine are responsible for which short- and long-term physiological effects is very difficult, yet absolutely necessary for developing prophylactic and therapeutic measures in children that were exposed to cocaine prenatally.

Both animal and human studies have lead to a growing consensus that impairment of attention regulation is a prominent long-lasting effect of prenatal cocaine exposure (PCE) (Accornero et al., 2007; A. Heffelfinfger et al., 1997; A. K. Heffelfinfger et al., 2002; Struthers & Hansen, 1992). The mechanism by which cocaine impairs attention is unknown. The results of our experiments described in Chapter 2 showed that cocaine exposure resulted in altered responsiveness to visual stimuli in two zebrafish brain

regions critical for attention regulation, TO and dTe. These results could provide a new approach to studying the mechanisms underlying the effects of PCE on attention.

The dopamine system, including DAT, plays a critical role in visual attention, active both in the retina and in areas of the brain responsible for visual and processing and cognition (Störmer, Passow, Biesenack, & Li, 2012). DAT is responsible for pumping dopamine out of the extracellular space and back into the cytosol. Inhibition of DAT by cocaine is now recognized to play a major, but not exclusive, role in the reinforcing effects of the drug (Sora et al., 2001). Thus, as a first choice, we targeted the DAT in our investigation into the mechanisms involved in the effects of cocaine on visual processing.

We examined the effects of cocaine in mutant zebrafish lacking DAT (DATKO). This is the first time such studies have been conducted in zebrafish. In rodents, DATKO animals have a broad array of physical, behavioral, cognitive and neurochemical abnormalities (Carpenter, Saborido, & Stanwood, 2012; Raul R Gainetdinov, Jones, & Caron, 1999; Raul R Gainetdinov, 2008; Rocha et al., 1998; Viggiano, Ruocco, & Sadile, 2003). Importantly, they are successfully utilized as a model of ADHD, linking DAT to regulation of attention. Furthermore, DAT knockout and PCE bear some important similarities, since they both result in elevated dopamine during early development. By testing whether or not specific effects of cocaine on the visual system are detectable in DATKO animals, we can begin to determine whether the effects of cocaine on visual processing are mediated by the dopamine system.

To address these questions, we developed a transgenic mutant zebrafish by cross-breeding a DAT knockout transgenic line (Foley et al., 2009) with our 21013t-GCaMP-HS transgenic line, as described in Chapter 1. We made use of the striking behavioral phenotype that DAT knockout animals display, and GCaMP-HS visualization, to select for both GCaMP-HS+ and DAT -/- animals. Both larval and adult DAT knockout zebrafish show a strong preference for swimming in the bottom of the water column. Employing the methods described in Chapter 2, we used calcium imaging in week-old DAT knockout larvae to determine whether cocaine exposure was capable of suppressing responses to light flash in the optic tectum (TO) and the telencephalon (Te). Then, we used a behavioral assay to test if cocaine could cause changes in larval swimming behavior in the presence and absence of DAT. Finally, to further investigate the consequences of DAT knockout on the dopamine system, and to compare DAT knockout to PCE, we used immunohistochemistry to visualize the location of dopamine receptors D1 (drd1) in the brains of wild types, wild types exposed to PCE, and DAT knockouts. Drd1 expression altered is after both DAT knockout (Raul R Gainetdinov et al., 1999) and prenatal cocaine exposure (L. B. Jones et al., 2000) in other species reflecting long-lasting dysregulation of the dopamine system following presumed hyperdopaminergic tone during early development.

We found that only some of the effects of cocaine on visual responsiveness were absent in DAT knockout animals, while others persisted. Drd1 expression was downregulated by PCE, but not DAT knockout, in certain brain regions. Unlike controls, DAT knockout animals did not respond behaviorally to cocaine. Taken together these

results suggested that the presence of an intact dopamine system is required for certain effects of cocaine on visual processing, but that other targets of cocaine are also likely to contribute to such effects. Those might include non-dopamine monoamine transporters, gene regulation pathways, and vascular systems.

Methods

Neuronal population imaging

As described in Chapter 1, we developed the s1013t-GCaMP-HS-DATKO transgenic line by crossing a DATKO line with the s1013t-GCaMP-HS line, expressing GCaMP-HS in TO and Te, which we used for our previous imaging experiments. Using the methodological approaches described in detail in Chapter 2, we recorded neuronal activity in the TO and Te of 7 dpf s1013t-GCaMP-HS-DATKO larvae using two-photon microscopy. The results described in this chapter were obtained from experiments consisting of two trials of 9 light stimulus presentations each, with one before and one after administration of cocaine or water, i.e. a modified version of the three trial structure used in Chapter 2 (Fig. 15).

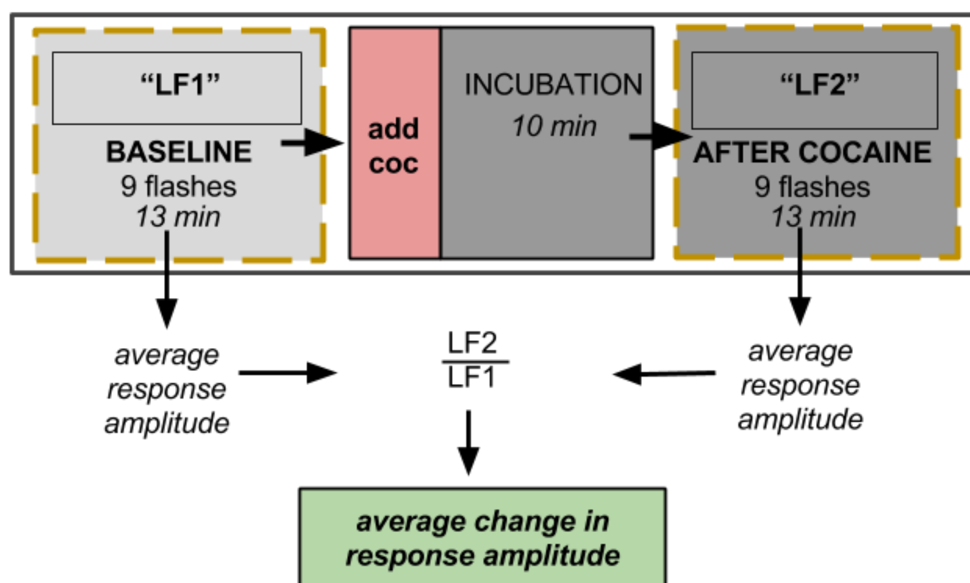

Figure 15. Schematic of 2-trial neuronal population imaging protocol. Each trial consisted of 9 stimulus presentations over 13 minutes. Change in signal amplitude was computed between trials. LF - light flash, LF1 – light flash trial 1, etc., RSP - repeated stimulus presentation.

Behavioral Assay

Our behavioral assay documented the vertical position of 7 dpf larvae in the water column. Specifically, we measured the number of larvae in the top half of the tank at each time point. We evaluated both s1013t-GCaMP-HS-GBC ("control") and s1013t-GCaMP-HS-DATKO ("DATKO") larvae. After being placed in a clear acrylic tanks (100mm x 50mm x 50mm) each containing 100 mL of egg water, larvae (n=20 per group) were allowed to acclimate for 15 minutes. Their baseline behavior was documented every 2 minutes for 1 hour. We then added 50 µL of fresh room-temperature 1 mM cocaine solution in egg water (0.5 µM final concentration) or 50 µL of vehicle (egg water). Larval behavior was then documented every 2 minutes for 2 hours. Cocaine and water treatments were conducted in parallel.

To analyze the data, we counted the number of larvae in the top half of the tank each frame. We also calculated the average number of larvae above the mark during each half-hour time period. To determine whether cocaine had a statistically significant effect on the level at which larvae were swimming in the water column we performed a two-way repeated-measures ANOVA ($p<0.05$).

To compare the baseline behavior of our control and DATKO larvae, we performed a t-test ($p<0.05$) between their respective baseline averages in the above behavioral assay. To measure the baseline swimming height of adult control and DATKO fish, the metric that we used to select for DATKO individuals, we allowed 1-year old fish ($n=8$ per group) to acclimate to 9-liter tanks filled with egg water for 15 minutes. Control and DATKO fish were evaluated side-by-side. After acclimation we recorded 2 minutes of video. To analyze, we counted the number of fish in the top half of the tank every 6 seconds. We used a t-test ($p<0.05$) to determine significance.

To document thigmotaxis, a tendency to stay close to the walls, in adult fish, we placed two 9-liter tanks side-by-side on top of gridded mats. Each tank was partially filled with 3 liters of water. We placed used the same 8 1-year-old fish from the behavioral assay described above into these tanks and recorded 2 minutes of video from above the tanks. To analyze, we counted the number of fish in the center region of the gridded mats every 6 seconds, and used a t-test to determine significance.

Prenatal cocaine treatment

For prenatal cocaine exposure (PCE), 0.5 μM cocaine was administered at 24, 48 and 72 hpf as described in detail in Chapter 2.

Immunohistochemistry

At 22 hours post fertilization, larvae were immersed in a solution of 75 µM N-Phenylthiourea (“PTU”, P7629, Sigma-Aldrich) in egg water at to prevent formation of melanophores. The remained in the solution until fixation. This concentration of PTU is not expected to affect development of the dopamine system (Holzschuh, Ryu, Aberger, & Driever, 2001). Larvae were fixed at 6.5 dpf by immersion in ice-cold 4% paraformaldehyde overnight. Beginning the next morning they were washed 5x for 5 min each time in PBS with 1% Triton-X-100 (PBT) at room temperature, then incubated for 30 min at 37C with 0.1% trypsin with 0.25% EDTA to permeabilize. After permeabilization they were again washed 5x for 5 min each time at room-temperature. Following the washes the larvae were incubated in a blocking solution of 3% donkey serum in PBT at room temperature for 2.5 hours, then placed in a 1:2500 dilution of the rabbit anti-drd1 primary antibody (ab81296, abcam, Cambridge, MA, USA) in 1% donkey serum in PBT overnight. The next day, the larvae were washed 3x for 45 min each time in PBT at room-temperature, then incubated with a 1:1000 dilution of the AF-555 donkey anti-rabbit secondary antibody (ab150073, abcam) in 1% donkey serum in PBT at room temperature for 2.5 hours, protected from light. After secondary antibody staining they were washed 3x for 45 min each time in PBT, then for 5 min in PBS, and placed in mounting solution containing DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA) overnight. The next day they were embedded in 1% low-melting agarose for imaging. All incubations except for permeabilization by trypsin took place on a rocker or orbital shaker.

Imaging of embedded larva was performed on a Zeiss LSM 710 NLO confocal microscope using 561 and 504 nm lasers.

Results

DAT knockout animals display anxiety-like behavior

Zebrafish larvae lacking DAT, s1013t-GCaMP-HS-DATKO, preferred to swim at the bottom of the tank and were present in the top half of the tank less than 5% of the time at the larval stage (7 dpf) (Fig. 16). Control larvae spent 41.64 ± 1.29% of their time in the top half of the tank, while DATKO larvae spent 3.13 ± 0.51% (t test, n=118, p< 0.0001). This striking behavior is very similar to that in the original DATKO mutant line lacking GCaMP-HS (Foley et al., 2009). Similar behavior is also observed in DATKO adult fish (Fig. 16). Control adults spend 54.44 ± 8.96% of their time in the top half of the tank, while DATKO adults spend 3.03 ± 1.56% (t test, n=22, p<0.0001). Notably, this swimming behavior does not reflect an inability to swim in the top half of the tank. When motivated, for example by food, DATKO fish swim at the top of the water column and do not appear to differ from controls. The s1013t-GCaMP-HS-GBC fish (controls), however, spend about 50% of their time in the lower half of the water column at larval and adult stages. This easily discernible phenotype served as a behavioral assay by which we separated DAT -/- from DAT +/- adults for breeding. The assay reliability was confirmed by measuring DAT mRNA expression using qPCR in adult fish selected by phenotype. We found that s1013t-GCaMP-HS-DATKO animals had a 21-fold reduction in DAT mRNA abundance when compared to control zebrafish (t test, n=8, p<0.0001), which suggests nonsense-mediated decay of mutated DAT mRNA.

Adult DATKO fish also display thigmotaxis, a tendency to remain close to the walls of the tank. DATKO adults spend 10.1 ± 1.92% of their time in the center region of a novel tank, compared with 21.42 ± 3.22% in controls (t test, n=44, p=0.03, Fig. 16d).

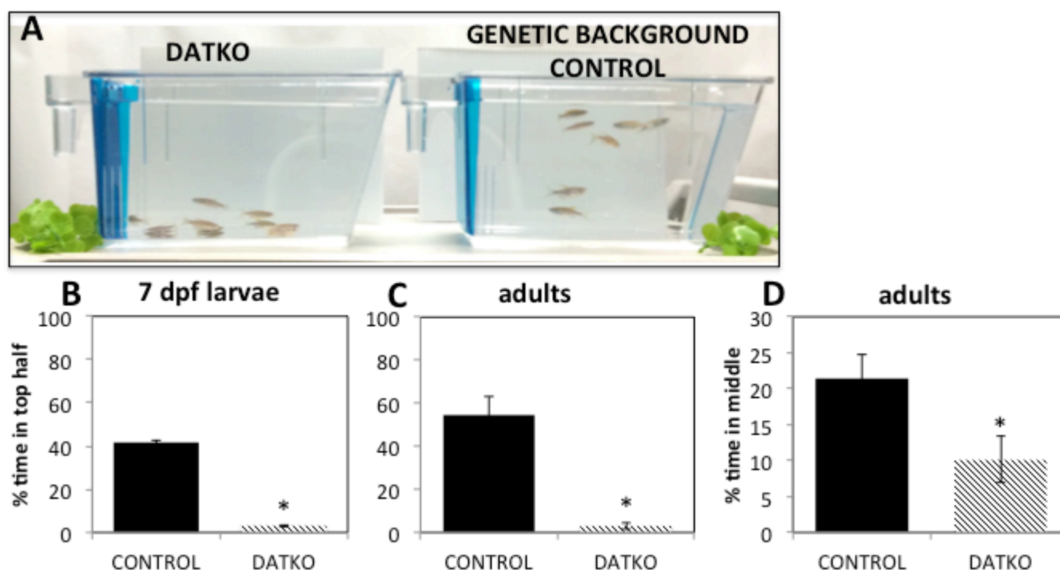

Figure 16. Characterization of the DATKO behavioral phenotype. (A) The DATKO phenotype in adults, swimming low in the water column. (B) and (C) Percentage of time spent swimming in the top half of the water column in larvae and adults. (D) Percentage of time adult fish spent in middle region of tank. All groups show mean ± SEM. *p<0.05.

DAT knockouts do not change swimming behavior during acute cocaine exposure, unlike control zebrafish

We tested the effect of acute exposure to 0.5 μM cocaine on swimming behavior in both DATKO and control larvae. In controls, larvae began to swim significantly lower in the water column 30 after cocaine administration (Fig. 17b,d). The largest effect was observed between 60-90 minutes after administration, when only 26 ± 1.85% of cocaine-treated larvae were swimming in the top half of the tank, compared with 41 ± 1.81% of water-treated larvae (repeated measures ANOVA, p=0.007, n=90). Cocaine-treated larvae were still swimming significantly lower than water-treated larvae at the conclusion

of the experiment 120 minutes after cocaine administration. In contrast to control animals, DATKO larvae did not display any effect of cocaine. Both water-treated and cocaine-treated DATKO larvae spent most of their time close to the bottom of the tank, with only 2.5 ± 0.41% of their time spent in the top of the tank (Fig. 17a,c).

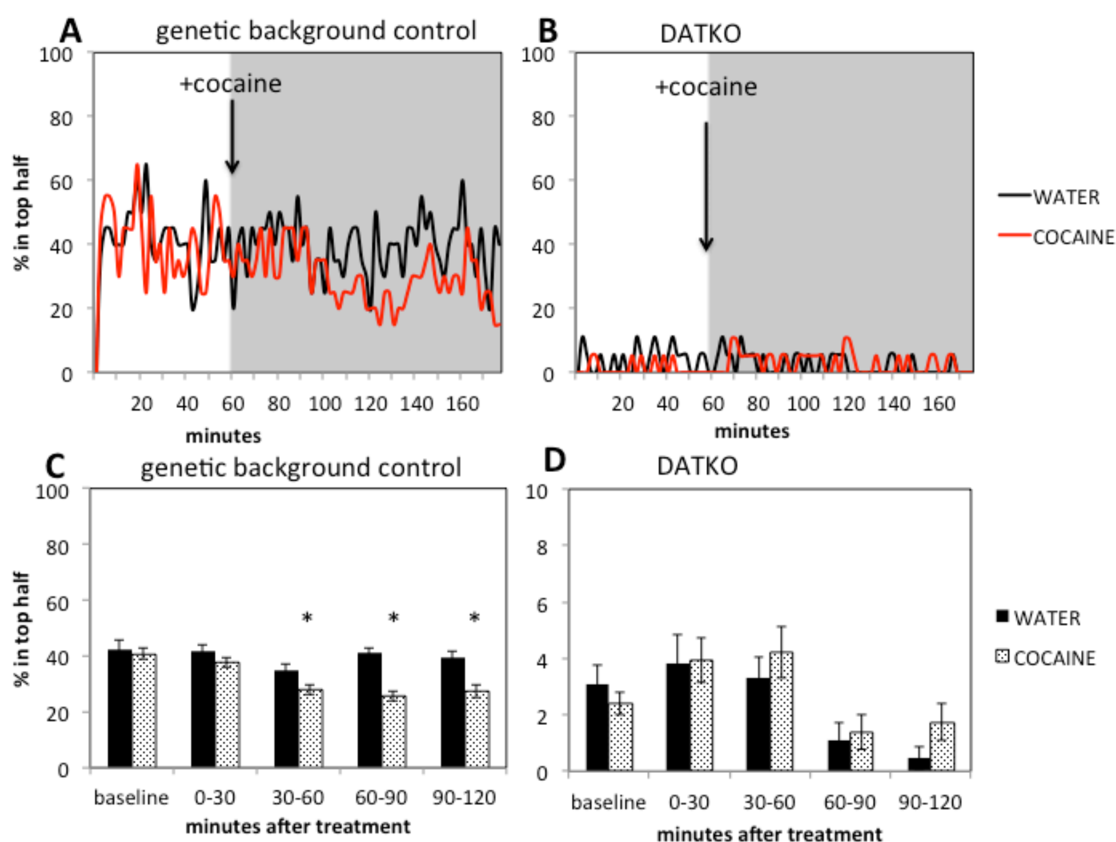

Figure 17. Effects of cocaine on swimming behavior in DATKO and control larvae. (A) and (B) Percent of larvae swimming in the top half of the tank throughout a 3h experiment, with 0.5 µM cocaine (red) or water (black) added at 60min. (C) and (D) Percent of larvae swimming in the top half of the tank during each 30min time period, mean ± SEM. * p<0.05.

Optic tectum neuropil responses to light flash are suppressed by cocaine in both DAT knockouts and controls

As described in detail in Chapter 2, we previously imaged live larvae using two-photon microscopy and demonstrated that acute cocaine exposure suppressed responses

to red light flash in both TO neuropil and dorsal Te in larvae with an intact dopamine system. To determine whether the presence of DAT is required for the effects we observed, we conducted similar experiments in DATKO larvae and their genetic background controls. Using a two-trial, within-subject design, we measured the average amplitude of calcium responses to red light flash (9 flashes per trial) before and after exposure to 0.5 uM cocaine or vehicle (water). In TO, control larvae treated with water showed an 18.65 ± 8.93% increase in response amplitude between trials (Fig. 18a). In contrast, a significant 22.2 ± 8.96% decrease in response amplitude was observed following cocaine treatment (t-test vs. water treatment, p=0.026, n=17, Fig. 18a). This was similar to the effects reported in Chapter 2 using fish of a different genetic background. The effect of cocaine was similar in DATKO larvae. They showed a 44.62 ± 14.63% increase (NS vs. control) in response amplitude between trials after water treatment, and a 9.86 ± 4.92% (t-test vs. water treatment, p=0.03, n=16, t-test vs. controls after cocaine, p=0.006, n=16) increase in response amplitude between trials after cocaine treatment (Fig. 18a). Although the DATKO larvae did not show an absolute decrease in response amplitude after cocaine treatment, the difference from controls was still significant (Fig. 18a). Thus, cocaine reduced response amplitude to light flash even in the absence of DAT.

Dorsal telencephalon responses to light flash are suppressed by cocaine in controls, but not DAT knockouts

In dTe, the control larvae showed a 16.9 ± 4.29% increase in response amplitude following water treatment, but responded to cocaine treatment with a 14.57 ± 11.93%

decrease in response amplitude between trials after cocaine treatment (t test vs. water treatment, p=0.02, n=11, Fig. 18b), again similar to our results reported in Chapter 2 in another zebrafish strain. In contrast, the DATKO larvae did not demonstrate any significant change in dTe neuronal activity following cocaine administration, with a trend toward increase in response amplitude after both water (+12.7 ± 8.99%) and cocaine (+10.13 ± 11.81%) treatment (Fig. 18b).

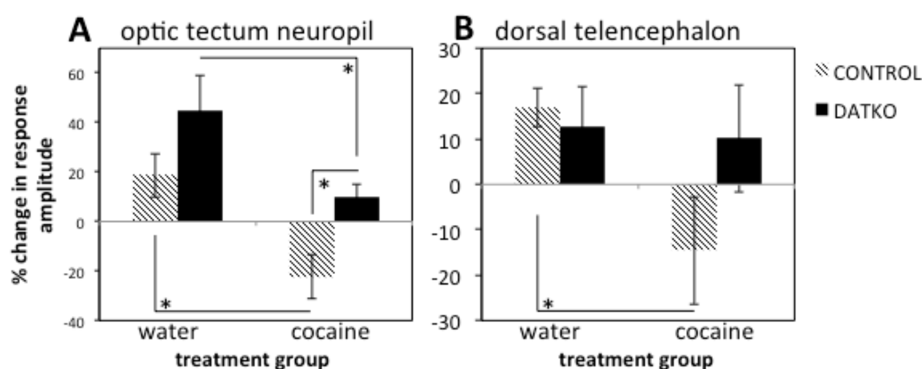

Figure 18. Effects of cocaine on responses to light flash in TOn and dTe in control and DATKO larvae. (A) TOn (B) dTe, mean ± SEM, n=8-10 per group. *p<0.05.

Drd1 expression is downregulated in certain brain regions after PCE

To determine whether expression of dopamine receptor D1 (drd1) is downregulated after PCE, or in DATKO zebrafish, we performed immunohistochemistry stains for drd1 in whole-mounted control, PCE and DATKO larvae. An additional DAPI stain allowed us to clearly discern the anatomical boundaries of the eye, telencephalon, tectum, and cerebellum (Fig. 19a). In controls, we found robust expression of drd1 in the retina, telencephalon, optic tectum and cerebellum (see Fig. 19b for example). Comparing the DAPI and drd1 stains, we confirmed that our antibody was detecting cell-surface drd1 expression (i.e. the two stains did not overlap) (Fig. 19b). To our

knowledge this is the first time that drd1 expression has been assessed using immunohistochemistry in zebrafish.

In PCE larvae, compared with control, drd1 expression was significantly reduced in the optic tectum (t test, n=14, p=0.026), cerebellum (t test, n=7, p=0.04) and brain overall (t test, n=39, p=0.006), but not in the eye or telencephalon (Fig. 20). In DATKO larvae, compared with control, drd1 expression was not significantly different in any brain region (Fig. 20).

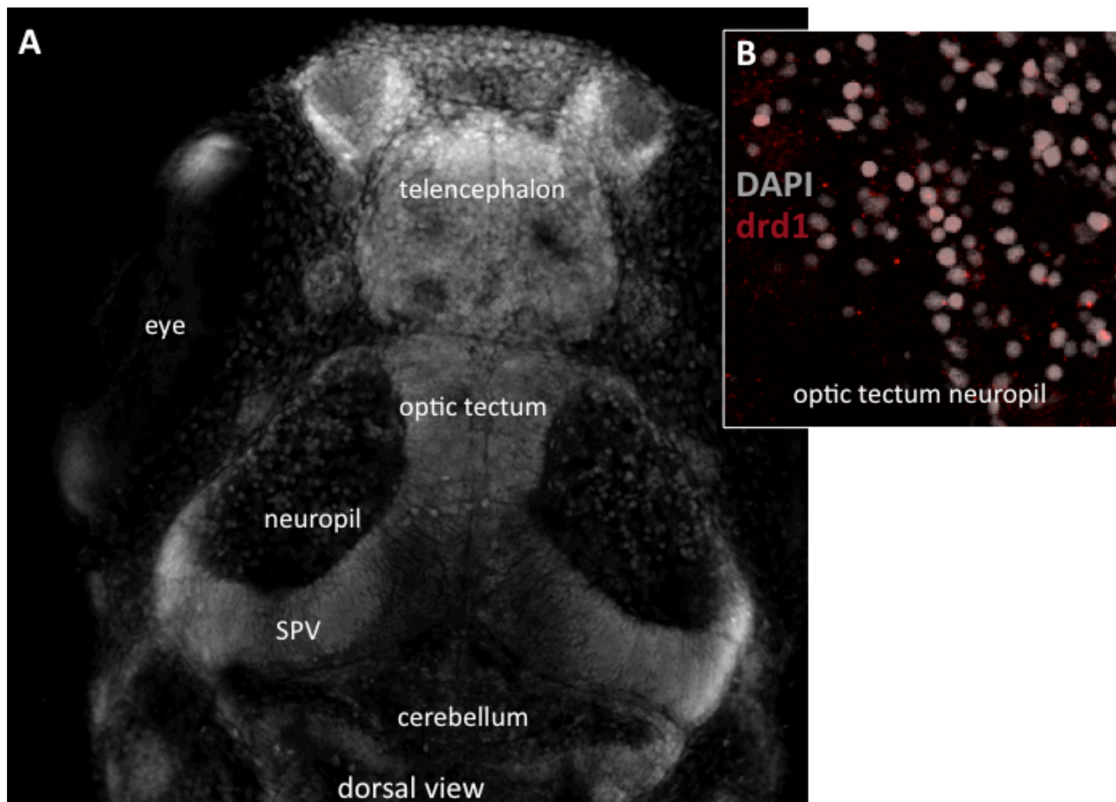

Figure 19. Dopamine receptor drd1 expression in whole-mount zebrafish larvae (7dpf). (A) DAPI expression in cell nuclei (confocal image). (B) Drd1 (red) and DAPI (gray) expression in the optic tectum neuropil.

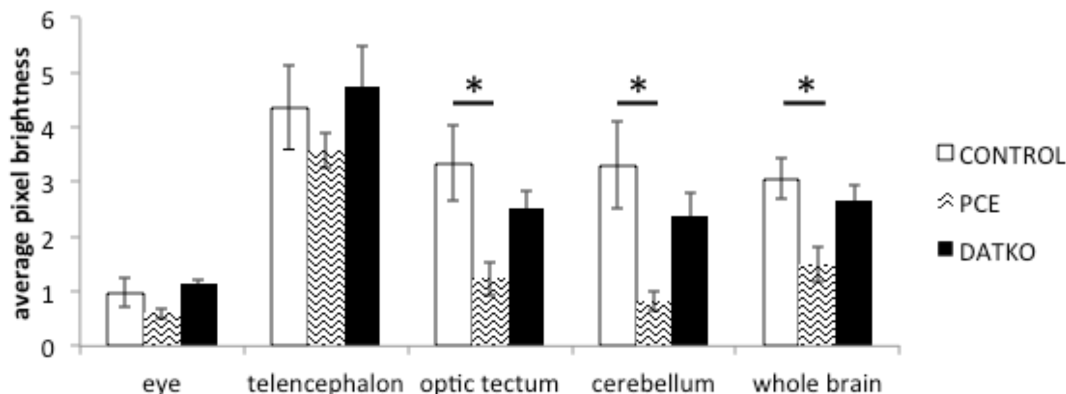

Figure 20. Changes in drd1 expression induced by PCE. Due to pigmentation of the retina which cannot be totally eliminated with PTU treatment, this is not an accurate measure of absolute drd1 expression in the eye. All bars – group mean ± SEM, n=4-6 per group. *p<0.05.

Discussion

Previously, we uncovered both stimulus- and region-specific effects of cocaine on visual processing in larval zebrafish with an intact dopamine system (see Chapter 2). In this study, we show that DAT knockout zebrafish larvae are susceptible to some, but not all of the effects of acute cocaine exposure present in control animals. We also show that drd1 expression is down regulated in some brain regions following prenatal cocaine exposure, but not DAT knockout. Our characterization of DAT knockout animals reveals abnormal, anxiety-like behavior that, in larvae, is not affected by cocaine. Together with the findings presented in Chapter 2, these results help us to understand some of the mechanisms by which cocaine interferes with visual processing and the role of the dopamine system in these effects.

The effects of cocaine in the telencephalon are dopamine-dependent

Our neuronal population imaging studies show that in DAT knockout animals, suppression of light flash responses by acute cocaine is abolished in the telencephalon,

though largely preserved in the optic tectum. This suggests that the mechanisms responsible for the effects of cocaine in these two structures differ, and that the effect in the telencephalon is dopamine-dependent. This could be related to the known role of dopamine in mediating the effects of cocaine on the mammalian telencephalon (Chen et al., 2013; Muñoz-Cuevas et al., 2013; G.D Stanwood, Washington, Shumsky, & Levitt, 2001). However, a better understanding of morphological and functional homology between the zebrafish and mammalian telencephalon is needed in order to provide more generalized conclusions.

The effects of cocaine in the optic tectum are likely due to both dopamine and norepinephrine

The optic tectum, in contrast to the telencephalon, shares direct structural, functional and neurochemical homology with the mammalian superior colliculus (Del Bene et al., 2010; Hunter, Lowe, Thompson, & Meyer, 2013; Isa, 2002; Krauzlis et al., 2013; Nevin et al., 2010), potentially helping to interpret the data we collected in this structure. Light flash responses in the optic tectum were suppressed by cocaine in DAT knockouts, suggesting that the effect is at least partially dopamine-dependent. However, because the effect of cocaine was significantly less pronounced in DAT knockouts than in controls, it is likely that another target of cocaine is also involved in mediating the effect. This is not surprising given other reports in the literature. In DAT knockout rodents, which display hyperactivity (Carpenter et al., 2012; Clements et al., 2014; Raul R Gainetdinov et al., 1999; Raul R Gainetdinov, 2008), amphetamine (selective for DAT and the norepinephrine transporter [NET]) is still able to reduce hyperactive behavior,

and in some cases is even more effective in the absence of DAT (R R Gainetdinov et al., 1999; R R Gainetdinov, Mohn, & Caron, 2001; Trinh, Nehrenberg, Jacobsen, Caron, & Wetsel, 2003). It is plausible that NET is involved in suppression of optic tectum responses to light flash. Suppression of visually evoked activity in superficial layers of the superior colliculus has already been demonstrated in hamsters (Zhang, Mooney, & Rhoades, 1999). Furthermore, application of norepinephrine to the superficial layers of rat superior colliculus reduces spontaneous firing rates (Sato & Kayama, 1983). Studies showing that the zebrafish optic tectum is preferentially innervated by noradrenergic fibers might also indirectly support this hypothesis (Parent et al., 1981; Wichmann & Starke, 1988), though it requires direct experimental evidence.

DAT knockout results in abnormal behavior

We found that DAT knockout leads to a striking change in swimming behavior. Both larval and adult fish swim predominantly at the bottom of the tank. Similar swimming behavior in zebrafish has been documented previously following exposure to alarm pheromone (Egan et al., 2009), chronic stress (Piato et al., 2011), methamphetamine (Kyzar et al., 2013), and caffeine (Egan et al., 2009). DAT knockouts also display thigmotaxis, preferentially swimming along the tank walls. Thigmotaxis has previously been documented in zebrafish, including larvae, after exposure to alcohol or stress (Blaser, Chadwick, & McGinnis, 2010; Lockwood, Bjerke, Kobayashi, & Guo, 2004). Low swimming behavior in the DAT knockouts, although more pronounced, strongly resembles the behavioral effects we observed following cocaine exposure in

control animals. This suggests that the effect of cocaine on swimming behavior could be due to increased synaptic dopamine levels.

An association between low DAT levels and abnormal psychiatric or behavioral conditions has been suggested but remains obscure. DAT knockout and knockdown mice, which have significantly increased synaptic dopamine levels, display abnormal behaviors that have been interpreted as decreased anxiety (Carpenter et al., 2012), increased depression (Perona et al., 2008), and marked hyperactivity (Raul R Gainetdinov, 2008; Russell, Sagvolden, & Johansen, 2005), based on specific tests. Mice with other components of the dopamine system knocked out, e.g. D1, D2, D3, D4 and D5 receptors and tyrosine hydroxylase, also display abnormal behavior, including altered locomotor activity and altered exploration of novel environments (Viggiano et al., 2003). In humans, polymorphisms in DAT and several dopamine receptors have been associated with ADHD (Bobb et al., 2005; Loo et al., 2003), borderline personality disorder (Joyce et al., 2006), and depression (Haeffel et al., 2008). While these observations strongly suggest that developmental perturbation of the dopamine system can profoundly impact cognition and behavior, the mechanisms and pathways by which specific alterations might generate psychological and behavioral phenomena are currently unknown.

To further characterize the consequences of PCE and DAT knockout on the dopamine system, we measured drd1 receptor expression in those two conditions. Because DAT knockout presumably induces long-term changes in dopamine levels, we expected that it would lead to a more profound disruption on the receptor level than three relatively short prenatal cocaine exposures. We were therefore surprised that drd1

expression was markedly reduced after PCE but appeared unchanged in DAT knockouts. That said, we cannot exclude the possibility that function of drd1, if not its expression, is affected in DAT knockouts. For example, a study in rabbits reported decoupling of drd1 receptors and their associated G-proteins, but no decrease in expression, following PCE (Gregg D Stanwood & Levitt, 2007) . In contrast, in DAT knockout mice drd1 expression is downregulated by approximately 50% in the striatum (Raul R Gainetdinov et al., 1999). Together, those findings and ours highlight the need for an in-depth characterization of the consequences of DAT knockout animals throughout development and in multiple brain regions.

Conclusion

The zebrafish model that we used in our studies provides an excellent opportunity to address the diverse consequences of an altered dopamine system throughout development. In this model, we have now reported two dopamine system perturbations, PCE and DAT knockout, that result in changes in neural activity and behavior. These results contribute to the growing understanding of the effects of early hyperdopaminergic tone on the development and function of the dopamine system, including possible effects on anxiety, attention, and visual processing. The zebrafish has great potential as a model in which to expand and clarify our knowledge of these effects, and our ability to treat unwanted consequences of disruption to the dopamine system.

APPENDIX

Appendix #1

Test for direct light sensitivity in dorsal telencephalon

In Chapter 2, we showed that the dorsal telencephalon (dTe) of the larval zebrafish is responsive to red light flash stimuli. It is uncertain, however, whether this region is directly or indirectly light sensitive. Light-sensitive neurons have been found in several places outside the eye in zebrafish brain (Kojima, Mano, & Fukada, 2000).

To determine whether dTe was directly light sensitive, versus receiving visual information from the eyes, we covered the left eye of an embedded larva (see Chapter 2 Methods) with a mix of graphite powder in agar. The objective was to block light transmission into the left eye while leaving the right eye, both optic tecta, and the entire telencephalon uncovered. We then proceeded to present red light flash stimuli to the larva as described in Chapter 2. Because zebrafish have no ipsilateral projection of retinal ganglion cells, information that is received by one eye is processed exclusively by the contralateral TO (Muto et al., 2013). Previously, we confirmed this by presenting light to only one eye using a laser pointer. Only the contralateral tectum showed responses to that stimulus. We therefore expected that the ratio between left and right TO response amplitudes would be significantly greater than one if the left eye were blocked. If dTe receives information from the eyes, via the TO or not, we would expect that the ratio between left and right dTe response amplitudes would also differ significantly from one (above or below, depending on how the two brain regions are connected). If,

however, dTe is independently light sensitive, the ratio between left and right response amplitudes should be very close to one.

For these experiments we used the s1013t-GCaMP-7a transgenic line, which expresses the improved GCaMP-7a fluorophore, to maximize our chances of detecting a difference in response amplitude ratio.

Our results showed that, with the left eye covered, the left/right ratio in TO was significantly greater than 1, as expected, while the left/right ratio in dTe was closer to 1 (Table 2). With the eyes uncovered, the left/right ratio in both regions was close to 1. The different between the groups was not statistically significant, likely because only 2 individuals were tested with their left eye covered. Nevertheless, the results suggest that the dTe may be directly light sensitive. More and better data could be acquired with a more reliable method for blocking light transmission into one eye.

Brain region	***TO***	***dTe***
Average left/right ratio with left eye covered	2.98 ± 1.17	0.87 ± 0.11
Average left/right ratio with eyes uncovered	1.06 ± 0.29	0.82 ± 0.07

Table 2. Ratio between left and right TO and dTe responses to light flash. With left eye covered, n=2. With eyes uncovered, n=6. All groups mean ± SEM.

Appendix #2

Prolonged responses to light in a caudal brain region

While imaging neural activity in the optic tectum and telencephalon as described in Chapters 2 and 3, we observed an additional GCaMP-HS-expressing light-responsive region in the larval zebrafish brain. In order to image this region, which is caudal, lateral

and ventral relative to the optic tectum (Fig. 21, top), it was necessary to embed the zebrafish in a tilted dorsal position. Unfortunately this is a difficult position in which to embed the fish. For this reason we have not characterized the light-responsive activity in this brain region. We present an example of its distinctive, very prolonged (>30 s) responses to light flash (Fig. 21, bottom).

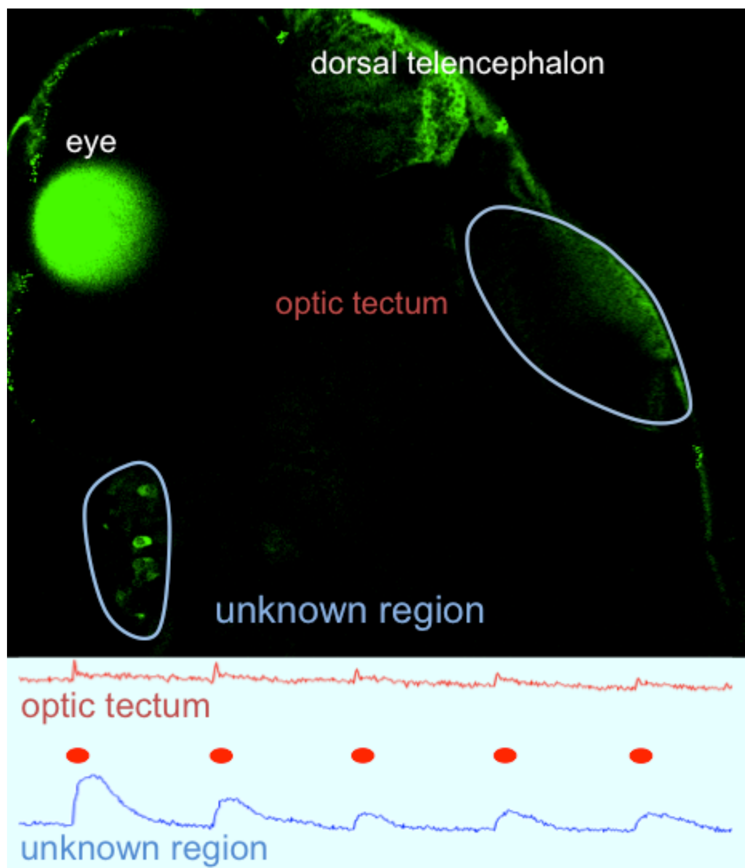

Figure 21. Light flash responses in an unknown caudal brain region. Top panel: dorso-lateral view of s1013t-GCaMP-HS larval brain, two-photon imaging. Bottom panel: Red and blue lines - traces of absolute fluorescence intensity during 5 presentations of red light flash, indicated by red ovals, for the optic tectum neuropil and the unknown region, respectively.

Appendix #3

Details of algorithm for analyzing neural responses to light stimuli

The algorithm we used to analyze the calcium imaging data in Chapters 2 and 3, written in Python, detects, measures and reports characteristics of peaks in fluorescence from a list of 1000 consecutive values in one or more regions of interest (ROIs). The actual code, and accompanying custom software (also written in Python) to display data and select and manage ROIs is available from the author or the Zhdanova Lab. Below, the outline of the algorithm is presented.

1. User can set stimulus frequency in frames (number of image frames between stimuli, default 100), and cutoff threshold in standard deviations (default 1.5)
2. Each frame is normalized to a running mean with a width of half of the inter-stimulus interval
3. Frame values above the cutoff threshold are detected
4. Groups of responses (more than two consecutive suprathreshold frame values) are detected
5. Groups which occur within 10 frames of a stimulus presentation (as defined by stimulus frequency variable) are marked as qualified responses
6. Normalized frame values are converted to percent above running mean
7. The following statistics are reported for qualified responses:
    a. Average response width (number of consecutive suprathreshold frames)
        i. All individual response widths
    b. Average response height (maximum percent above running mean)
        i. All individual response heights
    c. Percent of stimuli resulting in a qualified response
        i. Presence or absence of a qualified response following each stimulus

Appendix #4

Electronic circuit for generating dark flash stimuli

This circuit (Fig. 22) was built for generating dark flashes (interruptions in red light) during two-photon microscopy of live zebrafish larvae, as described in Chapter 2. It is designed to be controlled by the trigger system on a Zeiss LSM 710 microscope. In its default state it produces 450 lux red light, and when triggered the light turns off for 1-2 seconds (adjustable).

The following description of the circuit was provided by Brian Kardon, who assisted with circuit design. An SR latch (implemented with two NOR gates) holds a set of five transistor-controlled LEDs in an on state via the inverted output, until a 5V pulse from a microscope controller trigger output sets the main output of the SR latch, causing the LEDs to shut off. In an RC circuit (shown at the bottom), a capacitor simultaneously begins charging through a transistor. When the capacitor finishes charging (in an amount of time controlled by the potentiometer that forms part of the RC circuit), the SR latch is set back in the initial state, discharging the capacitor and turning the LEDs back on. The circuit remains in this on state until the next input pulse from the microscope controller is received.

Figure 22. Diagram of circuit for the production of dark flash stimuli

BIBLIOGRAPHY

Accornero, V. H., Amado, A. J., Morrow, C. E., Xue, L., Anthony, J. C., & Bandstra, E. S. (2007). Impact of prenatal cocaine exposure on attention and response inhibition as assessed by continuous performance tests. *Journal of Developmental and Behavioral Pediatrics : JDBP*, *28*(3), 195–205. doi:10.1097/01.DBP.0000268560.72580.f9

Ackerman, J. P., Riggins, T., & Black, M. M. (2010). A review of the effects of prenatal cocaine exposure among school-aged children. *Pediatrics*, *125*(3), 554–65. doi:10.1542/peds.2009-0637

Aizenberg, M., & Schuman, E. M. (2011). Cerebellar-dependent learning in larval zebrafish. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(24), 8708–12. doi:10.1523/JNEUROSCI.6565-10.2011

Azuma, S. D., & Chasnoff, I. J. (1993). Outcome of Children Prenatally Exposed to Cocaine and Other Drugs: A Path Analysis of Three-Year Data. *Pediatrics*, *92*(3), 396–402.

Bada, H. S., Bann, C. M., Bauer, C. R., Shankaran, S., Lester, B., LaGasse, L., … Higgins, R. (2011). Preadolescent behavior problems after prenatal cocaine exposure: Relationship between teacher and caretaker ratings (Maternal Lifestyle Study). *Neurotoxicology and Teratology*, *33*(1), 78–87. doi:10.1016/j.ntt.2010.06.005

Bandstra, E. S., Morrow, C. E., Anthony, J. C., Accornero, V. H., & Fried, P. A. (2001). Longitudinal investigation of task persistence and sustained attention in children with prenatal cocaine exposure. *Neurotoxicology and Teratology*, *23*(6), 545–559. doi:10.1016/S0892-0362(01)00181-7

Blaser, R. E., Chadwick, L., & McGinnis, G. C. (2010). Behavioral measures of anxiety in zebrafish (Danio rerio). *Behavioural Brain Research*, *208*(1), 56–62. doi:10.1016/j.bbr.2009.11.009

Block, S. S., Moore, B. D., & Scharre, J. E. (1997). Visual anomalies in young children exposed to cocaine. *Optometry and Vision Science : Official Publication of the American Academy of Optometry*, *74*(1), 28–36.

Bobb, A. J., Addington, A. M., Sidransky, E., Gornick, M. C., Lerch, J. P., Greenstein, D. K., … Rapoport, J. L. (2005). Support for association between ADHD and two candidate genes: NET1 and DRD1. *American Journal of Medical Genetics. Part B,*

Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics, *134B*(1), 67–72. doi:10.1002/ajmg.b.30142

Borla, M. A., Palecek, B., Budick, S., & O'Malley, D. M. (2002). Prey capture by larval zebrafish: evidence for fine axial motor control. *Brain, Behavior and Evolution*, *60*(4), 207–29. doi:66699

Buckingham-Howes, S., Berger, S. S., Scaletti, L. A., & Black, M. M. (2013). Systematic review of prenatal cocaine exposure and adolescent development. *Pediatrics*, *131*(6), e1917–36. doi:10.1542/peds.2012-0945

Burgess, H. A., & Granato, M. (2007a). Modulation of locomotor activity in larval zebrafish during light adaptation. *The Journal of Experimental Biology*, *210*(Pt 14), 2526–39. doi:10.1242/jeb.003939

Burgess, H. A., & Granato, M. (2007b). Sensorimotor gating in larval zebrafish. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(18), 4984–94. doi:10.1523/JNEUROSCI.0615-07.2007

Burgess, H. A., Schoch, H., & Granato, M. (2010). Distinct retinal pathways drive spatial orientation behaviors in zebrafish navigation. *Current Biology : CB*, *20*(4), 381–6. doi:10.1016/j.cub.2010.01.022

Campbell, K. J., Takada, M., & Hattori, T. (1991). Co-localization of tyrosine hydroxylase and glutamate decar□ylase in a subpopulation of single nigrotectal projection neurons. *Brain Research*, *558*(2), 239–244. doi:10.1016/0006-8993(91)90774-P

Carpenter, A. C., Saborido, T. P., & Stanwood, G. D. (2012). Development of hyperactivity and anxiety responses in dopamine transporter-deficient mice. *Developmental Neuroscience*, *34*(2-3), 250–7. doi:10.1159/000336824

Chen, B. T., Yau, H.-J., Hatch, C., Kusumoto-Yoshida, I., Cho, S. L., Hopf, F. W., & Bonci, A. (2013). Rescuing cocaine-induced prefrontal cortex hypoactivity prevents compulsive cocaine seeking. *Nature*, *496*(7445), 359–62. doi:10.1038/nature12024

Church, M. W., Crossland, W., Holmes, P. A., Overbeck, G. W., & Tilak, J. P. (1998). Effects of Prenatal Cocaine on Hearing, Vision, Growth, and Behaviora. *Annals of the New York Academy of Sciences*, *846*(1 COCAINE), 12–28. doi:10.1111/j.1749-6632.1998.tb09723.x

Clements, K. M., Devonshire, I. M., Reynolds, J. N. J., & Overton, P. G. (2014). Enhanced visual responses in the superior colliculus in an animal model of attention-

deficit hyperactivity disorder and their suppression by D-amphetamine. *Neuroscience*, *274*, 289–98. doi:10.1016/j.neuroscience.2014.05.054

Comoli, E., Coizet, V., Boyes, J., Bolam, J. P., Canteras, N. S., Quirk, R. H., … Redgrave, P. (2003). A direct projection from superior colliculus to substantia nigra for detecting salient visual events. *Nature Neuroscience*, *6*(9), 974–80. doi:10.1038/nn1113

Darland, T., & Dowling, J. E. (2001). Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(20), 11691–6. doi:10.1073/pnas.191380698

De Giovanni, N., & Marchetti, D. (2012). Cocaine and its metabolites in the placenta: a systematic review of the literature. *Reproductive Toxicology (Elmsford, N.Y.)*, *33*(1), 1–14. doi:10.1016/j.reprotox.2011.10.012

Del Bene, F., Wyart, C., Robles, E., Tran, A., Looger, L., Scott, E. K., … Baier, H. (2010). Filtering of visual information in the tectum by an identified neural circuit. *Science (New York, N.Y.)*, *330*(6004), 669–73. doi:10.1126/science.1192949

Delaney-Black, V., Chiodo, L. M., Hannigan, J. H., Greenwald, M. K., Janisse, J., Patterson, G., … Sokol, R. J. (2011). Prenatal and postnatal cocaine exposure predict teen cocaine use. *Neurotoxicology and Teratology*, *33*(1), 110–9. doi:10.1016/j.ntt.2010.06.011

Dommett, E., Coizet, V., Blaha, C. D., Martindale, J., Lefebvre, V., Walton, N., … Redgrave, P. (2005). How visual stimuli activate dopaminergic neurons at short latency. *Science (New York, N.Y.)*, *307*(5714), 1476–9. doi:10.1126/science.1107026

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., … Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*, *205*(1), 38–44. doi:10.1016/j.bbr.2009.06.022

Foley, J. E., Yeh, J.-R. J., Maeder, M. L., Reyon, D., Sander, J. D., Peterson, R. T., & Joung, J. K. (2009). Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN). *PloS One*, *4*(2), e4348. doi:10.1371/journal.pone.0004348

Gahtan, E., Tanger, P., & Baier, H. (2005). Visual prey capture in larval zebrafish is controlled by identified reticulospinal neurons downstream of the tectum. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(40), 9294–303. doi:10.1523/JNEUROSCI.2678-05.2005

Gainetdinov, R. R. (2008). Dopamine transporter mutant mice in experimental neuropharmacology. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *377*(4-6), 301–13. doi:10.1007/s00210-007-0216-0

Gainetdinov, R. R., Jones, S. R., & Caron, M. G. (1999). Functional hyperdopaminergia in dopamine transporter knock-out mice. *Biological Psychiatry*, *46*(3), 303–311. doi:10.1016/S0006-3223(99)00122-5

Gainetdinov, R. R., Mohn, A. R., & Caron, M. G. (2001). Genetic animal models: focus on schizophrenia. *Trends in Neurosciences*, *24*(9), 527–33.

Gainetdinov, R. R., Wetsel, W. C., Jones, S. R., Levin, E. D., Jaber, M., & Caron, M. G. (1999). Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science (New York, N.Y.)*, *283*(5400), 397–401.

Gaymard, B., François, C., Ploner, C. J., Condy, C., & Rivaud-Péchoux, S. (2003). A direct prefrontotectal tract against distractibility in the human brain. *Annals of Neurology*, *53*(4), 542–5. doi:10.1002/ana.10560

Gerlai, R., Lahav, M., Guo, S., & Rosenthal, A. (2000). Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacology Biochemistry and Behavior*, *67*(4), 773–782. doi:10.1016/S0091-3057(00)00422-6

Good, W. V., Ferriero, D. M., Golabi, M., & Kobori, J. A. (1992). Abnormalities of the Visual System in Infants Exposed to Cocaine. *Ophthalmology*, *99*(3), 341–346. doi:10.1016/S0161-6420(92)31967-0

Goodale, M. A., Foreman, N. P., & Milner, A. D. (1978). Visual orientation in the rat: A dissociation of deficits following cortical and collicular lesions. *Experimental Brain Research*, *31*(3). doi:10.1007/BF00237301

Gowan, J. D., Coizet, V., Devonshire, I. M., & Overton, P. G. (2008). D-amphetamine depresses visual responses in the rat superior colliculus: a possible mechanism for amphetamine-induced decreases in distractibility. *Journal of Neural Transmission (Vienna, Austria : 1996)*, *115*(3), 377–87. doi:10.1007/s00702-007-0858-6

Guo, S. (2004). Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? *Genes, Brain, and Behavior*, *3*(2), 63–74.

Haeffel, G. J., Getchell, M., Koposov, R. A., Yrigollen, C. M., Deyoung, C. G., Klinteberg, B. A., … Grigorenko, E. L. (2008). Association between polymorphisms in the dopamine transporter gene and depression: evidence for a gene-environment interaction in a sample of juvenile detainees. *Psychological Science*, *19*(1), 62–9. doi:10.1111/j.1467-9280.2008.02047.x

Hansen, R. L., Struthers, J. M., & Gospe, S. M. (1993). Visual evoked potentials and visual processing in stimulant drug-exposed infants. *Developmental Medicine and Child Neurology*, *35*(9), 798–805.

Hansen, R. L., Struthers, J. M., & Gospe, S. M. (2008). VISUAL EVOKED POTENTIALS AND VISUAL PROCESSING IN STIMULANT DRUG-EXPOSED INFANTS. *Developmental Medicine & Child Neurology*, *35*(9), 798–805. doi:10.1111/j.1469-8749.1993.tb11731.x

Heffelfinfger, A., Craft, S., & Shyken, J. (1997). Visual attention in children with prenatal cocaine exposure. *Journal of the International Neuropsychological Society*, *3*(03), 237–245.

Heffelfinfger, A. K., CRAFT, S., WHITE, D. A., & SHYKEN, J. (2002). Visual attention in preschool children prenatally exposed to cocaine: Implications for behavioral regulation. *Journal of the International Neuropsychological Society*, *8*(01), 12–21. doi:10.1017/S1355617701020021

Holzschuh, J., Ryu, S., Aberger, F., & Driever, W. (2001). Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing zebrafish embryo. *Mechanisms of Development*, *101*(1-2), 237–243. doi:10.1016/S0925-4773(01)00287-8

Hunter, P. R., Lowe, A. S., Thompson, I. D., & Meyer, M. P. (2013). Emergent properties of the optic tectum revealed by population analysis of direction and orientation selectivity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *33*(35), 13940–5. doi:10.1523/JNEUROSCI.1493-13.2013

Isa, T. (2002). Intrinsic processing in the mammalian superior colliculus. *Current Opinion in Neurobiology*, *12*(6), 668–677. doi:10.1016/S0959-4388(02)00387-2

Jones, A. W., Holmgren, A., & Kugelberg, F. C. (2008). Concentrations of cocaine and its major metabolite benzoylecgonine in blood samples from apprehended drivers in Sweden. *Forensic Science International*, *177*(2-3), 133–9. doi:10.1016/j.forsciint.2007.11.009

Jones, L. B., Stanwood, G. D., Reinoso, B. S., Washington, R. A., Wang, H.-Y., Friedman, E., & Levitt, P. (2000). In Utero Cocaine-Induced Dysfunction of Dopamine D1 Receptor Signaling And Abnormal Differentiation of Cerebral Cortical Neurons. *Journal of Neuroscience*, *20*(12), 4606–4614.

Joyce, P. R., McHugh, P. C., McKenzie, J. M., Sullivan, P. F., Mulder, R. T., Luty, S. E., … Kennedy, M. A. (2006). A dopamine transporter polymorphism is a risk factor

for borderline personality disorder in depressed patients. *Psychological Medicine*, *36*(6), 807–13. doi:10.1017/S0033291706007288

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *203*(3), 253–310. doi:10.1002/aja.1002030302

Kobat, D., Horton, N. G., & Xu, C. (2011). In vivo two-photon microscopy to 1.6-mm depth in mouse cortex. *Journal of Biomedical Optics*, *16*(10), 106014. doi:10.1117/1.3646209

Kojima, D., Mano, H., & Fukada, Y. (2000). Vertebrate Ancient-Long Opsin: A Green-Sensitive Photoreceptive Molecule Present in Zebrafish Deep Brain and Retinal Horizontal Cells. *Journal of Neuroscience*, *20*(8), 2845–2851.

Krauzlis, R. J., Lovejoy, L. P., & Zénon, A. (2013). Superior colliculus and visual spatial attention. *Annual Review of Neuroscience*, *36*, 165–82. doi:10.1146/annurev-neuro-062012-170249

Kyzar, E., Stewart, A. M., Landsman, S., Collins, C., Gebhardt, M., Robinson, K., & Kalueff, A. V. (2013). Behavioral effects of bidirectional modulators of brain monoamines reserpine and d-amphetamine in zebrafish. *Brain Research*, *1527*, 108–16. doi:10.1016/j.brainres.2013.06.033

Leech, S. ., Richardson, G. ., Goldschmidt, L., & Day, N. . (1999). Prenatal Substance Exposure. *Neurotoxicology and Teratology*, *21*(2), 109–118. doi:10.1016/S0892-0362(98)00042-7

Leinweber, M., Zmarz, P., Buchmann, P., Argast, P., Hübener, M., Bonhoeffer, T., & Keller, G. B. (2014). Two-photon calcium imaging in mice navigating a virtual reality environment. *Journal of Visualized Experiments : JoVE*, (84), e50885. doi:10.3791/50885

Lockwood, B., Bjerke, S., Kobayashi, K., & Guo, S. (2004). Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. *Pharmacology, Biochemistry, and Behavior*, *77*(3), 647–54. doi:10.1016/j.pbb.2004.01.003

Loo, S. K., Specter, E., Smolen, A., Hopfer, C., Teale, P. D., & Reite, M. L. (2003). Functional effects of the DAT1 polymorphism on EEG measures in ADHD. *Journal of the American Academy of Child and Adolescent Psychiatry*, *42*(8), 986–93. doi:10.1097/01.CHI.0000046890.27264.88

López Patiño, M. A., Yu, L., Yamamoto, B. K., & Zhdanova, I. V. (2008). Gender differences in zebrafish responses to cocaine withdrawal. *Physiology & Behavior*, *95*(1-2), 36–47. doi:10.1016/j.physbeh.2008.03.021

López-Patiño, M. A., Yu, L., Cabral, H., & Zhdanova, I. V. (2008). Anxiogenic effects of cocaine withdrawal in zebrafish. *Physiology & Behavior*, *93*(1-2), 160–71. doi:10.1016/j.physbeh.2007.08.013

Mabray, P. D. L. (2010). Circadian modulation of the vasoconstrictive properties of cocaine. (Unpublished doctoral dissertation). Boston University School of Medicine, Boston, MA.

McElligott, M. B., & O'Malley, D. M. (2005). Prey tracking by larval zebrafish: axial kinematics and visual control. *Brain, Behavior and Evolution*, *66*(3), 177–96. doi:10.1159/000087158

Milner, A. D., Foreman, N. P., & Goodale, M. A. (1978). Go-left go-right discrimination performance and distractibility following lesions of prefrontal cortex or superior colliculus in stumptail macaques. *Neuropsychologia*, *16*(4), 381–390. doi:10.1016/0028-3932(78)90062-3

Mitchell, J., & Vierkant, A. D. (1991). Delusions and Hallucinations of Cocaine Abusers and Paranoid Schizophrenics: A Comparative Study. *The Journal of Psychology*, *125*(3), 301–310. doi:10.1080/00223980.1991.10543294

Mueller, T., Dong, Z., Berberoglu, M. A., & Guo, S. (2011). The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). *Brain Research*, *1381*, 95–105. doi:10.1016/j.brainres.2010.12.089

Muñoz-Cuevas, F. J., Athilingam, J., Piscopo, D., & Wilbrecht, L. (2013). Cocaine-induced structural plasticity in frontal cortex correlates with conditioned place preference. *Nature Neuroscience*, *16*(10), 1367–9. doi:10.1038/nn.3498

Muto, A., Ohkura, M., Abe, G., Nakai, J., & Kawakami, K. (2013). Real-Time Visualization of Neuronal Activity during Perception. *Current Biology*, *23*(4), 307–311. doi:10.1016/j.cub.2012.12.040

Muto, A., Ohkura, M., Kotani, T., Higashijima, S., Nakai, J., & Kawakami, K. (2011). Genetic visualization with an improved GCaMP calcium indicator reveals spatiotemporal activation of the spinal motor neurons in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(13), 5425–30. doi:10.1073/pnas.1000887108

Nakai, J., Ohkura, M., & Imoto, K. (2001). A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nature Biotechnology*, *19*(2), 137–41. doi:10.1038/84397

Nevin, L. M., Robles, E., Baier, H., & Scott, E. K. (2010). Focusing on optic tectum circuitry through the lens of genetics. *BMC Biology*, *8*(1), 126. doi:10.1186/1741-7007-8-126

Ng, M.-C., Tang, T.-H., Ko, M.-C., Wu, Y.-J., Hsu, C.-P., Yang, Y.-L., & Lu, K.-T. (2012). Stimulation of the lateral division of the dorsal telencephalon induces synaptic plasticity in the medial division of adult zebrafish. *Neuroscience Letters*, *512*(2), 109–13. doi:10.1016/j.neulet.2012.01.070

Overton, P. G. (2008). Collicular dysfunction in attention deficit hyperactivity disorder. *Medical Hypotheses*, *70*(6), 1121–7. doi:10.1016/j.mehy.2007.11.016

Overton, P. G., & Devonshire, I. M. (2008). Cocaine facilitates craving via an action on sensory processing. *Bioscience Hypotheses*, *1*(2), 70–77. doi:10.1016/j.bihy.2008.03.002

Parent, A., Descarries, L., & Beaudet, A. (1981). Organization of ascending serotonin systems in the adult rat brain. A radioautographic study after intraventricular administration of [3h]5-hydroxytryptamine. *Neuroscience*, *6*(2), 115–138. doi:10.1016/0306-4522(81)90050-6

Parker, M. O., Brock, A. J., Walton, R. T., & Brennan, C. H. (2013). The role of zebrafish (Danio rerio) in dissecting the genetics and neural circuits of executive function. *Frontiers in Neural Circuits*, *7*, 63. doi:10.3389/fncir.2013.00063

Perona, M. T. G., Waters, S., Hall, F. S., Sora, I., Lesch, K.-P., Murphy, D. L., … Uhl, G. R. (2008). Animal models of depression in dopamine, serotonin, and norepinephrine transporter knockout mice: prominent effects of dopamine transporter deletions. *Behavioural Pharmacology*, *19*(5-6), 566–74. doi:10.1097/FBP.0b013e32830cd80f

Piato, Â. L., Capiotti, K. M., Tamborski, A. R., Oses, J. P., Barcellos, L. J. G., Bogo, M. R., … Bonan, C. D. (2011). Unpredictable chronic stress model in zebrafish (Danio rerio): behavioral and physiological responses. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *35*(2), 561–7. doi:10.1016/j.pnpbp.2010.12.018

Richardson, G. A., Larkby, C., Goldschmidt, L., & Day, N. L. (2013). Adolescent initiation of drug use: effects of prenatal cocaine exposure. *Journal of the American Academy of Child and Adolescent Psychiatry*, *52*(1), 37–46. doi:10.1016/j.jaac.2012.10.011

Ritz, M. C., Cone, E. J., & Kuhar, M. J. (1990). Cocaine inhibition of ligand binding at dopamine, norepinephrine and serotonin transporters: A structure-activity study. *Life Sciences*, *46*(9), 635–645. doi:10.1016/0024-3205(90)90132-B

Rocha, B. A., Fumagalli, F., Gainetdinov, R. R., Jones, S. R., Ator, R., Giros, B., … Caron, M. G. (1998). Cocaine self-administration in dopamine-transporter knockout mice. *Nature Neuroscience*, *1*(2), 132–7. doi:10.1038/381

Rose, T., Goltstein, P. M., Portugues, R., & Griesbeck, O. (2014). Putting a finishing touch on GECIs. *Frontiers in Molecular Neuroscience*, *7*, 88. doi:10.3389/fnmol.2014.00088

Russell, V. A., Sagvolden, T., & Johansen, E. B. (2005). Animal models of attention-deficit hyperactivity disorder. *Behavioral and Brain Functions : BBF*, *1*(1), 9. doi:10.1186/1744-9081-1-9

Sato, H., & Kayama, Y. (1983). Effects of noradrenaline applied iontophoretically on rat superior collicular neurons. *Brain Research Bulletin*, *10*(4), 453–457. doi:10.1016/0361-9230(83)90143-0

Schantz, E. J., & Lauffer, M. A. (1962). Diffusion Measurements in Agar Gel. *Biochemistry*, *1*(4), 658–663. doi:10.1021/bi00910a019

Schwartz, B. G., Rezkalla, S., & Kloner, R. A. (2010). Cardiovascular effects of cocaine. *Circulation*, *122*(24), 2558–69. doi:10.1161/CIRCULATIONAHA.110.940569

Scott, E. K., & Baier, H. (2009). The cellular architecture of the larval zebrafish tectum, as revealed by gal4 enhancer trap lines. *Frontiers in Neural Circuits*, *3*, 13. doi:10.3389/neuro.04.013.2009

Shang, E. H., & Zhdanova, I. V. (2007). The circadian system is a target and modulator of prenatal cocaine effects. *PloS One*, *2*(7), e587. doi:10.1371/journal.pone.0000587

Siegel, R. K. (1978). Cocaine hallucinations. *The American Journal of Psychiatry*, *135*(3), 309–14.

Sora, I., Hall, F. S., Andrews, A. M., Itokawa, M., Li, X. F., Wei, H. B., … Uhl, G. R. (2001). Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(9), 5300–5. doi:10.1073/pnas.091039298

Spiteri Cornish, K., Hrabovsky, M., Scott, N. W., Myerscough, E., & Reddy, A. R. (2013). The short- and long-term effects on the visual system of children following

exposure to maternal substance misuse in pregnancy. *American Journal of Ophthalmology*, *156*(1), 190–4. doi:10.1016/j.ajo.2013.02.004

Sprague, J. M., & Meikle, T. H. (1965). The role of the superior colliculus in visually guided behavior. *Experimental Neurology*, *11*(1), 115–146. doi:10.1016/0014-4886(65)90026-9

Stanwood, G. ., Washington, R. ., Shumsky, J. ., & Levitt, P. (2001). Prenatal cocaine exposure produces consistent developmental alterations in dopamine-rich regions of the cerebral cortex. *Neuroscience*, *106*(1), 5–14. doi:10.1016/S0306-4522(01)00256-1

Stanwood, G. D., & Levitt, P. (2007). Prenatal exposure to cocaine produces unique developmental and long-term adaptive changes in dopamine D1 receptor activity and subcellular distribution. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(1), 152–7. doi:10.1523/JNEUROSCI.4591-06.2007

Steenbergen, P. J., Richardson, M. K., & Champagne, D. L. (2011). Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: a pharmacological study. *Behavioural Brain Research*, *222*(1), 15–25. doi:10.1016/j.bbr.2011.03.025

Stolerman, I. P., & Price, L. H. (Eds.). (2010). *Encyclopedia of Psychopharmacology*. Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/978-3-642-27772-6

Störmer, V. S., Passow, S., Biesenack, J., & Li, S.-C. (2012). Dopaminergic and cholinergic modulations of visual-spatial attention and working memory: insights from molecular genetic research and implications for adult cognitive development. *Developmental Psychology*, *48*(3), 875–89. doi:10.1037/a0026198

Struthers, J. M., & Hansen, R. L. (1992). Visual recognition memory in drug-exposed infants. *Journal of Developmental and Behavioral Pediatrics : JDBP*, *13*(2), 108–11.

Thomas, L. T., Welsh, L., Galvez, F., & Svoboda, K. R. (2009). Acute nicotine exposure and modulation of a spinal motor circuit in embryonic zebrafish. *Toxicology and Applied Pharmacology*, *239*(1), 1–12. doi:10.1016/j.taap.2008.08.023

Trinh, J. V, Nehrenberg, D. L., Jacobsen, J. P. R., Caron, M. G., & Wetsel, W. C. (2003). Differential psychostimulant-induced activation of neural circuits in dopamine transporter knockout and wild type mice. *Neuroscience*, *118*(2), 297–310.

Viggiano, D., Ruocco, L. A., & Sadile, A. G. (2003). Dopamine phenotype and behaviour in animal models: in relation to attention deficit hyperactivity disorder. *Neuroscience and Biobehavioral Reviews*, *27*(7), 623–37.

Vorspan, F., Brousse, G., Bloch, V., Bellais, L., Romo, L., Guillem, E., … Lépine, J.-P. (2012). Cocaine-induced psychotic symptoms in French cocaine addicts. *Psychiatry Research*, *200*(2-3), 1074–6. doi:10.1016/j.psychres.2012.04.008

Vuilleumier, P. (2014). Affective and motivational control of vision. *Current Opinion in Neurology*, *28*(1), 29–35. doi:10.1097/WCO.0000000000000159

Weiss, F. (2010). Advances in Animal Models of Relapse for Addiction Research. CRC Press.

Weller, M. E., Rose, S., Jenner, P., & Marsden, C. D. (1987). in vitro characterisation of dopamine receptors in the superior colliculus of the rat. *Neuropharmacology*, *26*(4), 347–354. doi:10.1016/0028-3908(87)90187-0

Wichmann, T., & Starke, K. (1988). Uptake, release, and modulation of release of noradrenaline in rabbit superior colliculus. *Neuroscience*, *26*(2), 621–634. doi:10.1016/0306-4522(88)90169-8

Zhang, Y., Mooney, R. D., & Rhoades, R. W. (1999). Effects of norepinephrine on the activity of visual neurons in the superior colliculus of the hamster. *Visual Neuroscience*, *16*(3), 541–55.

Zhdanova, I. V, Wang, S. Y., Leclair, O. U., & Danilova, N. P. (2001). Melatonin promotes sleep-like state in zebrafish. *Brain Research*, *903*(1-2), 263–268. doi:10.1016/S0006-8993(01)02444-1

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