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Research article

Dopaminergic control of anxiety in young and aged zebrafish

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ABSTRACT

Changes in the expression of the dopamine transporter (DAT), or the sensitivity of dopamine receptors, are associated with aging and substance abuse and may underlie some of the symptoms common to both conditions. In this study, we explored the role of the dopaminergic system in the anxiogenic effects of aging and acute cocaine exposure by comparing the behavioral phenotypes of wild type (WT) and DAT knockout zebrafish (DAT-KO) of different ages. To determine the involvement of specific dopamine receptors in anxiety states, antagonists to D1 (SCH23390) and D2/D3 (sulpiride) were employed. We established that DAT-KO results in a chronic anxiety-like state, seen as an increase in bottom-dwelling and thigmotaxis. Similar effects were produced by aging and acute cocaine administration, both leading to reduction in DAT mRNA abundance (qPCR). Inhibition of D1 activity counteracted the anxiety-like effects associated with DAT deficit, independent of its origin. Inhibition of D2/D3 receptors reduced anxiety in young DAT-KO, and enhanced the anxiogenic effects of cocaine in WT, but did not affect aged WT or DAT-KO fish. These findings provide new evidence that the dopaminergic system plays a critical role in anxiety-like states, and suggest that adult zebrafish provide a sensitive diurnal vertebrate model for elucidating the molecular mechanisms of anxiety and a platform for anxiolytic drug screens.

1. Introduction

Dopamine (DA) is a critical neurotransmitter, involved in a broad range of physiological functions, including cognitive, visual and motor (Dunnett, 2005). Properties of the dopaminergic system, including DA synthesis and release from neurons, and the presence of both presynaptic and postsynaptic DA receptors, are highly conserved in vertebrates. DA levels in the synaptic cleft, and therefore its effects on receptors, depend on the rate of DA re-uptake by nerve terminals. The re-uptake rate is controlled by a presynaptic membrane-bound protein, dopamine transporter (DAT), which is critical for maintaining DA homeostasis and controlling the duration of DA signals (Kahlig and Galli, 2003).

As a result, genetic or pharmacologically-induced changes in DAT expression and function modulate the entire DA pathway, significantly affecting health and disease states. DAT polymorphisms are associated with extreme behavioral traits and disorders, including angry-impulsive personality and borderline personality disorder (Joyce et al., 2009, n.d.), binge-eating (Shinohara et al., 2004), disruptive behavior disorder (Lee et al., 2007), attention-deficit and hyperactivity disorder (Franke et al., 2008) and alcohol dependence (Ueno et al., 1999).

A variety of addictive drugs, from opiates to psychostimulants, inhibit DAT expression or function and thus extend the duration of DA effects on its receptors, both postsynaptic (e.g., D_1) and autoregulatory presynaptic (e.g., D_2) (Jia et al., 2005; Liang et al., 2014; Wilson et al., 1996; Yuan et al., 2015). This, in turn, affects drug-seeking behaviors and associated cognitive and emotional states (Wang and Deutch, 2007). Notably, DAT is a direct target of cocaine and both the reinforcing and drug-withdrawal effects of cocaine administration are linked to its ability to inhibit DAT function or modify DAT expression levels (Thomsen et al., 2009).

There is evidence that DAT expression in humans declines in normal aging. Imaging studies have revealed gradual and region-specific reductions in DAT density with age (Shingai et al., 2014; Volkow et al., 1998, 1996), supporting post-mortem findings of significant loss of nigrostriatal DA neurons even in the absence of neurodegenerative disease (Fearnley and Lees, 1991). Additionally, several major age-dependent disorders are known to be associated with DAT polymorphisms, such as Alzheimer's disease (Lin et al., 2012) and Parkinson's disease (le Couteur et al., 1997; Ritz et al., 2009). DAT polymorphisms

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have also been linked to reduced life-expectancy in humans (Hadi et al., 2015). However, despite a known correlation between decline in DAT or dopamine receptor markers and cognitive performance in the elderly (Bäckman et al., 2006; Li et al., 2010), the role of DAT deficiency in emotional and cognitive changes during normal aging remains to be fully elucidated.

To understand the DAT-dependent mechanisms involved in symptoms that are common to both aging and drug addiction, e.g., anxiety, and to develop ways to reverse these symptoms, the use of potent and preferably high throughput animal models is required. The recent and remarkable discovery that birds and reptiles lack the DAT transporter (Lovell et al., 2015) reduces the choice of vertebrate models to mammals and fish. Among those, the diurnal vertebrate zebrafish model offers multiple advantages, including its well-developed DA system (Panula et al., 2010), gradual aging (Kishi et al., 2009) and sensitivity to multiple drugs of abuse and their withdrawal (Bencan et al., 2009; López Patiño et al., 2008a; Riley et al., 2015; Rinkwitz et al., 2011).

In this study, we addressed the role of the dopaminergic system in the anxiogenic effects of both aging and cocaine by comparing the behavioral phenotypes of wild type (WT) and DAT knockout zebrafish (DAT-KO). We determined that DAT knockout results in an anxiety-like state, which is remarkably similar to that of aged zebrafish. It is also similar to the behavioral response of zebrafish to transient inhibition of DAT by acute cocaine exposure. Moreover, we found that such anxiety-like behavior in both DAT-KO and WT fish is under differential control of dopamine receptors. Together, this implies a major role of the dopaminergic system in anxiety and suggests that the zebrafish is a sensitive model for defining the mechanisms involved and designing effective treatments against anxiety associated with drug addiction or aging.

2. Materials and methods

2.1. Animals

Juvenile (3–4 months of age), adult (4–18 months), and aged (over 18 months) male zebrafish (*Danio rerio*) of both DAT knockout strain (DAT-KO, Foley et al., 2009) and genetic background wild type control (WT) were born and raised in the lab. Animals were housed in 14 L:10 D light:dark cycle, in 3 L tanks, 6–10 fish per tank, in a 26.5 °C, pH 7.0–7.4 controlled multi-tank recirculating water system (Aquaneering, San Diego, CA, USA). Animals were fed twice a day with live brine shrimp (BrineShrimpDirect, Ogden, Utah, USA) and flake food (TetraMin, Tetra, Blacksburg, VA, USA). Two weeks prior to the initiation of experimental procedures, fish were moved to individual 1 L housing tanks for adaptation, and separated from each other by opaque partitions. They remained in the 1 L tanks throughout the experimental period. All experiments were approved by Boston University Institutional Animal Care and Use Committee and were performed in accordance with the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Dopamine transporter mutation

The *dopamine transporter* (*DAT*, also known as *slc6a3*) mutant was generated using engineered zinc finger nucleases (Foley et al., 2009). This mutant line has a 4-basepair insertion in exon 12 (Fig. S1).

2.3. Behavioral assays

To document locomotor activity patterns, 1 L tanks containing individual fish were placed on an experimental rack, with the lateral wall of each tank facing the camera, to document the speed and depth at which fish were swimming. Activity in the entire tank (100 mm water column) and within 3 equally-spaced areas of the tank (the top, middle and bottom) was documented for up to 24h periods using VideoTrack image analysis software (ViewPoint, Canada). Fish were not able to see each other during recording. Fluorescent lighting at 100 lx was provided during the day only, with infrared illumination during the night. All recording was accomplished with cameras equipped with infrared pass filters, to ensure uniform images throughout the day and night. Consistent with our earlier studies (López Patiño et al., 2008a), high-speed swimming activity was defined as above 15.0 cm/s, low-speed as 0.1–15.0 cm/s, and inactivity as below 0.1 cm/s. To document thigmotaxis, a tendency to stay close to the walls, fish behavior in a 9 L tank (27.3 × 19.7 cm floor area) was video-recorded from above and the percent time spent within 5 cm of the walls versus the remaining central part of the tank was documented. To determine changes in locomotor activity across the lifespan, the swimming patterns of juvenile, adult and aged fish were recorded in parallel. Behavioral studies involved 6–22 fish per group.

2.4. Treatments

Stock solution of cocaine hydrochloride (NIDA) was prepared in water and, following baseline recording, added directly to the tanks containing fish, with final cocaine concentration in the tank ranging from 1 to 33 μM. Control groups received water. Behavioral assessment was conducted for 1 h for each dose (n = 6/group). The dose range was based on our earlier dose-dependency studies, in which cocaine-induced stereotypy or thigmotaxis was documented (López Patiño et al., 2008a).

For dopamine receptor antagonists, sulpiride (Sigma-Aldrich, D2/D3 receptor antagonist, dissolved in dimethyl sulfoxide) and SCH23390 (Tocris Bioscience, D1 receptor antagonist, dissolved in water) were used. Vehicle groups received either dimethyl sulfoxide or water. Following a baseline behavioral assessment (1 h), the appropriate receptor antagonist was added to tank water to achieve a final concentration of 10 μM (Darland et al., 2012). Behavioral assessment then continued for another hour.

Each DA receptor antagonist (10 μM) was also tested in combination with low (5 μM) or high (33 μM) cocaine dose (n = 6/group). Experiments had three 1 h phases. Following a baseline behavioral assessment (1 h), fish were treated with cocaine (1 h) and then appropriate receptor antagonist was added to tank water (1 h). Behavioral assessment continued throughout the experiment.

Diazepam (DzP, Abbott Laboratories, Chicago, IL, USA) was dissolved in 10% ethanol to produce a 17.5 mM working solution and administered directly into the fish tank, with final concentration of 5 μM. The benzodiazepine receptor inverse agonist, *N*-methyl-β-carboline-3-carboxamide (FG-7142, Tocris Cookson Inc., Ellisville, MO, USA) was dissolved in 10% ethanol to produce a 3.5 mM working solution, with final concentration in the tank of 0.5 μM. Both concentrations were based on earlier dose-dependence studies (López Patiño et al., 2008a). The control animals were exposed to a 0.003% final concentration of ethanol in tank water. Behavioral recording was conducted for 1 h, in parallel.

2.5. Real-time quantitative RT-PCR (qPCR)

Fish (n = 6/group) were dissected on dry ice and total RNA was extracted from individual brains using RNAeasy kit for high lipid content tissue (Qiagen, Chatsworth, CA, USA), according to the manufacturer's protocol. The quantity and quality of RNA was determined spectrophotometrically, at 260 nm and 260/280 nm respectively. The same amount of RNA from each sample was converted into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction. qPCR was performed using a TaqMan® Universal PCR Master Mix and ABI Prism 7300 Real Time PCR System (ABI, Foster City, CA, USA). The following TaqMan® primers and probes {5′ FAM, 3′ TAMRA} for