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Specific knockdown of the D2 Long dopamine receptor variant

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Abstract

Dopamine signaling in the nucleus accumbens is critical in mediating the effects of cocaine. There are two splice variants of dopamine D2 receptors, D2L and D2S, which are believed to have different functional roles. Here we show that knocking down D2L selectively using viral mediated shRNA led to a slight but significant decrease in basal locomotor activity with no significant change in cocaine induced stimulation of locomotion. The knockdown appears to produce a trend of reduced conditioned place preference to cocaine but the difference was not statistically significant. Our results demonstrated that the splice variants of D2 receptors can be selectively manipulated in vivo in specific brain regions allowing more specific studies of each D2 receptor isoform.

Keywords

Dopamine; cocaine; addiction; D2L; D2 receptors; shRNA; AAV

Introduction

Drug dependence is a persistent problem throughout the world. 22 million Americans were classified as having a significant degree of drug dependence in 2008 [1]. The health care cost for substance abuse was estimated to be comparable to that for cancer in 2002 [2]. All drugs of abuse increase striatal extracellular dopamine despite their widely varied mechanisms of action [3]. D2 dopamine receptor (D2R) abnormality in the striatum is associated with increased susceptibility to addiction [4,5]. D2R is expressed in the nucleus accumbens [6] and D2R signaling in this region is involved in the initiation of addiction to substances of abuse [7].

There are two splice variants of D2R, a long isoform (D2L) which contains the 29 amino acid sequence derived from exon 6 and a short isoform (D2S) without exon 6 [8]. These isoforms have different functions and localizations, with D2S acting primarily as a presynaptic autoreceptor [9,10]. Taq1A, a polymorphism in D2R gene in the human population, has been associated with increased risk to substance abuse [11,12]. Most individuals with Taq1A also have several intronic single nucleotide polymorphisms

affecting the ratio of D2L and D2S [8], which might be responsible for the increased risk of addiction associated with Taq1A.

The majority of what is known about D2R was acquired from studies using agonists, antagonists and transgenic mice [13]. At the time of writing, no agonist or antagonist could fully differentiate between D2, D3 and D4 receptors, let alone the individual isoforms of D2R. D2L knockout mice show overexpression of D2S, making conclusions pertaining to D2L difficult to interpret [14]. Therefore, we utilized viral-mediated delivery of short-hairpin RNA (shRNA) to knockdown D2L without affecting D2S. This specific manipulation was used to investigate the role of D2L within the nucleus accumbens in cocaine induced behaviors.

Materials and Methods

Animals

Male C57 BL/6 mice (8 to 10 weeks of age) were acquired through Jackson Labs (Bar Harbor, ME). They were group housed and kept on a 12-h day and 12-h night cycle, provided *ad libitum* access to food and were treated in accordance with the Institutional Laboratory Animal Care and Use Committee, the Ohio State University.

Viral vector preparation

Several shRNAs against exon 6 of the D2R, present only in D2L, were designed using siRNA wizard 3.1 (Invitrogen, San Diego, CA) and evaluated in vitro [15]. The most effective one, shRNA-D2L (CTGGGAGTTTCCCAGTGAACA), was cloned into an AAV vector. Replication deficient AAV1 serotype viral vectors were prepared at a concentration of 1.5×10^{14} viral genome units per microliter (vgu/ μ l) [16]. A virus expressing shRNA against GFP (shRNA-GFP) was used as a negative control for all experiments.

Surgical procedures

Mice were anesthetized with ketamine (100 mg/kg), xylazine (30 mg/kg) and stereotactically injected with the AAV1 vector (1.0×10^{12} vgu/ μ l). Unilateral double injection (either right or left): 0.5 μ l and 0.75 μ l were injected into the ventral and dorsal striatum (coordinates in mm: 0.75 anterior, 1.75 lateral; 4.25 and 3.00 ventral to Bregma). Bilateral injection: 0.5 μ l was injected into nucleus accumbens at a 10° angle toward the mid-sagittal plane (coordinates in mm: 1.3 anterior, 1.3 lateral; 4.4 ventral to Bregma). All injections were at a rate of 0.1 μ l per minute. Further experiments were done at least 2 weeks after surgery.

qPCR analysis

Animals for qPCR received unilateral injection of either shRNA-D2L or shRNA-GFP virus. After a 3 week recovery animals were sacrificed via cervical dislocation, followed immediately by rapid brain removal. Hole-punches were taken from a 1 mm brain section at the injection site and at the corresponding site of the non-injected hemisphere. mRNA was extracted according to the protocol of the RNeasy Lipid Tissue Minikit (Qiagen, Valencia, Ca.). Reverse transcriptase reactions were performed with the iscript cDNA synthesis kit (Thermo Scientific, Waltham, Ma). Duplex qPCR was performed with Maxima Hotstart Taq Polymerase (Thermo Scientific, Waltham, Ma) using β -actin as a within sample control. All reactions were run in triplicate with probes conjugated to either Fam or Hex (Sigma, St. Louis, MO) using a Mastercycler ep Realplex² (Eppendorf, westbury, NY). The levels of tested mRNAs relative to β -actin mRNA were determined. The primer and probe sequences, as well as their efficiency and R² values are included as Supplemental Digital Content 1.

Immunohistochemistry

Drug-naïve animals were used for immunohistochemical staining. Serial sections of paraformaldehyde fixed brain were cut (40µm) and stained using anti-D2 primary (1:500; Millipore, Billerica, Ma), goat anti-rabbit secondary (1:600; Sigma, St. Louis, Mo.) and rabbit anti-goat Pap-conjugated antibodies (1:300; Jackson Labs, Bar Harbor, Maine). D2 staining area was quantified using ImageJ software (NIH, Bethesda, MD) by experimenters who were blind of the injection groups.

Drugs

Cocaine was provided by the National Institute on Drug Abuse drug supply program. Cocaine (10mg/kg) was dissolved in 0.9% saline and injected interperitoneally in a volume of 0.1 ml/10 g of body weight.

Conditioned Place Preference and locomotor activity

We used an unbiased conditioned place preference (CPP) paradigm. During the preconditioning day, mice freely explored a 3-chamber box and video tracking software (ANYmaze, Stoelting Company, Wood Dale, IL) was used to record the time mice spent in each chamber and their basal locomotor activities simultaneously. The acrylic CPP boxes consisted of a smaller middle chamber (12.5 × 7.5 cm) and two larger conditioning chambers (12.5 x 17.5 cm) which have distinct cues. Based on pretest results, the drug-paired chambers were assigned so that the drug and saline groups were both counterbalanced and unbiased toward the cues.

During conditioning, all 3 chambers contained either the saline or cocaine paired cue set. Animals were injected with saline on days 1, 3, 5, 7, 9, and with cocaine (10mg/kg) on days 2, 4, 6, 8, 10. Immediately after injection, mice were allowed to freely explore all 3 chambers with the saline or cocaine-paired cue set for 30 minutes while locomotor activity was recorded. During the postconditioning test, animals were placed into the CPP boxes with a setup identical to the preconditioning day, and time spent in each chamber was recorded over 30 minutes. Mice in the control group received saline on all conditioning days and had each viral injection group represented (shRNA-GFP and shRNA-D2L). Saline groups did not differ and were pooled.

Statistics

Statistics were performed using SPSS (SPSS 18.0, Chicago, IL.). Two-way analysis of variance (ANOVA) was used to assess overall effects of shRNA-D2L × shRNA-GFP on CPP score or total locomotor activity, followed by one-way ANOVA to determine significance. Post-hoc Tukey tests were performed after one-way ANOVA to look for specific differences between groups. Locomotor differences across time between shRNA groups were assessed via multivariate ANOVA. All qPCR data sets were analyzed via independent-sample two-tailed T tests.

Results

Striatal expression changes after D2L knockdown

To confirm D2L knockdown, we unilaterally injected shRNA-D2L (n=8) into the striatum and compared it to shRNA-GFP (n=8) injections. mRNA expression after virus injection was presented as a ratio to that of the uninjected hemisphere. Expression profiles within the non-injected hemispheres of both groups of mice did not differ, and were pooled. Injection of shRNA-D2L into the striatum led to a significant reduction (~50%) in D2L mRNA expression, as compared to both the uninjected and shRNA-GFP treated hemispheres

(Figure 1A). Importantly, D2S mRNA expression was not affected (Figure 1B). A trend of reduction (not reaching significant level) in overall D2 protein expression was seen in the nucleus accumbens after shRNA-D2L injection (Figure 2C) compared to shRNA-GFP injected mice despite the fact that the primary antibody cannot differentiate between D2L and D2S. Regulators of G protein signaling (RGS), especially RGS4 and RGS9, are known negative regulators of intracellular D2 receptor signaling. It would be interesting to know whether knocking down D2L have an impact on RGS proteins. Figure 1C shows D2L knockdown increased RGS4 mRNA significantly but did not alter RGS9 mRNA level (Figure 1D).

Conditioned place preference behavior after D2L knockdown

To test the role of D2L in cocaine reward, we knocked down D2L with bilateral injections of shRNA-D2L into the nucleus accumbens. Figure 2A shows a representative expression pattern of red fluorescent protein (RFP) following virus injection. RFP is co-expressed with shRNA from the viral genome following viral infection [15]. RFP expression was examined for all animals after behavioral testing and those with patterns different from that shown in Figure 2A were removed from the study (<10%). Figure 3A shows that cocaine induced significant CPP in both shRNA-D2L injected mice (n=15) and shRNA-GFP injected mice (n=20) compared to saline conditioned control mice (6 shRNA-D2L mice and 5 shRNA-GFP mice, which were pooled). There is a trend towards reduction of CPP score after D2L knockdown compared to the shRNA-GFP group, but the difference was not significant (p=0.16).

Locomotor differences after D2L knockdown in the nucleus accumbens

Locomotor activity was measured during the pretest and conditioning periods of the CPP procedure. Significantly less total baseline locomotor activity was seen in shRNA-D2L animals as compared to the shRNA-GFP group during the CPP pretest (Supplemental Digital Content 2). When looked at in 5 min bins (Figure 3B), the baseline locomotor activity difference between the two groups was most prominent after 20 and 25 minutes. At these two time points, animals with D2L knockdown within the nucleus accumbens showed significantly decreased basal locomotor activity. However, no significant difference in locomotor activity was seen between viral groups after the first saline, or cocaine injection (Figure 3C). The remainder of the conditioning days also did not show a difference in activity across viral groups (data not shown).

Discussion

We present here the first published report of nucleus accumbens specific knockdown of the D2L receptor. We were able to confirm appropriate bilateral injection of the virus by visualizing RFP expression which were localized to the core of the nucleus accumbens, with some animals also displaying expression in the shell of this area (Figure 2A). This region of the brain is involved in addiction-like behavior. Our results indicate that viral-mediated delivery of shRNA was able to significantly knockdown D2L mRNA expression by approximately 50%, without altering the expression of D2S. D2 protein was also found to show a trend of reduction which did not reach statistical significance. The primary antibody for D2 is not able to differentiate between D2L and D2S, which might have contributed to the discrepancy between knockdown results at the mRNA and protein levels.

D2R has been implicated in both animal and human studies of addiction. While many groups attribute the hedonic and locomotor affects of cocaine to D1 dopamine receptor activation, a growing body of evidence has suggested the involvement of D2R [6,7]. We observed that animals with D2L knockdown in the nucleus accumbens showed a trend

toward reduced rewarding effect of cocaine as measured in CPP. However, this was not found to be significantly different from control virus injected animals ($p = 0.16$). Since we are only able to knockdown D2L expression by approximately 50%, further studies are needed to determine D2L's involvement in addiction-like behaviors.

Our results indicate a mild, but significant effect on basal locomotor activity after D2L knockdown. This may reflect expedited habituation to the novel environment, or it may imply a reduction in exploratory behavior. D2L knockout mice showed reductions in locomotor activity that were greater than what was seen in our study, but those mice also had subsequent overexpression of D2S, indicating adaptive changes to the knockout [14,17]. Our manipulation did not significantly change D2S expression, and thus these mild but significant locomotor changes are due specifically to reduced D2L mRNA expression within the nucleus accumbens.

RGS can inhibit G-protein coupled signaling by accelerating intrinsic GTPase activity of G α proteins [13]. RGS4 and RGS9-2 appear to most notably regulate D2R signaling [13,18,19]. Our results showed a significant up regulation of RGS4 mRNA in animals with D2L expression knocked down. The potential importance of these results lies in the fact that RGS4 also regulates the signaling of metabotropic glutamate receptors, μ -opioid receptors and serotonin receptors [20]. It is possible that D2L signaling is able to regulate other G-protein coupled receptors by altering the expression of RGS4. Future studies focusing on the interaction between different isoforms of the D2 receptor and RGS4 may elucidate novel regulatory pathways involved in addiction.

Conclusion

We present here that virus mediated shRNA expression in the nucleus accumbens knocked down D2L dopamine receptors without affecting D2S, which slightly, but significantly, reduced locomotor activity, and increased RGS4 mRNA expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Results from the 2008 National Survey on Drug Use and Health: National Findings. U.S. Department of Health and Human Services, Office of Applied Studies; 2008.
2. Office of National Drug Control Policy. Washington, DC: Executive Office of the President; 2004. The Economic Costs of Drug Abuse in the United States, 1992–2002. (Publication No. 207303)
3. Volkow ND, Wang GJ, Telang F, Fowler JS, Logan J, Childress AR, et al. Cocaine cues and dopamine in dorsal striatum: mechanism of craving in cocaine addiction. *J Neurosci*. 2006; 26:6583–6588. [PubMed: 16775146]
4. Thompson D, Martini L, Whistler JL. Altered ratio of D1 and D2 dopamine receptors in mouse striatum is associated with behavioral sensitization to cocaine. *PLoS One*. 5:e11038. [PubMed: 20543951]

5. Dalley JW, Everitt BJ. Dopamine receptors in the learning, memory and drug reward circuitry. *Semin Cell Dev Biol.* 2009; 20:403–410. [PubMed: 19560045]
6. Centonze D, Grande C, Usiello A, Gubellini P, Erbs E, Martin AB, et al. Receptor subtypes involved in the presynaptic and postsynaptic actions of dopamine on striatal interneurons. *J Neurosci.* 2003; 23:6245–6254. [PubMed: 12867509]
7. Giordano TP 3rd, Satpute SS, Striessnig J, Kosofsky BE, Rajadhyaksha AM. Up-regulation of dopamine D(2)L mRNA levels in the ventral tegmental area and dorsal striatum of amphetamine-sensitized C57BL/6 mice: role of Ca(v)1. 3 L-type Ca(2+) channels. *J Neurochem.* 2006; 99:1197–1206. [PubMed: 17026527]
8. Moyer RA, Wang D, Papp AC, Smith RM, Duque L, Mash DC, et al. Intronic polymorphisms affecting alternative splicing of human dopamine D2 receptor are associated with cocaine abuse. *Neuropsychopharmacology.* 36:753–762. [PubMed: 21150907]
9. Boundy VA, Lu L, Molinoff PB. Differential coupling of rat D2 dopamine receptor isoforms expressed in *Spodoptera frugiperda* insect cells. *J Pharmacol Exp Ther.* 1996; 276:784–794. [PubMed: 8632351]
10. Guiramand J, Montmayeur JP, Ceraline J, Bhatia M, Borrelli E. Alternative splicing of the dopamine D2 receptor directs specificity of coupling to G-proteins. *J Biol Chem.* 1995; 270:7354–7358. [PubMed: 7706278]
11. Munafo M, Clark T, Johnstone E, Murphy M, Walton R. The genetic basis for smoking behavior: a systematic review and meta-analysis. *Nicotine Tob Res.* 2004; 6 :583–597. [PubMed: 15370155]
12. Munafo MR, Matheson IJ, Flint J. Association of the DRD2 gene Taq1A polymorphism and alcoholism: a meta-analysis of case-control studies and evidence of publication bias. *Mol Psychiatry.* 2007; 12:454–461. [PubMed: 17453061]
13. Beaulieu JM, Gainetdinov RR. The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev.* 63:182–217. [PubMed: 21303898]
14. Wang Y, Xu R, Sasaoka T, Tonegawa S, Kung MP, Sankoorikal EB. Dopamine D2 long receptor-deficient mice display alterations in striatum-dependent functions. *J Neurosci.* 2000; 20:8305–8314. [PubMed: 11069937]
15. Naughton BJ, Han Dawn D, Gu Howard H. Fluorescence based evaluation of shRNA efficacy. *Analytical Biochemistry.* 2011
16. During MJ, Young D, Baer K, Lawlor P, Klugmann M. Development and optimization of adeno-associated virus vector transfer into the central nervous system. *Methods Mol Med.* 2003; 76:221–236. [PubMed: 12526166]
17. Holmes A, Lachowicz JE, Sibley DR. Phenotypic analysis of dopamine receptor knockout mice; recent insights into the functional specificity of dopamine receptor subtypes. *Neuropharmacology.* 2004; 47:1117–1134. [PubMed: 15567422]
18. Taymans JM, Leysen JE, Langlois X. Striatal gene expression of RGS2 and RGS4 is specifically mediated by dopamine D1 and D2 receptors: clues for RGS2 and RGS4 functions. *J Neurochem.* 2003; 84:1118–1127. [PubMed: 12603835]
19. Celver J, Sharma M, Kovoov A. RGS9-2 mediates specific inhibition of agonist-induced internalization of D2-dopamine receptors. *J Neurochem.* 114:739–749. [PubMed: 20477943]
20. Schwendt M, McGinty JF. Regulator of G-protein signaling 4 interacts with metabotropic glutamate receptor subtype 5 in rat striatum: relevance to amphetamine behavioral sensitization. *J Pharmacol Exp Ther.* 2007; 323:650–657. [PubMed: 17693584]

Figure 1.

Relative mRNA expression in animals unilaterally injected with either the shRNA-D2L or the shRNA-GFP control virus. Expression of the D2L receptor (A), D2S receptor (B), RGS4 (C), and RGS9 (D) within the striatum. The quantity of mRNA is presented as the ratio to the uninjected hemisphere. A significant reduction of D2L mRNA occurred as compared to both the uninjected hemisphere and the shRNA-GFP control virus injected mice (A). A significant increase of RGS4 mRNA was seen, as compared to both controls (C). shRNA-D2L had no significant effect on the mRNA levels of D2S (B) or RGS9 (D).

Figure 2.

A) A representative figure showing RFP expression from a mouse with bilateral virus injection. A coronal section of the mouse striatum (40µm thick, +1.0mm anterior of Bregma) is visualized at 1x with fluorescence microscope. RFP expression is confined to the nucleus accumbens with little expression occurring in the dorsal striatum. B) Representative immunohistochemistry of D2 receptor protein within the nucleus accumbens (scale bar indicates 150 µm). C) D2 receptor expression levels were quantitated by ImageJ analysis of immunohistochemically stained tissue. The results are presented as the percent of the areas of D2 receptor staining (Black spots in panel B) over the total area examined. A trend towards decreased D2 protein was seen ($p = 0.15$).

Figure 3.

Behavioral tests in mice with bilateral nucleus accumbens injection of either the shRNA-D2L or the shRNA-GFP control AAV virus. A) Conditioned place preference (CPP) to 10mg/kg cocaine by mice injected with shRNA-GFP (middle bar) and shRNA-D2L (right bar). The left bar is for mice conditioned with saline only. Both the shRNA-GFP and the shRNA-D2L groups showed a significant (* $p < 0.05$) CPP compared to animals only injected with saline. B) Time course of basal locomotor activity in 5 min intervals. While the distance traveled did not differ between groups during the first 15 minutes, a significant difference (* $p < 0.05$) occurred between the two groups after 20 and 25 minutes. C) Total locomotor activity after saline or cocaine injection. Cocaine significantly increased locomotor activity (** $p < 0.01$) in both groups but there was no difference between viral treatments.