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A CHOICE BEHAVIOR FOR MORPHINE REVEALS EXPERIENCE-DEPENDENT DRUG PREFERENCE AND UNDERLYING NEURAL SUBSTRATES IN DEVELOPING LARVAL ZEBRAFISH

S. BRETAUD,[a] Q. LI,[a] B. L. LOCKWOOD,[a] K. KOBAYASHI,[a] E. LIN[a] AND S. GUO[a,b,c]*

[a]*Department of Biopharmaceutical Sciences, University of California, San Francisco, CA 94143-2811, USA*

[b]*Programs in Human Genetics, Biological Sciences, Neuroscience, University of California, San Francisco, CA 94143-2811, USA*

[c]*Wheeler Center for the Neurobiology of Addiction, University of California, San Francisco, CA 94143-2811, USA*

Abstract—Transparent larval zebrafish offer the opportunity to unravel genetic and neuronal networks underlying behavior in a developing system. In this study, we developed a choice chamber paradigm to measure reward-associated behavior in larval zebrafish. In the chamber where larval zebrafish have a choice of spending their time in either a water- or morphine-containing compartment, larvae that have previously experienced morphine spend significantly more time in the compartment containing morphine. This behavior can be attentuated by pre-treatment with antagonists of the opioid receptor or the dopamine receptor, and furthermore, is impaired in the *too few* mutant, which has a genetic deficiency in the production of specific groups of dopaminergic and serotonergic neurons in the ventral forebrain. These results uncover a choice behavior for an addictive substance in larval zebrafish that is mediated through central opioid and monoaminergic neurotransmitter systems. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: choice behavior, zebrafish, reward, addiction, opioid system, dopamine system.

Like humans, most animals display preference when confronted with alternatives, so that they acquire or avoid one or more alternatives because of their rewarding or aversive properties. The genetic and neuronal networks mediating choice are incompletely understood, and dysfunction in this process is implicated in disorders ranging from addiction to obesity.

One way to study this process is to analyze a simple preference behavior in animals that are amenable to molecular genetic analysis. For example, *Drosophila* was shown to display choice behavior when facing contradictory visual cues (Tang and Guo, 2001). In rodents, conditioned place preference (CPP) and self-administration are behavioral paradigms widely used to assess reward-associated choice making. In CPP, animals spend more time in the compartment where they have previously received a reward (for instance, food or drugs of abuse) (Tzschentke, 1998). In self-administration, a reward reinforces operant behavior by which the animal can directly obtain the reward (Spealman and Goldberg, 1978). All of these behaviors involve complex and yet poorly understood circuitries that fulfill sensory perception, associative learning, decision-making, and finally motor outputs. Interestingly, disruption of the mesolimbic dopaminergic (DA) pathway in rodents blocks these behaviors induced by most reinforcing agents, suggesting that dopamine plays an important role in regulating these behaviors (Tzschentke, 1998).

Larval zebrafish [from 5 days postfertilization (dpf), to 2–3 weeks old] are small-sized (~2–10 mm in length) and obtainable in large quantities (each adult zebrafish mating can usually produce ~200 to ~800 progeny). These features make them highly suitable for large-scale screening (Grunwald and Eisen, 2002). Moreover, larval zebrafish are optically transparent. Together with transgenic tools available in zebrafish, this makes it possible to visualize neural circuitry in living and possibly behaving animals (Megason and Fraser, 2003; Guo, 2004). Compared with the wide use of embryonic and larval zebrafish in developmental studies, they are infrequently used for behavioral genetic analysis because of the relative scarcity of established behavioral paradigms, although behaviors related to sensory perception (Baier, 2000) or locomotor activity (Drapeau et al., 2002) have been described in larval zebrafish. In addition, alcohol can modify locomotor behavior of larval zebrafish in a manner similar to its effect in adult zebrafish and mammals (Gerlai et al., 2000; Lockwood et al., 2004). Seizures are inducible in larval zebrafish in a model of epilepsy by treatment with the convulsant agent pentylenetetrazole (Baraban et al., 2005). Studies of preference behavior for addictive drugs have been investigated in adult zebrafish (Darland and Dowling, 2001; Lau et al., 2006; Ninkovic et al., 2006). To date, no choice behavior has ever been described in larval zebrafish. Given the nice features of larval zebrafish, availability of such behavioral paradigms would facilitate the understanding of molecular and cellular basis of choice making and of the influence of addictive substances on the developing brain.

Here we describe a choice behavior for morphine in larval zebrafish. Morphine is an opiate drug that is widely used as a pain medication, and has demonstrated rewarding and reinforcing effects in mammals (Shippenberg and

*Correspondence to: S. Guo, Department of Biopharmaceutical Sciences, University of California, San Francisco, CA 94143-0446, USA. Tel: +1-415-502-4949.
E-mail address: su.guo@ucsf.edu (S. Guo).

Abbreviations: ANOVA, analysis of variance; CPP, conditioned place preference; DA, dopaminergic; LC/MS/MS, high performance liquid chromatography–tandem mass spectrometry; MOR, μ-opioid receptor; MS, morphine sulfate; SCH 23390, Schering compound 23390.

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Elmer, 1998). We found that larval zebrafish pre-exposed to morphine preferred the morphine-containing compartment in a "choice chamber paradigm." Such morphine preference behavior is significantly reduced by pre-treatment with the opioid receptor antagonist naloxone, or with the dopamine receptor antagonist SCH Schering compound 23390 (23390). Furthermore, the preference behavior is significantly attenuated in the *too few* mutant, which has a genetic deficiency in the production of specific groups of DA and serotonergic neurons in the ventral forebrain (Guo et al., 1999; Levkowitz et al., 2003; Rink and Guo, 2004). These findings reveal an experience-dependent choice behavior in larval zebrafish, which requires central opioid and monoaminergic neurotransmitter systems. Our results establish a behavioral paradigm for assessing genetic and neuronal networks underlying reward-associated choice making, and for testing the impact of a variety of addictive substances on the developing vertebrate brain.

EXPERIMENTAL PROCEDURES

Rearing

Adult zebrafish were maintained following standard procedures (Westerfield, 1995). AB is a laboratory-bred strain from the University of Oregon. EK is a commercially available strain from Ekkwill, Gibsonton, FL, USA. Larvae of AB wild type or AB/EK hybrid strain (backcrossed to AB for more than five generations) were raised by groups of 50 until the age of 2 weeks at 28 °C in a rearing tank (25×16.5×6.5 cm), with 1 l of water containing 3 g of artificial Instant Ocean salts (Aquatic Ecosystem, Apopka, FL, USA), and under a photoperiod of 8 a.m.–10 p.m. They were fed twice a day with a spawn powder (Aquatic Ecosystem) from day 6 to day 13.

Mutation analysis

The *too few* mutant was originally isolated in a AB/EK hybrid background, and backcrossed more than five generations into AB background. Homozygous *too few* mutant versus wildtype siblings were identified by extracting genomic DNA from tail fins, PCR with the *too few* gene specific primers, and sequencing. The reduction of DA and 5-HT neurons was further confirmed by immunostaining progeny of genotyped zebrafish with TH and 5-HT antibodies as previously described (Guo et al., 1999). Two-week-old progeny from *too few* homozygous mating versus their wildtype sibling mating were used for behavioral and immunocytochemical analyses.

Choice chamber paradigm

We devised a choice chamber to measure reward in larval zebrafish: it is a continuous tank with a drain located in the center; a flow of chemical (morphine)-containing solution can be applied to one end of the tank, and a countervailing water flow can be applied to the other end of the tank (Fig. 1A). The choice chamber apparatus is in the same dimensions as the zebrafish rearing tank and filled with 600 ml of salted water. A chemical solution and water (30 ml) were introduced at each extremity of the system, at the surface of the water, with a flow rate of 4.3 ml/min. A drain at the center ensured minimal mixing between liquids from the left and right sides. We tested the tank with Methylene Blue, and noted that the dye does not cross to the other side of the tank.

For the study of morphine, larvae were pre-exposed for 1 h to a solution of morphine sulfate (MS, a gift from NIDA) at 0.0

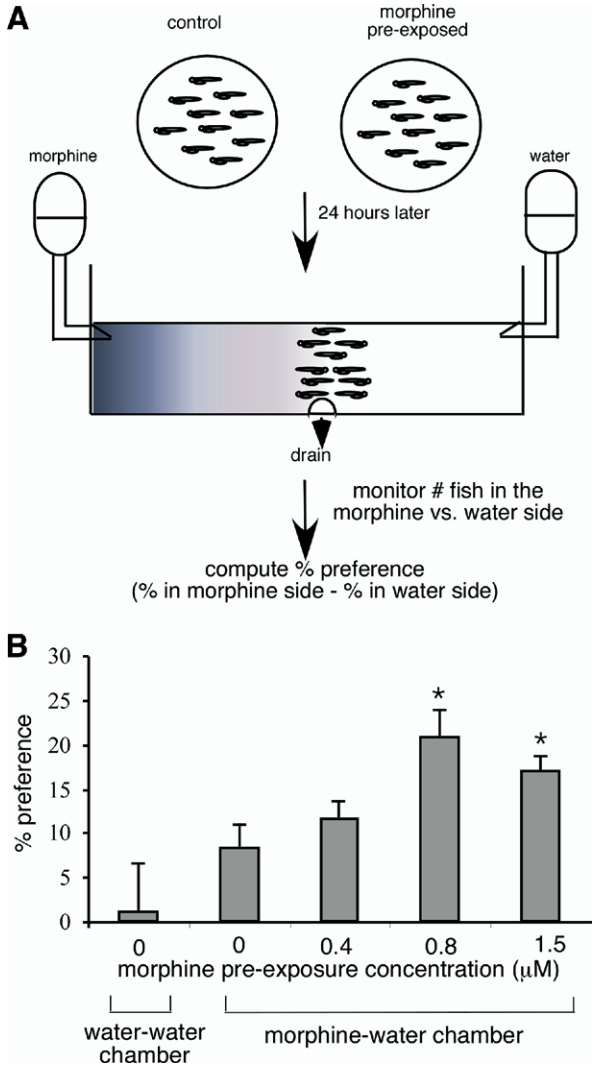

Fig. 1. Morphine preference behavior of larval zebrafish measured in the choice chamber. (A) Schematic diagram of the choice chamber test. (B) Larval zebrafish exhibited a significant preference for MS, which depended on the pre-exposure concentration (ANOVA, $F(4,145)=7.049$, $P<0.001$). Non-pre-exposed (naïve) larvae showed a slight preference toward MS; however this preference was not significantly different from larval zebrafish tested in water-water chamber (Dunnett's T3 post hoc multiple comparison, $P>0.50$). Larvae pre-exposed to 0.8 and 1.5 μM MS showed a significantly higher preference for MS than naïve larvae (Dunnett's T3 post hoc multiple comparison, * $P<0.05$). Data are presented as mean percent preference ± S.E.M. $N=30$ (groups).

(control), 0.4 μM, 0.8 μM, and 1.5 μM, directly dissolved into their tank. The next day, the larvae were transferred into the choice chamber in a group of 10, since we did not observe apparent schooling behavior in 2-week-old larvae. After habituation for 5 min, a solution of 15 μM morphine and system water were introduced at each end of the choice chamber. The number of larvae in the side containing the solution of morphine was monitored for 10 min after the initiation of the morphine and water flow, by noting every 10 s the number of larvae in either the morphine side or the water side. The total number of animals present in the morphine side during the 10-min observation period divided by the total number of expected animal counts (10 larvae×60=600) gives rise to the % larvae in the morphine side. Percent preference (the