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Viral Gene Delivery Selectively Restores Feeding and Prevents Lethality of Dopamine-Deficient Mice

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Summary

Dopamine-deficient mice ($DA^{-/-}$), lacking tyrosine hydroxylase (TH) in dopaminergic neurons, become hypoactive and aphagic and die by 4 weeks of age. They are rescued by daily treatment with L-3,4-dihydroxyphenylalanine (L-DOPA); each dose restores dopamine (DA) and feeding for less than 24 hr. Recombinant adeno-associated viruses expressing human TH or GTP cyclohydrolase 1 (GTPCH1) were injected into the striatum of $DA^{-/-}$ mice. Bilateral coinjection of both viruses restored feeding behavior for several months. However, locomotor activity and coordination were partially improved. A virus expressing only TH was less effective, and one expressing GTPCH1 alone was ineffective. TH immunoreactivity and DA were detected in the ventral striatum and adjacent posterior regions of rescued mice, suggesting that these regions mediate a critical DA-dependent aspect of feeding behavior.

Introduction

The nigrostriatal, mesolimbic, and mesocortical pathways are the major dopaminergic pathways within the central nervous system (CNS). DA-producing cells in the substantia nigra (SN) and ventral tegmental area (VTA) project topographically to specific brain regions, and dopamine (DA) released in these regions is postulated to regulate distinct behaviors (Koob, 1992; Self and Nestler, 1995). For example, the nigrostriatal dopaminergic pathway, neurons that project from the SN to the striatum (caudate putamen), is thought to be intimately involved in the control of locomotion because selective 6-hydroxydopamine (6-OHDA) or N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesions of this pathway in rodents and nonhuman primates result in defects in initiation and coordination of locomotion (Ungerstedt, 1971; Langston et al., 1984; Kopin and Markey, 1988). In addition, patients with Parkinson's disease exhibit motor defects such as tremors and difficulty in initiation of locomotion that result from a gradual degeneration of the nigrostriatal pathway with symptoms becoming apparent when DA concentrations fall below ~20% of normal levels (Hornykiewicz, 1966). Reward-related behaviors appear to involve the mesolimbic dopaminergic system, which is comprised of the group A10 dopaminergic neurons that project from the VTA to limbic structures, including the nucleus accumbens (NAc) and amygdala (Dahlstrom and Fuxe, 1964; Lindvall and Bjorklund, 1983; Koob, 1992; Koob and Nestler, 1997). DA is released in the NAc when animals perform tasks that are postulated to be rewarding. For example, extracellular dopamine is detected in this brain region when rodents self-administer drugs such as cocaine and amphetamine (Hurd et al., 1989; Pontieri et al., 1995).

DA is also released in the NAc and amygdala when food-deprived animals are given a palatable meal, and bilateral injection of DA into the NAc can stimulate feeding (Martel and Fantino, 1996a; Hajnal and Lenard, 1997; Swanson et al., 1997; Taber and Fibiger, 1997). In addition, bilateral 6-OHDA lesions of midbrain dopaminergic neurons results in aphagia, and complete removal of DA from dopaminergic neurons abolishes ingestive behaviors (Ungerstedt, 1971; Zhou and Palmiter, 1995). Thus, there are strong indications that DA is necessary for feeding, but the neural pathways involved are not established. Furthermore, the effects of DA depletions on feeding behavior could be nonspecific because DA signaling impinges on many brain functions (Salamone et al., 1990).

We used gene-targeting techniques to generate $DA^{-/-}$ mice that cannot synthesize TH, the initial, rate-limiting enzyme in catecholamine biosynthesis, in dopaminergic neurons but can in noradrenergic neurons (Zhou and Palmiter, 1995). $DA^{-/-}$ mice are born normally but gradually stop nursing and die. These mice can be rescued to adulthood with daily administration of L-DOPA, the biosynthetic product of TH. This treatment causes them to become hyperactive for ~6 hr and consume sufficient quantities of food and water to survive, but then they become inactive and stop eating until the next injection. Analysis of brain catecholamines reveals that norepinephrine levels are normal, but DA concentrations only reach 10% to 40% of wild-type levels at the peak of their activity (1 hr following L-DOPA treatment) and gradually decline to basal levels by 24 hr (Zhou and Palmiter, 1995; our unpublished data). When this daily regimen is terminated, $DA^{-/-}$ mice stop eating and drinking, lose weight, and expire within 3 days of their last injection. Surprisingly, they display locomotor activity between 24 to 48 hr following L-DOPA removal that is equivalent to wild-type mice, but they eat very little (our unpublished data). This suggests that movement and feeding are separable. The dopamine D_1, D_2, D_3, and D_4 receptors and the dopamine reuptake transporter (DAT) knockout mice display locomotor deficits but do not exhibit serious feeding deficits (Drago at al., 1994; Xu et al., 1994; Baik et al., 1995; Accili et al., 1996; Giros et al., 1996; Maldonado et al., 1997; Rubinstein et al., 1997; Xu et al., 1997). Thus, unbalanced DA signaling affects locomotion without disrupting food consumption.

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Several groups have recently demonstrated that recombinant adenovirus (rAd), adeno-associated viruses (rAAV), and herpes simplex virus can transduce neurons and glial cells in the CNS and promote ectopic gene expression of TH in these cell types. TH expression decreases apomorphine-induced rotational behavior that occurs upon unilateral 6-OHDA destruction of nigrostriatal neurons. This suggests that exogenous L-DOPA production is able to partially ameliorate deficits that occur with destruction of dopaminergic neurons (Davidson et al., 1993; During et al., 1994; Horellou et al., 1994; Kaplitt et al., 1994).

Here, we report that coinjection of two rAAVs expressing either human TH or GTP cyclohydrolase 1 (GTPCH1) rescues the lethality of DA$^{-/-}$ mice by selectively restoring feeding behavior to previously aphagic DA$^{-/-}$ mice. These viruses were injected into brain regions containing projection fields of dopaminergic neurons. GTPCH1 is the rate-limiting enzyme in the synthesis of tetrahydrobiopterin (BH4), an essential cofactor for TH function (Nichol et al., 1985; Levine et al., 1990). It is expressed preferentially in cells that produce monoamines or nitric oxide (Lentz and Kapatos, 1996). Thus, GTPCH1 virus was included because neurons and glia in the projection fields were not expected to have adequate BH4. Previous experiments with 6-OHDA lesioned rats indicated that both rAAV-TH and rAAV-GTPCH1 were required for efficient L-DOPA production (Mandel et al., 1998). We have used this approach to begin to map where DA acts in the brain to influence locomotor and feeding behaviors.

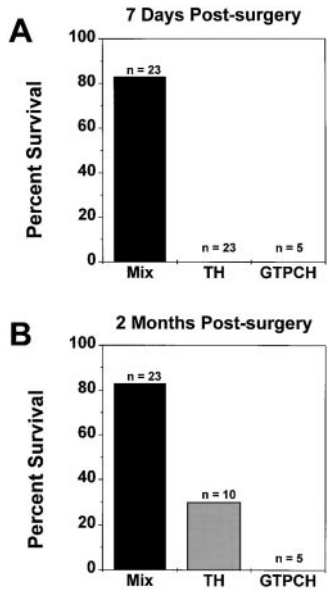


Figure 1. Viral Gene Therapy Promotes Survival of DA$^{-/-}$ Mice

DA$^{-/-}$ mice were injected with two rAAV vectors expressing either human TH or human GTPCH1, and survival was monitored after removal of L-DOPA. Three groups were generated via bilateral direct injection; mix (rAAV-TH + rAAV-GTPCH1), TH (rAAV-TH), and GTPCH1 (rAAV-GTPCH1). L-DOPA was removed 7 days following surgery. Food consumption and body weight measurements were recorded daily as criteria for survival. Mice that lost >20% body weight in 3 days and looked moribund were considered as nonsurvivors.

(A) Survival following termination of L-DOPA treatment 7 days postsurgery.

(B) Survival following termination of L-DOPA treatment 2 months postsurgery.

Results

Coinjection of rAAV Expressing Human TH and GTPCH1 Rescues the Lethality of DA$^{-/-}$ Mice

Recombinant AAV engineered to express either human TH (rAAV-TH) or GTPCH1 (rAAV-GTPCH1) were injected bilaterally into the ventral caudate putamen of adult DA$^{-/-}$ mice. Groups of mice were generated via direct injection of rAAV expressing TH alone, GTPCH1 alone, or a 1:1 mixture of the two viruses (designated as mixed vector). Because gene expression from transgenic rAAV is delayed for several days after viral transduction, daily L-DOPA treatment was continued for 6 to 7 days after viral delivery to allow for expression of TH and GTPCH1 (Kessler et al., 1996; Miao et al., 1998). Then the ability to survive without L-DOPA treatment was assessed by monitoring food intake and body weight. Mice that consumed greater than 50 g/kg body weight and lost less than 20% of their starting body weight were monitored for an additional 24 hr without L-DOPA treatment. To avoid casualties, animals below this threshold were kept alive with daily L-DOPA treatment and were tested for survival at regular intervals thereafter. Most of the mixed vector group (20 of 23 injected mice) survived when L-DOPA was removed 7 days following surgery; however, neither the TH nor GTPCH1 groups met the above criteria and were, therefore, treated with L-DOPA (Figure 1A). A few of the TH alone group (three of ten) challenged with L-DOPA removal 2 months following surgery survived for 2 weeks before they were sacrificed (Figure 1B). None of the GTPCH1 group survived with this challenge.

Because BH4 appears to be required for L-DOPA production in rescued mice, we attempted to stimulate feeding in the TH alone group with intraperitoneal injections of either BH4 or a more stable methylated derivative, DL-6-methyl-5,6,7,8-tetrahydropterine (PH4). Several concentrations (25, 50, and 100 mg/kg body weight) of both drugs were tested, but none were successful at stimulating feeding over a 2 hr period (data not shown). Because an effective concentration of BH4 or PH4 reaching the brain was not achieved with a single injection, we also attempted to reach a therapeutic dose by using an osmotic minipump delivering a solution of PH4 at a rate of 100 mg/kg/hr over 7 days. Minipumps were implanted into a subset of the TH alone group ($n = 4$), but this treatment was also ineffective at promoting survival when L-DOPA was removed (data not shown).

Feeding Behavior of Rescued DA$^{-/-}$ Mice

On the first day after L-DOPA removal, the average amount of food ingested by the mixed vector group (65 ± 11.2 g/kg body weight) was greater than quantities

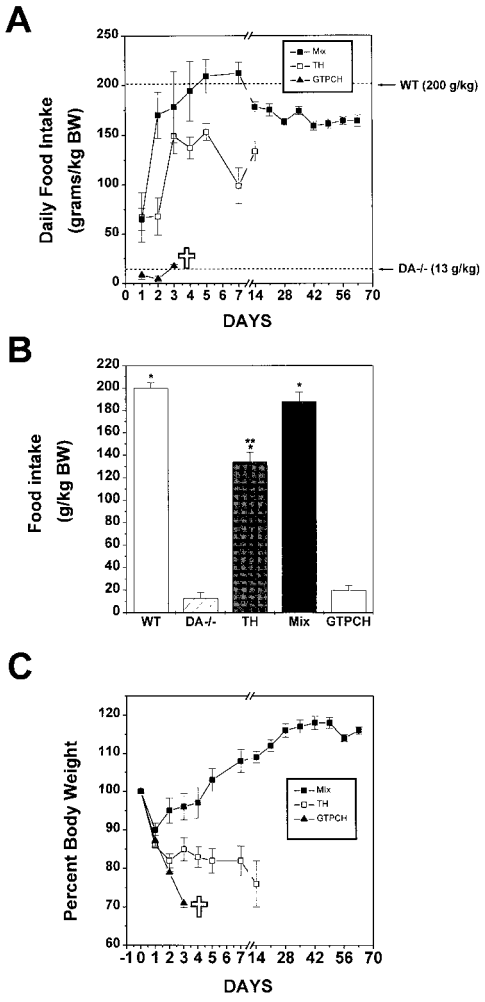


Figure 2. Food Intake and Body Weight Measurements of Virally Transduced DA$^{-/-}$ Mice following Termination of L-DOPA Treatment

(A) Daily food intake of mice injected with rAAV-TH + rAAV-GTPCH1 (mix), rAAV-TH alone (TH), and rAAV-GTPCH1 alone (GTPCH1) was monitored following removal of L-DOPA. Average food consumption by wild-type and DA$^{-/-}$ mice are indicated by dotted lines. Nonsurvivors are indicated by the cross.

(B) Comparison of daily food intake of wild-type (WT), DA$^{-/-}$, and virally transduced mice (TH, Mix, and GTPCH1) averaged over a period of a week. *$p < 0.05$ compared with DA$^{-/-}$ mice and **$p < 0.05$ compared with wild-type and mixed vector mice (one way ANOVA followed by Post Hoc analysis).

(C) Body weight measurements of virally transduced mice.

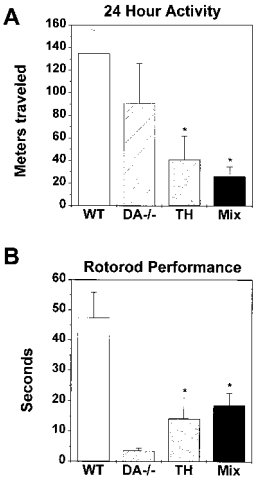


Figure 3. Ambulatory Activity and Coordinated Locomotion of Wild-Type, DA$^{-/-}$, and Virally Rescued Mice

(A) Activity of wild-type (n = 8), DA$^{-/-}$ (n = 17), and virally rescued mice (TH, n = 3; Mix, n = 20) was monitored over a 24 hr period in an activity chamber. *$p < 0.05$ compared to wild-type and DA$^{-/-}$ mice assessed 24 to 48 hr after their last L-DOPA injection.

(B) Time spent on a rotarod by wild-type (n = 15), DA$^{-/-}$ (n = 15), and virally rescued mice (TH, n = 3; Mix, n = 20). *$p < 0.05$ compared to wild-type mice.

consumed by DA$^{-/-}$ mice removed from L-DOPA treatment but was less than wild-type mice (Figure 2A). The 24 hr food consumption increased over the next 7 days, and by the end of the week, 87% of the mixed vector group (20 of 23 mice injected) consumed quantities of food that were equivalent to wild-type mice. Beginning at ~2 weeks following removal of L-DOPA, their food consumption fell to ~80% of wild-type levels, but all the mice appeared healthy, and their body weights continued to increase (Figure 2C).

Only 30% of the TH alone group challenged with L-DOPA removal 2 months following surgery consumed quantities of food that were similar to the mixed vector group on the first day following L-DOPA removal. Thereafter, they began to eat less than the mixed vector group (Figure 2A). However, the amount of food consumed was significantly greater than GTPCH1 injected mice or untreated DA$^{-/-}$ mice monitored 24 to 48 hr following their last L-DOPA injection (Figure 2B).

Body weight measurements were consistent with food intake (Figure 2C). The initial weight loss in the mixed vector group was followed by a rebound in body weight that exceeded their body weight on the day of the surgery. Three of mixed vector group mice have been monitored for survival without L-DOPA treatment for 13 months, and they continued to consume sufficient quantities of food for survival and maintenance of body weight. The partially rescued TH alone mice (n = 3), lost ~18% of their body weight during the first 2 days, maintained that weight for a few days, but then gradually lost more weight (Figure 2C). The experiment was terminated because of their weight loss, unkempt appearance, and shakiness.

Ambulatory and Motor Activity in Rescued Mice

In the process of monitoring food intake and body weight, we noticed that most rescued mice were inactive at the time of data collection regardless of whether measurements were taken during the light or dark cycle. Therefore, we measured their ambulatory activity over 24 hr (Figure 3A). The ambulatory activity of rescued mice (mixed vector, n = 20; TH alone, n = 3) was significantly lower than that observed in wild-type mice (n = 8) and DA$^{-/-}$ mice 24 to 48 hr following removal of L-DOPA (n = 17). Although the majority of mixed vector

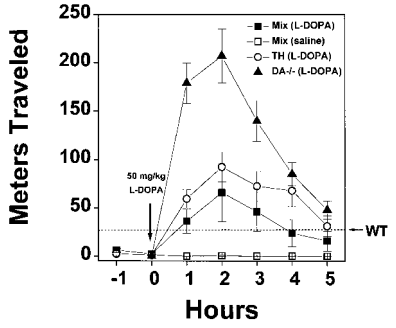


Figure 4. Activity Measurements of $DA^{-/-}$ and Virally Rescued Mice Treated with L-DOPA

$DA^{-/-}$ (n = 17) and rescued mice (Mix, n = 15; TH, n = 3) were placed in an activity chamber and allowed to acclimate to the environment for 24 hr. Mice were then injected with L-DOPA (50 mg/kg body weight) or saline, and ambulations and food intake were monitored for 5 hr following administration of L-DOPA. Average activity of wild-type mice is indicated by dotted line; they do not respond to L-DOPA treatment.

mice traveled less than 30 m/day, a large range of activity was observed (5.5 to 78 m/day). In fact, one of the mixed vector group displayed ambulatory activity (78 m/day) that was similar to the least active wild-type mouse tested (70 m/24 hr). The distances traveled in 24 hr by the mixed vector and TH alone groups were also lower than those traveled by $DA^{-/-}$ mice monitored 24 to 48 hr following their last injection of L-DOPA. This is because $DA^{-/-}$ mice exhibit a DA-independent wave of activity that occurs during this time (our unpublished data).

During routine handling, rescued mice exhibited deficits in initiation and coordination of motor movement that were similar to $DA^{-/-}$ mice 24 hr after removal of L-DOPA. Thus, to examine coordinated motor activity, the performance of the mice on a rotating wheel (rotarod) was examined. Wild-type mice perform the rotarod task reasonably well, staying on the rod for an average of 47 s during a 90 s trial (Figure 3B). The mixed vector group and the TH alone group were deficient in this test but were better than $DA^{-/-}$ mice tested 24 hr following their last injection of L-DOPA.

Rescued Mice Exhibit Increased Locomotion with L-DOPA Treatment

L-DOPA treatment of $DA^{-/-}$ mice results in hyperactivity that lasts for ~6 hr following drug administration (Zhou and Palmiter, 1995). Because deficiencies in ambulatory activity and coordinated locomotion were observed in rescued mice, we asked whether they would respond to L-DOPA treatment. Rescued mice were habituated in activity chambers for 24 hr and then injected with a standard dose of L-DOPA or saline, and their ambulatory activity and food intake were monitored for 5 hr following treatment. Ambulatory activity increased significantly following L-DOPA treatment, peaked at 2 hr, and gradually declined so that by 5 hr, activity was nearly back to basal levels (Figure 4). The magnitude of this increase was less than that observed after treatment of $DA^{-/-}$ mice with L-DOPA, but the duration of the effect was identical. A slight increase in feeding over the 5 hr period was observed with L-DOPA treatment (L-DOPA = 64 ± 9 g/kg body weight, saline = 31 ± 11.3 g/kg body weight). However, when rescued mice were given L-DOPA, they traveled ~100 times farther than when they were treated with saline.

Unilateral Injection of the Mixed Vector Causes Rotational Behavior but Does Not Rescue Feeding

We also attempted to rescue $DA^{-/-}$ mice by unilaterally injecting the mixed vector at identical coordinates into the right hemisphere of the brain (n = 5). None of these animals consumed enough food to survive when L-DOPA treatment was terminated, and therefore, they were maintained with daily injections (data not shown). Interestingly, two weeks after viral injection, we noticed that all surviving animals exhibited spontaneous rotational behavior 21 hr following L-DOPA treatment that was exclusively to the left, which was counterclockwise from the site of injection (Figure 5A). This rotational behavior of unilaterally injected mice persisted until the experiment was terminated (23 days after surgery). Treatment with L-DOPA produced clockwise rotations and increased the number of turns (Figure 5B). In some cases (mice #23 and #24), the rotational behavior switched completely from counterclockwise (21 hr following their last injection) to clockwise (1 hr following injection). After 2 hr, rotational behavior evened out, with most animals exhibiting rotations in both directions (Figure 5C).

TH Immunohistochemistry Reveals Differences in Anatomical Distribution between Mixed Vector and rAAV-TH Alone Groups

TH immunohistochemistry was performed to determine the extent of viral transduction and to ascertain if behavioral differences between groups could be attributed to variation in TH expression. With the exception of the locus ceruleus, endogenous TH is not synthesized within the brains of $DA^{-/-}$ mice, and therefore, TH antibody could be used to identify areas that were virally transduced. Sections 20 μm thick were cut from the brains of virally injected $DA^{-/-}$ mice, and multiple sections (spaced ~100 μm apart) surrounding the injection site and encompassing the majority of the brain were probed with TH antibody. Low-powered views of three representative coronal sections from a mixed vector mouse (designated as 18 in Figure 7) rescued for feeding but not locomotion are shown in Figures 6A–6C). Note extensive TH staining indicated by arrows within the caudate putamen (CPu) but also within ventral structures in the brain (marked with arrowheads). Additional staining was also detected in posterior brain regions (Figure 6C). A higher magnification view of one of the anterior brain regions (0.9 mm bregma) depicting TH staining within ventral structures is shown in Figure 6D. Cell bodies and fibers stained with TH antibody can be visualized under higher magnification within the insular (Ins), dorsal endopiriform (DEn), and piriform (Pir) corticies (Figures 6E and 6F). This is the typical pattern of staining observed in TH-positive areas; however, regions where only fibers exhibited staining were also observed (Figure 6G). These fiber-containing regions were routinely found in cortical areas dorsal and lateral to regions that exhibited intense TH staining. The specific nature of this staining was

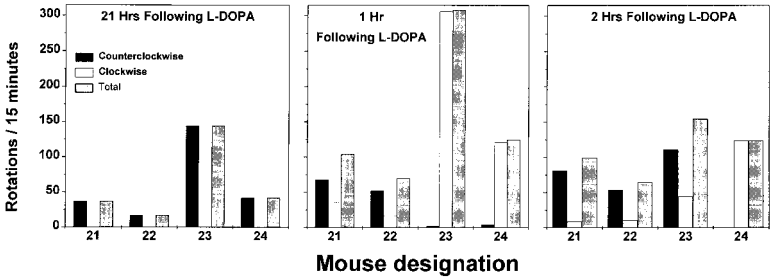


Figure 5. Rotational Behavior of DA$^{-/-}$ Mice Injected Unilaterally with Mixed Vector

Unilaterally injected mice (n = 4) were treated with L-DOPA (50 mg/kg body weight) and returned to their homecage, and their activity over 15 min was videotaped at 1, 2, and 21 hr following treatment. The number of 360° clockwise or counterclockwise turns that occurred during these intervals was counted.

confirmed in unilaterally injected mice because cortical fiber staining was only observed on the side of the brain where rAAV-TH was injected. GTPCH1 immunostaining revealed a pattern that overlapped with TH expression. An example of this overlap is shown in Figures 6H and 6I.

Regions exhibiting TH immunoreactivity in rAAV-TH alone and mixed vector mice are summarized in Figure 7. These overlays were created by systematically mapping TH-positive regions within coronal sections of virally transduced mice. Brain regions described in this study are shown in the color-coded diagrams in the top panel of Figure 7. Regions transduced by rAAVTH in each mouse are shown in green and red. Because bilateral transduction in ventral structures correlates with rescue, these regions are shown in red. TH was detected in the CPu and in ventral brain structures of all rescued and partially rescued mice (Figure 7, TH + GTPCH1, mice #17, #18, #19, and #57 and TH alone, mice #1, #4, and #5). The anterior-most brain region (bregma, 0.9 mm) consistently exhibited TH staining in ventral structures including several cortical areas. Analysis of a more posterior brain region (bregma, 0.3 mm) also revealed staining in ventral brain regions of rescued mice, notably, the ventral pallidum (VP), olfactory tubercle (Tu), and medial preoptic area (MCPO). The mixed vector mice

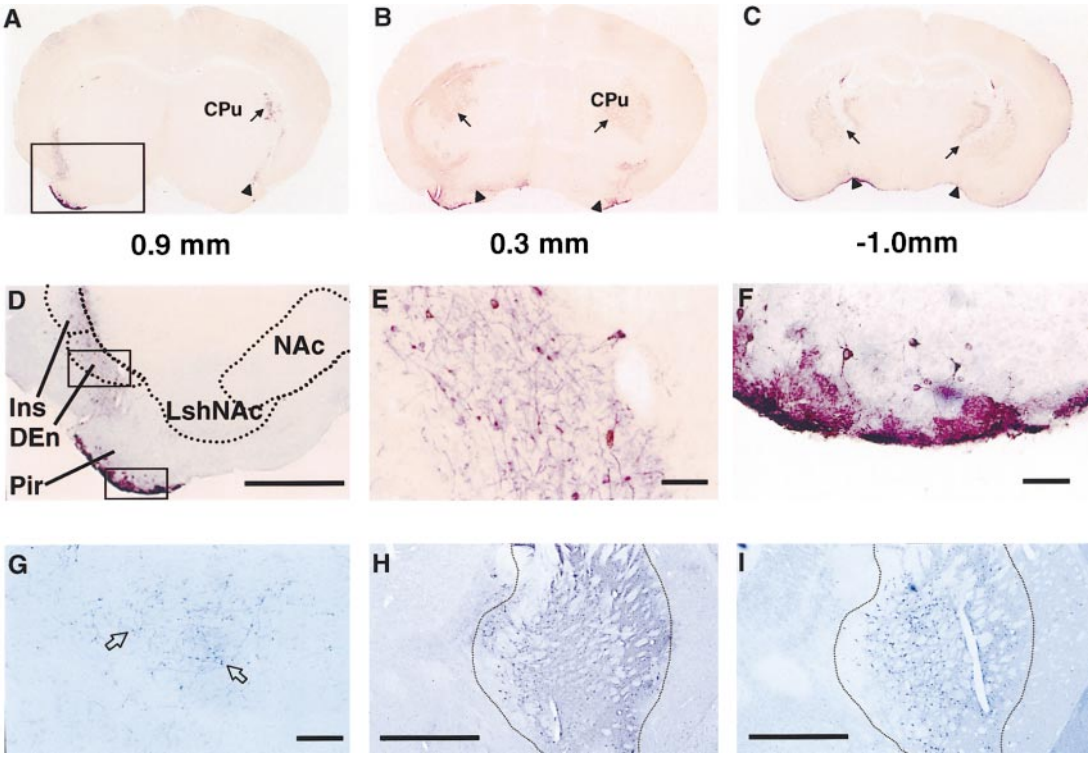


Figure 6. TH Immunohistochemistry in Various Brain Regions of Virally Transduced DA$^{-/-}$ Mice

(A–C) Low-powered view of TH immunostaining in coronal sections of a rescued mouse transduced with rAAV-TH + rAAVGTPCH1 (Mouse #18, Figure 7). Arrows denote TH-positive areas within dorsal structures, and arrowheads demarcate TH staining in ventral structures.

(D) A high-powered view of boxed area in (A) shows TH immunostaining within ventral brain structures. TH-positive brain regions include the insular (Ins), dorsal endopiriform (DEn), and Piriform (Pir) corticies. Other areas of interest that are not stained include the lateral shell (LshNAc) and core (NAc) of the nucleus accumbens; bar represents 500 μm.

(E and F) TH staining of cell bodies and fibers within the DEn (upper box from [D]), and Pir (lower box from [D]); bar = 100 μm.

(G) A high-power view of TH-positive fibers found within the dorsal neocortex of a rescued mouse; bar = 100 μm.

(H) TH immunostaining within the CPu of a mouse transduced with mixed virus (mouse #17 in Figure 7); bar = 500 μm.

(I) GTPCH1 immunostaining in an adjacent section from mouse #17 demonstrates a similar pattern of GTPCH1 expression; bar = 500 μm.

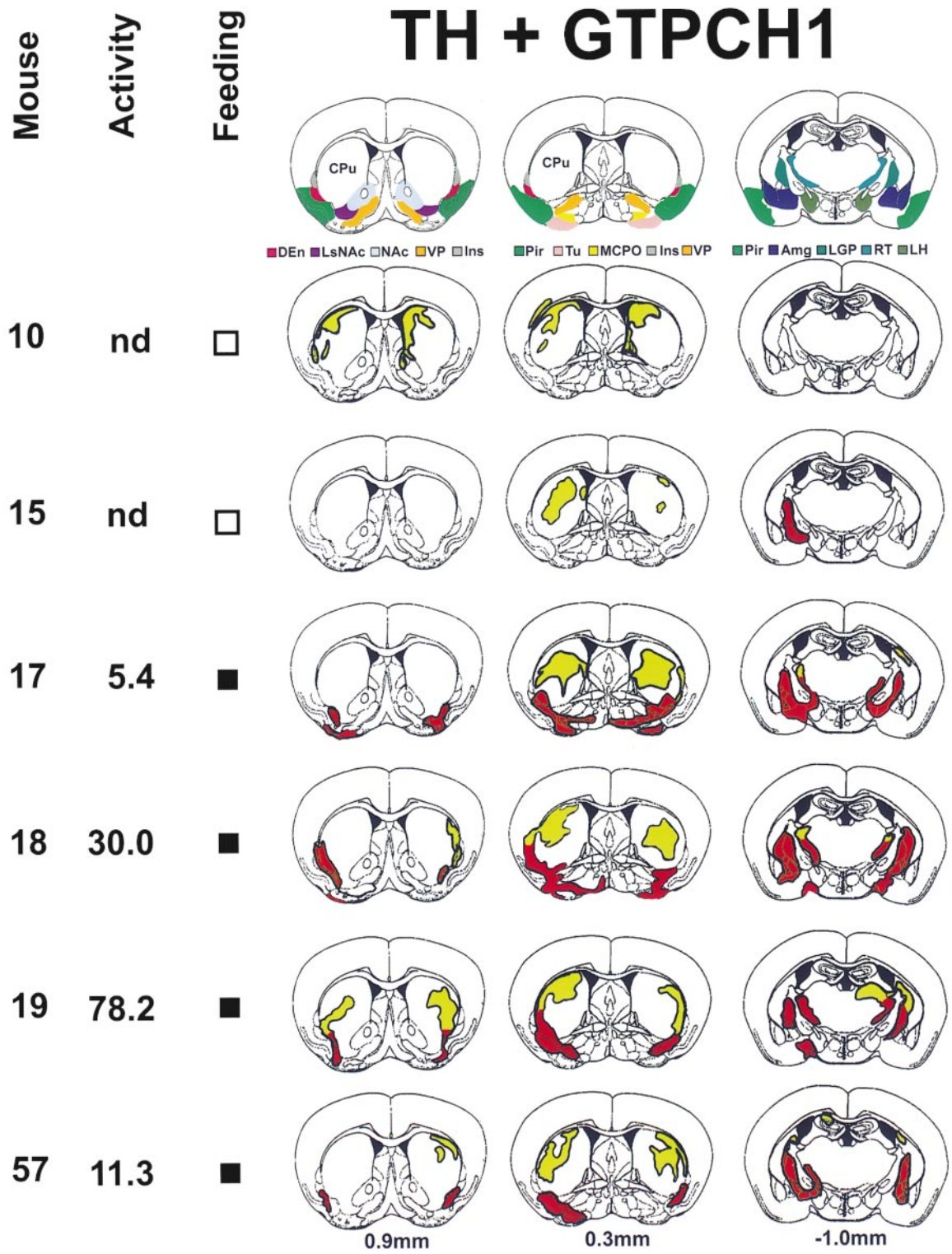
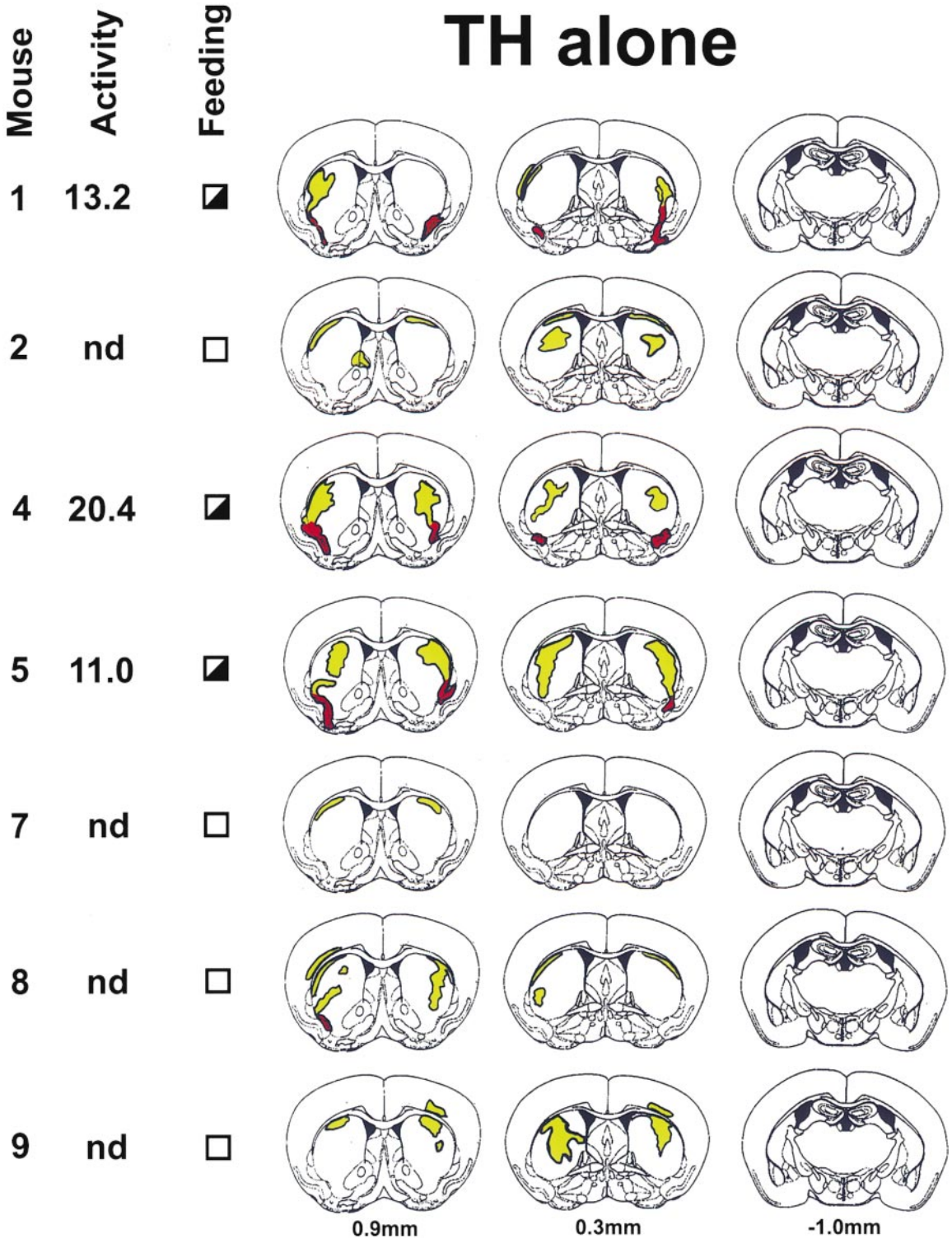


Figure 7. Mapping of TH-Positive Areas in Virally Transduced Mice

A color-coded key to various brain regions depicted in these sections is shown at top of left panel. Below are shown analyses of six mice treated with mixed virus. Right panel shows analysis of seven mice treated with rAAV-TH alone. Free-floating coronal sections spaced at 100 μm intervals and encompassing the entire brain were stained with TH antibody, and TH-positive areas were identified with a microscope under low magnification. Putative areas were then viewed under higher power to verify the presence of cell bodies. Regions containing cell bodies were scored as positive and are shown in green (dorsal structures) and red (ventral structures). Values for activity are reported in meters traveled over 24 hr (nd = not determined). Open boxes denote virally transduced mice that were not rescued for feeding, partially filled boxes represent partial rescue of feeding, and filled boxes full rescue. Diagrams of coronal sections were modified from Franklin and Paxinos (1997).


TH alone
Mouse
Activity
Feeding
1
13.2
2
nd
4
20.4
5
11.0
7
nd
8
nd
9
nd
0.9mm
0.3mm
-1.0mm

also exhibited TH staining in the other posterior brain regions (-1.0 mm bregma) that did not appear in any of the TH alone group including the partially rescued mice. Within this region, TH staining was observed in the lateral globus pallidus (LGP), amygdala (Amg), and the reticular thalamus (RT). Although TH staining was detected within the CPu of nonrescued mice, none was detected in either ventral or posterior brain regions.

Feeding was not restored in a few mice that received bilateral injections of mixed vector or in any of the mice that were unilaterally injected (Figure 7, mice #10 and #15). Presumably, critical brain regions were not transduced in these mice. Bilateral TH staining was observed in the anterior CPu of one of the bilaterally injected nonrescued mice (mouse #10), but not in ventral or posterior brain nuclei. Another bilaterally injected mouse (mouse #15) also exhibited bilateral TH staining in the anterior CPu, but not within ventral (Ins, VP, DEn, and Pir) regions and only unilaterally within posterior (LGP, RT, and Amg) brain regions. The anterior to posterior pattern of TH immunostaining observed on the right side of three of five unilaterally injected mice was similar to that observed in most bilaterally injected mice (data not shown). In general, the distribution of TH immunostaining in the mixed vector group was greater than that observed in the TH alone group, but TH was observed in cell bodies and dendritic processes of both groups.

Catecholamine Measurements Correlate with TH Immunostaining

Catecholamine measurements were performed on 2 mm diameter punches taken from 2 mm thick coronal sections of brains from mice injected with TH alone or mixed virus. Three samples, designated as anterior, posterior, and midbrain, were obtained from each mouse. The anterior punch contained most of CPu and NAc, the posterior punch contained the tail end of the CPu, LGP, and the entire amygdala, and the midbrain punch contained the substantia nigra. DA was detected in the anterior CPu (DA = 298.1 ± 36.2 ng/mg protein), posterior brain nuclei (DA = 28.4 ± 4.9 ng/mg protein), and substantia nigra (DA = 14.5 ± 1.4 ng/mg protein) of wild-type mice, but none was detected in $DA^{-/-}$ mice. DA content in the CPu of the mixed vector group ($n = 5$) was 24% of wild-type levels, and the TH alone group ($n = 5$) was 5.8% of wild-type levels in this brain region. DA was also present in the posterior brain regions of all mixed vector mice (36% of wild type) but was undetectable in the TH alone group. In addition, DA was detected in the substantia nigra of one mixed vector mouse (77% of wild type). These results support the conclusion that differential TH expression and subsequent production of DA from L-DOPA is responsible for the behavioral differences observed between the two groups.

The DOPAC/DA ratio, which is an index of DA turnover rate, was 0.09 in wild-type mice, 0.118 in the mixed vector group, but was 0.283 in the TH alone group, indicating that turnover was elevated in the TH alone group. Thus, both DA content and turnover rates were closer to wild-type values in the mixed vector group, which is consistent with the behavioral differences observed between the two groups.

Discussion

Dopamine signaling in the CNS affects locomotion, neuroendocrine secretion, cognition, feeding, and reward-related behaviors. Pharmacology and electrophysiology experiments have begun to identify specific dopaminergic pathways responsible for the regulation these distinct processes. It has been demonstrated that DA plays a role in regulating locomotion through its connections to the basal ganglia, and experimental evidence suggests that the mesolimbic dopaminergic pathway is involved in regulating other behaviors including drug self administration. However, the extent to which these behaviors are attributable to these distinct pathways is not clear.

Although DA is known to regulate a number of behaviors, its role in feeding is less certain. Anand and Brobeck (1951) found that electrolytic lesions of the lateral hypothalamus (LH) caused aphagia, suggesting that this brain region is involved in the control of feeding behavior. The coincident loss of ingestive behavior and depletion of catecholamines in the LH suggested that DA might function to stimulate feeding. Destruction of dopaminergic neurons that pass through the LH with 6-OHDA in adult rats initially confirmed this hypothesis (Ungerstedt, 1971; Fibiger et al., 1973). However, subsequent studies with this model suggested that the aphagia might result from nonspecific sensorimotor deficits rather than a suppressive effect on feeding caused by DA depletion (Salamone et al., 1990).

Pharmacological experiments point to an inhibitory or stimulatory role of DA in feeding, depending on the location of DA delivery. For example, chronic bilateral infusion of DA into the LH of obese Zucker rats decreased meal size and promoted weight loss (Yang et al., 1997). Likewise, injection of amphetamine or DA agonists into the LH inhibited fast-induced feeding by rats (Leibowitz and Brown, 1980). However, DA release in the shell of the nucleus accumbens (NAc) correlates with increased feeding behavior (Heffner et al., 1980; Hoebel et al., 1989). Furthermore, low doses of DA or DA agonists injected into the NAc or CPu stimulate feeding (Winn et al., 1982; Swanson et al., 1997; Taber and Fibiger, 1997). Microdialysis experiments demonstrate that DA release and turnover are increased in the NAc during feeding. Extracellular DA and its metabolites are increased in anticipation of a meal, in response to presentation of food and during ingestive behavior (Hoebel et al., 1992; Wilson et al., 1995; Martel and Fantino, 1996b). This suggests that DA release in the NAc may play a direct role in stimulating ingestive behavior; however, other brain regions innervated by dopaminergic neurons might also be involved. In particular, the amygdala and insular cortex have been implicated in the regulation of taste-motivated behaviors and the acquisition of taste (Mogenson and Wu, 1982; Fernandez-Ruiz et al., 1993).

We have previously shown that $DA^{-/-}$ mice are aphagic and expire at 2 to 4 weeks of age without treatment but can be kept alive by daily L-DOPA injections (Zhou and Palmiter, 1995). By 9 hr after their last injection with L-DOPA, they cease to eat enough to maintain body weight even though they are able to run, jump, and climb on cage tops in a fashion that is quantitatively similar

to wild-type mice (our unpublished data). The $DA^{-/-}$ mice grasp chow pellets and ingest small amounts of food during the second day following L-DOPA treatment, but they do not continue to eat even though they are nutritionally deprived. These observations suggest that $DA^{-/-}$ mice possess the motor capabilities to eat, but they lack a drive to initiate and maintain feeding and thus terminate feeding prematurely.

Mechanism of Gene Therapy in $DA^{-/-}$ Mice

In wild-type animals, L-DOPA is synthesized from tyrosine in dopaminergic neurons by action of TH and then converted to DA by L-aromatic amino acid decarboxylase (L-AADC). DA is transported into synaptic vesicles by a vesicular transporter (VMAT2) and released upon neuronal excitation. Extracellular DA is then cleared from the synaptic cleft by transport into presynaptic terminals via the DAT. Inactivation of the TH gene in dopaminergic neurons precludes L-DOPA biosynthesis, but it does not appear to affect survival of dopaminergic neurons, their ability to innervate the CPu, or the ability of postsynaptic neurons in the CPu to synthesize characteristic marker proteins (Zhou and Palmiter, 1995). Thus, unlike 6-OHDA-lesioned models, dopaminergic terminals are distributed normally in $DA^{-/-}$ mice, but they cannot synthesize or release DA unless L-DOPA is provided. For routine maintenance of these mice, L-DOPA is provided daily by intraperitoneal injection. L-DOPA readily crosses the blood–brain barrier and is presumably taken up by all cells via amino acid transporters. All cells that express L-AADC would be able to convert it to DA (this would include all monoamine-producing cells), and those cells that express VMAT2 would be able to package and release the DA. After release, DA could be taken up by dopaminergic terminals via the DAT. DA is also readily metabolized by monoamine oxidases and catecholamine methyl transferase; thus, after a single injection of L-DOPA, DA levels peak at about 1 hr and decline to barely detectable levels 9 hr later.

Our strategy was to inject the rAAVs into the projection fields of dopaminergic neurons in the ventral CPu and NAc. The most likely scenario for rescue is that nondopaminergic neurons at the site of injections are transduced by rAAV. Most of the cells probably do not express GTPCH1; thus, with rAAV-TH alone, these cells probably rely on subthreshold levels of BH4, and consequently, they would synthesize very little L-DOPA.

It is readily apparent that TH immunoreactivity in the CPu is within cells with cell bodies and projections that resemble neurons. They are most likely medium spiny GABAergic neurons because they are the most abundant neurons in this region, but various interneurons are probably also transduced. These cells would not be expected to have either L-AADC or VMAT2; therefore, we suspect that the L-DOPA they produce (or DA if they have L-AADC) crosses the plasma membrane and is taken up by dopaminergic terminals in the vicinity, packaged into vesicles, and released appropriately. We speculate that diffusion of L-DOPA and/or DA released by these cells is limited because dopaminergic neurons that project to virally transduced regions contain normal levels of DAT, which would facilitate DA uptake into dopaminergic neurons (our unpublished data). Furthermore, the DOPAC/DA ratio in rescued mice is similar to wild-type mice, suggesting that normal DA metabolism has been established. It is also possible that some dopaminergic neurons are transduced through their axons and that the viral DNA is transported retrogradely to the cell bodies where it is transcribed. In this case, rAAV expressing TH alone would be sufficient. The fact that the mixed vector was much more effective at rescuing $DA^{-/-}$ mice than rAAV-TH expressing TH alone argues that this is not the major mechanism involved, but it may contribute.

Separate Dopaminergic Pathways Contribute to Feeding and Locomotion

Coinjection of TH and GTPCH1 vectors into $DA^{-/-}$ mice fully rescued ingestive behaviors in most of the mice, but activity and coordinated movement were only partially restored. Rescued mice exhibited decreased ambulatory activity and performed at suboptimal levels on a rotating wheel (Figure 3B) and in a pole climbing test (Ogawa et al., 1985; our unpublished results). In addition to these deficits, rescued mice also display deficiencies in nest building behavior (Aubert et al., 1997; Sherwin, 1997; M. S. S. and R. D. P., unpublished data). Taken together, these three tests demonstrate that not only is coordinated behavior compromised (rotarod and pole climbing) but also certain aspects of motivated behavior (nest building). Analysis of TH expression in the mice that were and were not rescued by the mixed virus, as well as the nonrescued mice injected with rAAV-TH alone, indicate that bilateral transduction of cells in ventral brain regions is critical for normal feeding. Although the shell of the NAc has been implicated in feeding by other techniques (Maldonado-Irizarry et al., 1995), we did not observe high levels of TH expression within this nucleus. Instead, we detected extensive bilateral transduction in regions ventral and lateral to the NAc. It is possible that DA diffuses a short distance to the NAc or that neuronal projections of virally transduced cells project to the NAc and release DA, but close analysis of the NAc of rescued mice did not reveal TH-immunoreactive fibers.

Our data indicate that cotransduction with TH and GTPCH1 is essential for early and prolonged rescue. GTPCH1 not only provides an essential cofactor for enzymatic activity but may also stabilize TH against degradation. The fact that GTPCH1 is required indicates that most of the transduced cells are not capable of making adequate BH4. Surprisingly, none of our attempts to supply BH4 peripherally were able to restore feeding in mice transduced with rAAV-TH only. Thus, the mixed vector approach (Mandel et al., 1998), or bicistronic expression vectors, are likely to be more effective as possible treatments of humans with DA deficiencies.

Although most $DA^{-/-}$ mice treated with the mixed virus eat enough to gain and maintain body weight, they do not display normal motor behaviors. There are several possible explanations for these observations. This might be expected if restoration of feeding requires less DA than restoration of movement. However, lower

doses of L-DOPA are required to restore locomotion than feeding in DA$^{-/-}$ mice. This may reflect supersensitivity of pathways specifically required for locomotor behavior (our unpublished data). Another possibility is that a subpopulation of neurons in DA$^{-/-}$ mice are affected by development in the absence of DA. These changes might preclude the ability to restore movement and coordinated behavior to wild-type levels even if DA is replaced in critical brain regions at appropriate levels. However, a range of locomotor activity was observed in mice that exhibited total rescue of feeding behavior, and one mouse displayed motor activity that was similar to wild-type mice. Yet another possibility is that dopaminergic projection fields involved in feeding are distinct from those involved in movement. Many previous studies have suggested that the dorsal lateral striatum and its direct and indirect output pathways are involved in locomotion, whereas the ventral striatum is involved in directing motivated behaviors (Kopin and Markey, 1988; Wise and Hoffman, 1992). In most of the mice treated with the mixed vector, TH expression was prominent in ventral structures of the anterior brain and also in more caudal brain nuclei (Figure 7). Depletion of DA results in a locomotor supersensitivity that is revealed upon activation of DA receptors (Ungerstedt, 1971). The observation that supersensitivity persists but is attenuated in rescued mice, argues that DA has not been restored to normal levels in brain regions that control locomotion. One mouse that was not rescued for feeding by the mixed virus was incredibly active (mouse #15). Subsequent analysis revealed bilateral TH expression in the dorsal regions of the caudate putamen. Thus, we favor the idea that the sites of viral integration and the location of local L-DOPA production are determinants of behavioral correction. Consequently, with more refined placement and more restricted areas of transduction, it should be possible to delineate more precisely specific brain regions responsible for various DA-mediated behaviors. This does not preclude the possibility of L-DOPA production affecting several behaviors.

We have demonstrated that gene therapy rescues the aphagia of DA$^{-/-}$ mice and presented evidence of a partial separation of movement and feeding. These data suggest that this technique can be used to map dopaminergic pathways that specifically regulate feeding and movement. Application of this technique provides a new approach to identify dopaminergic pathways that are required for specific behaviors. Furthermore, this work demonstrates that rAAV-based gene therapy has potential application for the treatment of human Parkinson's disease.

Experimental Procedures

Generation of DA$^{-/-}$ Mice

Mice were housed on a 12 hr light-dark cycle with the dark cycle occurring from 19:00 to 07:00. Food and water were provided ad libitum. All injections were performed on 3- to 6-month-old DA$^{-/-}$ mice; they were third and fourth generation hybrids of 129 Sv/CPJ × C57BL/6J. All behavioral tests were performed during the light cycle. Genotype was confirmed by Southern blot analysis as described (Zhou and Palmiter, 1995).

Recombinant AAV Vector Production

Recombinant AAV vectors rAAV-TH, rAAV-GTPCH1, and rAAV-GFP were prepared as described by Snyder et al. (1997) with the following modifications. The helper plasmid pUC19.ACG was used to supply AAV rep and cap functions. This construct harbors the XbaI fragment of pACG2–1 described by Li et al. (1997), which was isolated by PCR to change the 5′ XbaI site to HindIII and the 3′ XbaI site to BamHI. This fragment was inserted into the HindIII and BamHI sites in pUC19; it does not harbor the adenoviral terminal repeats. rAAV virions were purified on two sequential isopycnic CsCl gradients formed in a Beckman NVT65 rotor at 60,000 rpm for 6 hr minimum, and fractions were collected using a Beckman Fraction Recovery System. Following CsCl banding, the fractions containing rAAV were dialyzed against PBS. The vector preparations had particle titers between 10^{11}–10^{12}/ml. Wild-type AAV contamination was less than 2 particles/10^8 particles, and there was no contaminating infectious adenovirus.

Intracerebral Injection of Recombinant AAV Vectors

Mice were placed in a stereotax (Kopf), and the head was leveled in the x, y, and z planes using the sagittal suture and lambda and bregma as landmarks. Recombinant AAV vectors in PBS were injected into ventral striatum (0.8 anterior-posterior, 2.2 and −2.2 medial-lateral, and 3.6 dorsal-ventral using bregma as the reference point [Slotnick and Leonard, 1975]). Virus (2 μl) was injected through a 5 μl Hamilton syringe fitted with a 30 gauge needle at a rate of 0.5 μl/min. Injection rate was controlled using a KD scientific infusion pump. Following each 4 min injection, the needle remained stationary for an additional 4 min and was then raised 0.5 mm and maintained in this position for an additional 2 min before being removed. Injections were performed sequentially into the right and left sides at identical coordinates. DA$^{-/-}$ mice were injected bilaterally with 2 μl of: rAAV-TH (n = 23), rAAV-GTPCH1 (n = 5), or a 1:1 mixture of rAAV-TH and rAAV-GTPCH1 (n = 23). An additional four mice were injected unilaterally with 2 μl of the mixed virus (n = 4).

Behavioral Analysis

Ambulatory activity of wild-type or rescued mice was measured in transparent plexiglass cages (40 × 20 × 20 cm) placed into an activity chamber equipped with infrared beams (San Diego Instruments Inc.). The number of consecutive beam breaks that occurred each hour during a 48 hr period was measured and converted to meters using the distance between beams (8.8 cm) as a conversion factor. Response of DA$^{-/-}$ and rescued mice to L-DOPA was monitored as above after they were allowed to habituate for 24 hr. Activity was monitored for 1 hr, and then they were injected with either 50 mg/kg L-DOPA (dissolved in PBS) or PBS, and their activity and food intake were monitored over the next 5 hr. Rotarod experiments were performed with Jones and Roberts apparatus (Rod diameter = 4 cm). Wild-type (n = 16), DA$^{-/-}$ (n = 10), TH alone (n = 3), and mixed vector mice (n = 13) were placed on a rotarod for 1 min. The rotarod was accelerated to a speed of 10 RPM over ~40 s and was maintained at 10 RPM for an additional 50 s. Each animal was trained four times; the time spent on the rod during the 4th trial is reported. Activity and rotarod measurements were performed 3 to 5 days and 2 months following removal of L-DOPA.

Rotational behavior of unilaterally injected mice (n = 4) was quantitated following L-DOPA treatment. Mice were injected with 50 mg/kg body weight of L-DOPA and returned to their homecages, and then they were filmed for 15 min periods at 1, 2, and 21 hr following treatment. The number of 360° turns that occurred during each 15 min period in either the clockwise or counterclockwise directions were tallied by viewing videotape of each mouse.

Histology

TH and GTPCH1 immunohistochemistry were performed as follows. Mice were killed by CO_2 inhalation, systemic blood was cleared by perfusion with PBS (PBS was 100 mM phosphate, 150 mM NaCl [pH 7.0]), then mice were perfused with 4% paraformaldehyde in PBS and postfixed overnight at 4°C. Brains were sunk in 30% sucrose and frozen on dry ice. Sections (10–20 μm) were then cut on a freezing microtome and stored at 4°C in PBS containing 0.1% azide. The following manipulations were performed at room temperature. Free-floating sections were washed briefly in PBS containing 0.1% Triton X-100 (PBS-TX) and then incubated for 20 min in PBS-TX + 3% H_2O_2. Sections were rinsed in water, washed 3 times in

PBS-TX (5 min each wash), and then incubated in PBS-TX containing 8% normal goat serum (NGS) for 1 hr to block nonspecific binding. Sections were incubated for 15 to 24 hr in PBS-TX + 1% NGS containing rabbit polyclonal TH antibody (Eugene Tech, 1:1000 dilution), washed 3 times in PBS-TX, and then incubated for 2 hr with biotinylated secondary antibody in PBS-TX + 1% NGS (Vector Laboratories, 1:500). Sections were washed and stained for TH according to standard streptoavidin immunohistochemical procedures. GTPCH1 immunohistochemistry was performed using identical conditions. Primary antibody against GTPCH1 was used at a 1:3000 dilution (Mandel et al., 1998).

Mapping TH Expression

Anterior, posterior, medial, lateral, dorsal, and ventral regions referred to in this study were designated as follows using the albino mouse atlas as a guide for map coordinates (Slotnick and Leonard, 1975). Anterior is defined as an area extending from 1.3 to −0.7 with respect to bregma. Posterior is defined as an area encompassing from −0.7 to −2.7 with respect to bregma. The anterior CPu is divided into medial-lateral and dorsoventral portions. Medial extends from midline to a 2.2 mm distance from bregma, and lateral was designated from 2.2 mm to the outer most portion of the brain (∼5.0 mm). Dorsal extends 2.2 mm from bregma, and ventral extends from 2.5 mm to the ventral most portion of the brain (∼5.6 mm).

Dopamine Content

The brains of five rescued (mixed vector), five nonrescued (rAAV-TH alone), two wild-type, and two $DA^{-/-}$ mice were dissected into three regions, and 2 mm tissue punches were collected as above. Catecholamine content was determined as described (Mandel et al., 1998).

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