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D2 dopamine receptor subtype-mediated hyperactivity and amphetamine responses in a model of ADHD

Xueliang Fan [a], Ming Xu [b], Ellen J. Hess [a,*]

[a] Departments of Pharmacology and Neurology, Emory University School of Medicine, 101 Woodruff Circle, Suite 6300 Atlanta, GA 30322, USA
[b] Department of Anesthesia and Critical Care, University of Chicago, Chicago, IL 60637, USA

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ABSTRACT

Low doses of psychostimulants produce beneficial behavioral effects in ADHD patients but the mechanisms underlying the response are not understood. Here we use the hyperactive mouse mutant *coloboma* to identify D2-like dopamine receptor subtypes that mediate the hyperactivity and response to amphetamine; we have previously demonstrated that D1-like dopamine receptors are not involved. Targeted deletion of the D2, but not the D3 or the D4, dopamine receptor in *coloboma* mice eliminated the hyperactivity; depleting D2 dopamine receptors also restored the excess dopamine overflow that may drive the hyperactivity to normal concentrations. Similar to its effects on ADHD patients, amphetamine reduced the hyperactivity of *coloboma* mice. The D2 dopamine receptor-selective antagonist L-741,626, but not D3 or D4 dopamine receptor-selective antagonists, blocked the amphetamine-induced reduction in locomotor activity. Thus, the D2 dopamine receptor subtype mediates both the hyperactivity and response to amphetamine, suggesting a specific target for novel therapeutics in ADHD.

A familiar feature of attention deficit hyperactivity disorder (ADHD) is the response to psychostimulants such as methylphenidate and amphetamine. In ADHD patients, stimulants reduce excess motor activity and enhance concentration. The reduction in physical activity in ADHD patients after psychostimulant treatment is supported by studies using subjective rating scales and objective measures such as actometers, respiration calorimetry and microwave motor activity detectors (Arnold et al., 1972, 1978; Butte et al., 1999; Elia et al., 1991; Evans et al., 1986; Rapoport et al., 1978).

Because the primary treatment for ADHD is stimulant medication, research has focused on dopamine. Imaging experiments have identified dopamine transporters predominantly in the caudatoputamen as methylphenidate's site of action (Wang et al., 1995). SPECT and PET studies in ADHD patients have also demonstrated decreased metabolic activity in the basal ganglia (Lou et al., 1989, 1990), a region that contains high concentrations of dopamine and dopamine receptors. Assessments of catecholamine metabolites in cerebral spinal fluid of ADHD children support the imaging studies, demonstrating a positive correlation between the dopamine metabolite homovanillic acid and the degree of hyperactivity (Castellanos et al., 1994).

Although dopamine is implicated in the pathophysiology and treatment of ADHD, there is little evidence implicating specific dopamine receptor subtypes. The lack of identified receptor targets is due primarily to the entirely non-selective action of psychostimulants, which increase the extracellular concentration of monoamines, resulting in the broad activation of many receptor subtypes. In the absence of an unambiguous therapeutic mechanism of action, specific treatment strategies are not forthcoming. Although determining the receptors mediating the positive behavioral effects of psychostimulants in humans would require years of correlative experiments, animal models of ADHD provide an opportunity to explore directly both pathogenic and therapeutic mechanisms.

It is not feasible to reproduce the entire spectrum of a neuropsychiatric disorder in an animal because of the complexity of the behavioral pathology and because some phenotypes are not credibly mimicked in animals. Therefore, animal models of psychiatric disorders, including ADHD, focus on specific quantifiable behavioral features. The pathophysiological insight gained through the analysis of discrete quantifiable behaviors then provides a foundation for understanding more complex features of the disorder. For ADHD, hyperactivity and the response to psychostimulants are features of the disorder convincingly modeled in rodents. All models of ADHD exhibit hyperactivity, including *coloboma* mice. These mice exhibit inattention, impulsivity and hyperactivity attributable to a hemizygous

* Corresponding author. Fax: +1 404 712 8576.
E-mail address: ejhess@emory.edu (E.J. Hess).
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deletion of *Snap25* (Bruno et al., 2007; Hess et al., 1996), a gene that is also associated with ADHD in humans (Barr et al., 2000a; Brookes et al., 2006; Faraone et al., 2005). Similar to its effects on ADHD patients, amphetamine reduces the excess motor activity in *coloboma* mice. We have previously determined that the broad class of D2-like dopamine receptors, but not D1-like dopamine receptors, mediates the effects of amphetamine in *coloboma* mice (Fan and Hess, 2007). Here we dissect the D2 dopamine receptor subtypes (D2, D3 and D4) to define receptor-specific regulation of hyperactivity and response to amphetamine.

Materials and methods

Mice

All mouse strains were bred and group-housed at Johns Hopkins University. *Coloboma* (*Cm*/+) mice on the C3H/HeSnJ strain were originally obtained from JAX (Bar Harbor, ME). All dopamine receptor knockout mice were originally generated using 129/SV-derived embryonic stem cells and subsequently backcrossed onto the C57BL/6J strain. Dr. Greg Elmer (University of Maryland) generously provided D2 dopamine receptor knockout mice (Kelly et al., 1997). D3 dopamine receptor knockout mice were as described previously (Xu et al., 1997). D4 dopamine receptor knockout mice (Rubinstein et al., 1997) were obtained from Dr. David Grandy (Oregon Health and Science University).

The *coloboma* mutation is semidominant. Therefore, *coloboma* mice lacking D2, D3 or D4 dopamine receptors were bred in a two-generation cross. First, male *coloboma* mice were mated with female mice carrying the D2, D3 or D4 null alleles. F1 progeny were genotyped and then male *coloboma* mice that were also heterozygous for the knockout allele were bred with heterozygous knockout females. The F2 generation therefore consisted of all experimental control and mutant genotypes. For all experiments, mutant and control mice were 2–4 months of age. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Genotyping

Mice were screened for the D2, D3 or D4 dopamine receptor targeted deletions by PCR of genomic DNA extracted from tail samples. Neo primers were used to identify the null allele. For D2 and D4 dopamine receptor PCRs, a single reverse neo primer was embedded in the reaction to differentiate normal from null alleles. The primer sequences were as follows: D2, forward 5′-TGATGACTGG-GAATGTTGGTGTGC-3′, reverse 5′-CCGAGCCAAGCTAACACTGCAGAG-3′ and Neo reverse 5′-AGGATTGGGAAGACAATAGCAG-3′ (Diaz-Torga et al., 2002); D3, forward 5′-GCTCACCACTAGGTAGTTG-3′, reverse 5′-ACCTCTGAGCCAGATAAGC-3′, Neo forward 5′-CAAGATGGATTGCACG-CAGG-3′, Neo reverse 5′-AGCAAGGCGAGATGACAGGA-3′ (Pritchard et al., 2003); D4, forward 5′- TCTCACATAACCAAAGAAGA-3′, reverse 5′-CACTGGCGAAGCCACCGCGG-3′, Neo forward 5′-CAAGATGGATTG-CACGCAGG-3′. PCR was performed in a volume of 12.5 μl containing 0.2 mM dNTP, 4 mM $MgCl_2$, 0.4 –0.8 μM primer, 50 ng template, and 1.25 U Taq polymerase. Reactions were denatured at 94 °C for 5 min prior to 15 cycles consisting of 94 °C for 1 min, 67 °C (decreasing 1 °C per cycle) for 2 min, and 72 °C for 3 min followed by 25 cycles of 94 °C for 1 min, 52 °C for 2 min, and 72 °C for 3 min with a final 7-min extension at 72 °C.

Mice were screened for the *Snap25* gene dose by semi-quantitative PCR using the *Il1b* gene (interleukin 1β), which is also located on mouse chromosome 2, as a within reaction reference. Primers for *Snap25* were as follows: forward 5′-CGAAGAAGGCATGAACCATAT-CAACC-3′ and reverse 5′-GCCCGCAGAATTTTCCTAGGTCCG-3′. Primers for *Il1b* were as follows: forward 5′-CCTGAACTCAACTGTGAAATGC-CAC-3′ and reverse 5′-GTCCGTCAACTTCAAAGAACAGGTC-3′. PCR was performed in a volume of 12.5 μl containing 0.2 mM dNTP, 5 mM $MgCl_2$, 0.5 μM primer, 50 ng template and 1.25 U Taq polymerase. Reactions were denatured at 94 °C for 3 min, prior to 23–29 cycles of 15 s at 94 °C, 30 s at 68 °C, 30 s at 72 °C, plus a final 7 min at 72 °C extension. Each sample was subjected to at least three PCRs, each with a different number of cycles (23–29). *Il1b* and *Snap25* PCR products from normal (+/+) mice were comparable in intensity on an agarose gel with ethidium bromide visualization; the *Snap25* PCR product from *coloboma* mice was considerably less intense than the *Il1b* product.

Drugs

Drugs were injected intraperitoneally in a volume of 10 ml/kg. S33084 was a generous gift from Dr. Mark Millan (Servier, France). L-745,870 and L-741,626 were purchased from Tocris (Ellisville, MS). All other drugs were purchased from Sigma (St. Louis, MO).

Locomotor activity

Mice were tested in photocell activity cages (29×50 cm) equipped with 12 infrared beams arranged in a 4×8 grid (San Diego Instruments, San Diego, CA). Beam breaks were recorded every 10 min. Control and mutant mice were tested simultaneously and were habituated to the cages for at least 4 h prior to start of the test. Mice had access to food and water *ad lib* during the entire habituation and test period. Test sessions started 3 h after the start of the dark cycle.

For amphetamine-antagonist challenge experiments, mice were tested in a repeated measures design. The order of drug doses and vehicle was pseudorandom with each mouse receiving every dose only once within an experiment. Mice were given a 4-day drug holiday between challenges to avoid supersensitivity, as described previously (Fan and Hess, 2007). For dopamine receptor knockout experiments, vehicle and amphetamine were delivered in pseudo-random order.

Stereotypy

During the locomotor activity tests, mice were rated for stereotypy under red light illumination every 10 min for 30 s A 0–5 behavioral scale was used: 0 = sleeping; 1 = awake, inactive; 2 = active or exploring; 3 = hyperactive; 4 = hyperactive with bursts of stereotypic behavior; and 5 = continuous persistent stereotypy.

Microdialysis

No-net-flux microdialysis was performed in alert, freely moving mice, as previously described (Fan and Hess, 2007). Briefly, concentric microdialysis probes were constructed as described (Kasim et al., 2006). Mice were anesthetized with Avertin and a microdialysis probe was implanted in the striatum (+0.6 AP, +1.7 ML, 4.5 DV). The probe was perfused overnight with artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 3.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, pH 7.0–7.4) at a flow rate of 0.6 μl/min. No-net-flux microdialysis commenced the following morning. The probe was perfused with aCSF plus 250 μM ascorbic acid and 0, 2, 10 or 20 nM dopamine (C_{in}) presented in pseudorandom order. After a 25 min equilibration period for each concentration of dopamine, three samples (20 min each) were collected (C_{out}). After completion of the experiment, brains were removed and probe location was confirmed; only animals with probes in the striatum were included.

Samples were stored at −80 °C until HPLC analysis. Dopamine concentrations (C_{out}) were determined by HPLC consisting of an MD-150 column (150 mm length; 3.2 mm I.D.; ESA, Chelmsford, MA), a 5014B coulometric microdialysis cell plus guard cell (5020) with a