Simultaneous absence of dopamine D1 and D2 receptor-mediated signaling is lethal in mice


Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 Rue Laurent Fries, 67404 Illkirch, France; Laboratory of Mammalian Genes and Development, National Institutes of Health, Bethesda, MD 20892; and Howard Florey Institute, University of Melbourne, Parkville, Victoria 3010, Australia

Edited by Richard D. Palmiter, University of Washington School of Medicine, Seattle, WA, and approved June 21, 2004 (received for review March 23, 2004)

Dopamine (DA) controls a wide variety of physiological functions in the central nervous system as well as in the neuroendocrine and gastrointestinal systems. DA signaling is mediated by five cloned receptors named D1–D5. Knockout mouse models for the five receptors have been generated, and, albeit impaired for some important DA-mediated functions, they are viable and can reproduce. D1 and D2 receptors are the most abundant and widely expressed DA receptors. Cooperative/synergistic effects mediated by these receptors have been suggested, in particular, in the control of motor behaviors. To analyze the extent of such interrelationship, we have generated double D1/D2 receptor mutants. Interestingly, in contrast to single knockouts, we found that concurrent ablation of the D1 and D2 receptors is lethal during the second or third week after birth. This dramatic phenotype is likely to be related to altered feeding behavior and dysfunction of the gastrointestinal system, especially because major anatomical changes were not identified in the brain. Similarly, in the absence of functional D1, heterozygous D2 mutants (D1r−/−;D2r+/−) showed severe growth retardation and did not survive their postweaning period. The analysis of motor behavior in D1r/D2r compound mutants showed that loss of D2-mediated functions reduces motor abilities, whereas the effect of D1r ablation on locomotion strongly depends on the experimental paradigms used. These studies highlight the interrelationship between D1 and D2 receptor-mediated control of motor activity, food intake, and gastrointestinal functions, which has been elusive in the single-gene ablation studies.

Knockout mice | motor function | feeding behavior | gastrointestinal system

The diverse physiological functions of dopamine (DA) are mediated by five distinct receptors, which by structural and pharmacological means have been grouped into two families: the D1-like and D2-like receptors. The D1-like family comprises the D1 and D5 receptors, whereas D2, D3, and D4 receptors form the D2-like family. Pharmacological studies have not allowed a full understanding of the specific role of each DA receptor in vivo because of the lack of ligands with absolute receptor specificity. Knockout mice for each DA receptor have now been reported (1–9), substantially increasing our knowledge of the dopaminergic system (10, 11).

Among DA receptors the most widely and abundantly expressed in the central nervous system are the D1 and D2 receptors, whereas D3, D4, and D5 have lower abundance and a very restricted localization. Mutation of the D1 gene (D1r) in mice resulted in growth retardation, failure to respond to the motor stimulant effects of addictive drugs, and poor learning performance (2, 3, 12–14). Moderate growth retardation was also reported in mice lacking D2 receptors (D2r−/−), which in addition are hypoactive, fail to experience the rewarding properties of morphine, and lose DA autoreceptor function (4, 15, 16).

Importantly, neuroanatomical as well as pharmacological studies have proposed that a concomitant activation of D1r and D2r is crucial in the control of diverse physiological functions regulated by DA (17–19). To obtain insight into the interaction between D1r and D2r, we generated D1/D2 double knockout (DKO) mice. Interestingly, concurrent ablation of D1r and D2r is not compatible with life; DKO mice do not survive after the second to third week after birth. These mutants are severely growth-retarded, a phenotype similar to that of mice lacking DA (20). These results indicate that, among DA receptors, D1r and D2r are key components of the dopaminergic system and reveal the presence of functional interactions between these two receptors.

Materials and Methods

Animals. D1r (3) and D2r (4) heterozygous (75% C57BL/6, 25% 129/Sv) mice were mated to obtain double heterozygous mice (D1r−/−;D2r+/−). Mating of these generated DKO and compound mutant mice. Litters were controlled twice daily for appropriate nursing and feeding conditions. Age- and sex-matched mice were group-housed with free access to mouse breeder diet [Scientific Animal Food and Engineering (SAFE)]; DO3, 2,800 kcal/kg (1 kcal = 4.18 KJ). Average analysis: minerals, 6%; moisture, 12%; lipids, 8%; proteins, 24%; fibers, 4% and water under a 12-h light/dark cycle. D1r−/−;D2r−/− mice were fed a semiliquid diet of food pellets ground into powder and mixed with water. Food was served into dishes on the cage floor and changed twice per day.

Tube feeding was performed on entire litters (n = 3) from double heterozygote matings, starting at postnatal day 11. Mice were group-housed and gavaged with Calorie Mate (Otsuka Pharmaceutical, Tokyo; 1,000 kcal/liter. Average analysis: proteins, 5%; lipids, 2.2%; sugar, 15%; fibers, 1%; minerals, 0.4%; vitamins, 0.04%) twice daily. Genomic DNA was digested with HindIII or EcoRI to genotype D1r or D2r alleles, respectively (3, 4).

Immunohistochemistry. Cryostat sections were postfixed in ice-cold 4% paraformaldehyde for 15 min and preincubated for 1 h in 5% normal goat serum and 0.05% Tween 20 in PBS, followed by incubation with rabbit anti-tyrosine hydroxylase (TH) antibody (1:400) (Chemicon) at 4°C overnight. Slides were incubated for 1 h with goat anti-rabbit IgG antibody conjugated with biotin and revealed by the ABC kit (Vector Laboratories).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DA, dopamine; DKO, double knockout; TH, tyrosine hydroxylase; Enk, enkephalin; SP, substance P; Dyn, dynorphin; NPY, neuropeptide Y; AGRP, agouti-related protein; GI, gastrointestinal.

†Present address: Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., Nagazumi-cho, Shizuoka 411-8731, Japan.
‡Present address: Istituto di Neuroscienze del Consiglio Nazionale delle Ricerche, Pisa 56100, Italy.
§To whom correspondence should be addressed. E-mail: eb@igbmc.u-strasbg.fr.

© 2004 by The National Academy of Sciences of the USA
In Situ Hybridization. In situ hybridizations were performed as described (4, 21). Sections were incubated with antisense probes for enkephalin (Enk), substance P (SP), or dynorphin (Dyn) (4), washed and exposed to Kodak NTB emulsion (Eastman Kodak, Rochester, NY) for 24 h (Enk) or 1 week (SP and Dyn). cDNA probes for neuropeptide Y (NPY) (22), agouti-related protein (AGRP) (23), and orexin (24) were obtained by RT-PCR from mouse mRNA. After hybridization, sections were exposed to imaging plates for 5 days and signals were quantified with the BAS2000 system (Fuji Film, Tokyo).

Histology. Histological analyses of digestive tracts were performed on paraffin-embedded tissue after fixation in Bouin’s fixative and examination by hematoxylin/cosin coloration (21).

Behavioral Studies. Behavioral assays were performed on 2- to 3-month-old mice. Data were combined across genders because there was no statistically significant interaction between gender and behavioral scores obtained from ANOVA testing. Activity in home cage was assessed in photo-beam-equipped cages (Viewpoint, Lyon, France) during the dark phase of the light/dark cycle. Daily food intake and body weight for each animal was measured during this period. Open-field test was conducted in 30 × 30-cm chambers with controlled illumination (80 lux). Distance traveled and rearing events were assessed during the first 60 min with a video tracking system (Viewpoint). In rotarod tests, mice were placed on a 5-cm-diameter rod and left for 30 s to habituate. The rod was set in motion at 8 rpm. Each animal was tested for a maximum of 2 min and given a maximum of three trials in the same day (4).

Statistical Analyses. Statistical comparisons between WT and compound mutants were performed by using one-way ANOVA followed by Tukey’s post hoc test. P < 0.05 was considered statistically significant between groups.

Results

DKO Mice Are Not Viable. Single ablation of either D1r or D2r resulted in viable animals able to reach adulthood. The progeny obtained from D1r+/−D2r+/− mice followed the expected Mendelian ratio, suggesting that the embryonic development of the DKO mice is normal. However, D1r/D2r dual deficiency caused postnatal lethality, which occurred just before weaning. Newborn DKO mice were indistinguishable from their littermates. Growth retardation occurred at the end of the first postnatal week (Fig. 1A). The growth rate of DKO mice stopped after postnatal days 7–10, followed by marked hypoactivity. DKO mice eventually died by the third postnatal week (Fig. 1B). This phenotype is strongly reminiscent of that of DA-deficient mice (20). Routine macroscopic and microscopic analysis of the brain of DKO mice did not show any abnormality. The pattern of the TH immunostaining suggested that the development of midbrain DA neurons, as well as their projections to the striatum, is normal in DKO mice as compared with WT littermates (Fig. 1C).

A characterization of the influence of double-receptor ablation on the expression of striatal peptides showed that SP, Dyn, and Enk expression is modified in a manner that mirrors their expression in D1r−/− or D2r−/− simple mutants (3, 4); SP and Dyn are down-regulated, whereas Enk is up-regulated (Fig. 1D). No cooperation/synergy was observed between D1r- and D2r-mediated signaling in the control of the expression of these neuropeptides at the mRNA level.

DKO Mice Have Altered Gastrointestinal (GI) and Hypothalamic Functions. The GI tract of DKO mice was assessed in an effort to explain the failure-to-thrive phenotype. Milk was always found in the stomach of WT mice, whereas the stomach of DKO mice only contained traces of it, indicating that DKO mice cease feeding during their postnatal development. In an effort to rescue DKO mice from dying, we tried hand-feeding the animals once the runt phenotype became evident (postnatal day 11). However, none of the DKO mice fed by gavage survived, and all eventually died by day 15. Their littermates, similarly fed, were still alive at day 17, the time at which the experiment was interrupted.

Notably, among 45 DKO mice observed between the ages of 7 and 15 days, five were found with hemorrhage in the digestive tract. Because DA acts as an inhibitory modulator of GI motility (25), this might also suggest that the stomach of DKO mice...
Poor intestinal development and hypothalamic dysfunctions in DKO. (A) Hematoxylin/eosin stainings of paraffin-embedded intestinal sections (duodenum) from WT (Left) and DKO (Right). (Scale bar, 400 μm.) (B) In situ hybridizations of brain sections from WT and DKO using probes specific for NPY, AGRP, and orexin. Animals were killed at the beginning of the dark phase when feeding behavior is nearly at its highest level. Experiments were repeated three times. WT values (black bars) were arbitrarily taken as 100%. * P < 0.05; ** P < 0.01 versus WT (Student’s t test). (Scale bar, 1.5 mm.)

Phenotypes of DTr/Dd2r Compound Mutant Mice. DTr−/− and Dd2r−/− consumed less food than their WT littermates (3, 4) (daily food intake: male WT, 3.9 ± 0.2 g; DTr−/−, 2.3 ± 0.2 g; and Dd2r−/−, 3.1 ± 0.1; n = 8 per genotype), leading to a moderate reduction of body weight. These results underscore the relevance of intact DTr- and Dd2r-mediated signaling in the regulation of food intake. Interestingly, food intake was strongly affected by loss of Dd2r-mediated signaling on a DTr−/− background. Indeed, although DTr−/−:Dd2r−/− animals do not survive the postweaning period, DTr−/−:Dd2r−/− mutants are viable and able to reach adulthood (Fig. 3C). Thus, DTr-mediated signaling is necessary and sufficient to ensure survival when Dd2r signaling is reduced or absent.

In contrast to DKO mice, DTr−/−:Dd2r−/− mice can be rescued from death by serving them highly hydrated food (Fig. 3A and B). Once this feeding regimen was discontinued, they lost weight and eventually died in 2–3 days. Analyses of the GI tract of 21-day-old DTr−/−:Dd2r−/− mice (n = 6) under a normal diet showed the presence of hemorrhages in the digestive tract (Fig. 3C). No food was ever found in their stomach, despite food ingestion. At the macroscopic level, severe ulcerative jejunoileitis, characterized by multiple chronic small intestine ulcers and bleeding, was observed in DTr−/−:Dd2r−/− mice consuming normal chow as compared with WT animals. As for DKO mice, these data suggest that absence of DTr and Dd2r might accelerate gastric emptiness.

At the microscopic level, the histological changes were most marked in the duodenum and upper jejunum of DTr−/−:Dd2r−/− animals. This phenotype was less prominent in the lower jejunum and minimal or absent in the ileum. Duodenal sections of DTr−/−:Dd2r−/− mice on normal diet revealed a severe villous atrophy. Morphologic integrity was preserved in animals fed...
alleles impaired locomotor activity in a gene-dosage-dependent manner. In agreement with previous data (4), loss of functional D2r alleles in compound mutants appeared to be prevented by semiliquid diet. (Scale bar, 200 μm.)

with a semiliquid diet (Fig. 4) with a subsequent acute relapse when mice were rechallenged with a solid diet. Compound mutants of the other genotypes did not show any of these signs.

Motor Activity of D1r/D2r Compound Mutant Mice. The motor activity of D1r/D2r compound mutants was analyzed in 2- to 3-month-old mice, in the home cage and open field (Fig. 5A–C). In agreement with previous data (4), loss of functional D2r alleles impaired locomotor activity in a gene-dosage-dependent manner (compare WT versus D1r+/+;D2r+/+ and D1r++;D2r–/–). D1r+/– mice had a significant decrease of motor activity in the home cage, comparable with that of D2r–/–. Interestingly, deletion of one functional allele of either D2r or D1r did not further reduce motor activity in D1r–/– or D2r-null background (i.e., D1r+/–;D2r+/+ versus D1r–/–;D2r+/+ and D1r+/+;D2r–/– versus D1r+/+;D2r+/+). Conversely, a moderate but significant increase of motor activity was observed in D1r+/+;D2r+/+ mutants as compared with WT littermates (Fig. 5A). This activation was brought down by the deletion of one D2r allele. Indeed, D1r+/+;D2r+/+ mice had a level of locomotion similar to WT animals, whereas D1r+/+;D2r–/– mice had the same level of locomotion as D1r–/– or D2r–/– mice.

In the open field (Fig. 5B and C), D2r–/– mice showed reduced activity in horizontal locomotion and rearing, as previously observed (4). In contrast, D1r–/–;D2r+/+ and D1r–/–;D2r–/– mice showed hyperlocomotion in this test. Moreover, both D1r compound mutants appeared to move along stereotyped pathways, which resulted in a reduced number of rearing events (Fig. 5C). The motor behavior of D1r–/–;D2r+/+ mice did not significantly differ from that of their WT littermates in this paradigm. Conversely, the locomotor activity of D2r–/– mice has values intermediate to those of D2r–/– and WT animals, unless D1r was also deleted. Altogether, these results underline the cooperative/synergistic activity of D1r and D2r in the regulation of locomotion.

Balance and motor coordination were tested in D1r/D2r compound mutants by the rotarod assay (Fig. 5D). In agreement with previous data (4), D2r–/– mice performed poorly in this test. In addition, animals on a D1r–/– background showed a markedly reduced latency to fall compared with WT or compound heterozygote mice. Increased locomotion of D1r–/–;D2r+/+ and D1r–/–;D2r–/– mice in the open-field test confirms that the reduced latency to fall in the rotarod test was caused by a coordination problem rather than weakness or bradykinesia. These results suggest that the ability to coordinate movements requires functional D1r as well as D2r.

Discussion

Development of Dopaminergic and Striatal Neurons in DKO Mice. In the developing rat brain, D1r and D2r mRNAs appear as early as embryonic day 14, after the establishment of midbrain dopaminergic neurons and before the maturation of striatal
projections, which continue to increase after birth (28–30). In DKO mice, TH immunoreactivity in both midbrain and striatum (Fig. 1C), as well as expression of striatal neuropeptides (Fig. 1D), suggests that establishment of the nigrostriatal system is not reliant on developmental expression of D1r and D2r D1 and D2 DA receptors, in agreement with data obtained in DA-deficient mice (20). This finding suggests that the dopaminergic signal is not required for striatal neurogenesis and the establishment of the nigrostriatal pathway. The dissociation constants (Kd) of D1r and D2r for specific ligands are not modified in compound mutants, and no functional compensation by other DA receptors at the level of expression is observed in these mice (data not shown). In addition, the altered striatal neuropeptide expression levels in DKO mice perfectly mirror those observed in both D1r−/− and D2r−/− (3, 4), suggesting the absence of synergy/ cooperation between these receptors in the control of neuropeptide expression.

**DA and the GI System.** Central as well as peripheral DA has been reported to modulate GI functions in mammals and protect against ulcer formation induced by chemicals or stress (31–34). Notably, loss of DA in Parkinson’s disease is accompanied by a high incidence of duodenal ulcers (35) and by impaired GI motility (36). DA inhibits gastric acid secretion, enhancing submucosal blood flow and regulating GI motility. Both D1- and D2-like receptors are involved in these functions (25, 37–39). D1r expression has been reported in the GI tract; D1r was found in the lamina propria submucosal vessels as well as smooth muscle cell layers (40, 41), whereas D2r was found in the myenteric ganglia (41), where TH and DA transporter (DAT) are also expressed (42). Lack of DAT results in hyperdopaminergia and altered regulation of colonic motility (43). DA has been shown to inhibit contraction of isolated distal colonic smooth muscles, and these effects are blocked by D1- and D2-like antagonists (43), suggesting that the colonic phenotype of our DΚΟ mice is partly due to the peripheral loss of DA-mediated signaling through D1r and D2r. It is thus conceivable to suggest that DKO mice might die by dysregulation of DA-regulated functions at the peripheral level.

Importantly, in DKO mice the GI phenotype is progressively worsened in the transition from single knockout to DKO, and at least one functional allele of either D1r or D2r is able to help overcome GI dysfunctions. However, loss of D1r could be rescued by one D2r allele only in animals fed a semiliquid diet, suggesting a preponderant D1-mediated role in the control of GI activity. Interestingly, another DA receptor, D5r, is also expressed in the GI mucosa and suggested to mediate cytoprotective effects (41, 44, 45). This expression suggests that D5r might compensate for loss of D1r in the presence of D2r-mediated signaling but is unable to compensate for loss of both receptor-mediated functions. It thus appears that D1- and D2-mediated signaling act cooperatively with respect to the regulation of GI functions (46). These receptors might activate the same path, although at different levels, or converging pathways leading to a common physiological effect. Future studies are required to dissect the sites of this cooperation.

**DA and Food Intake.** Eating is a complex process occurring in response to sensory cues that are associated with food availability and with satiety/hunger states. The dopaminergic signaling is clearly involved in feeding behavior. Hyperphagia induced by electrical stimulation of lateral hypothalamus is reversed by systemic administration of D2-like antagonists (47), whereas D1-like antagonists decrease the ingestion of palatable food in normal rats (48). DA depletion by 6-hydroxydopamine (6-OHDA) treatment results in reduced feeding behavior (49, 50). Moreover, severe aphagia and adipsia are present in mice lacking DA, in which L-dopa treatments restore feeding (20, 51). Thus, the reduction of body weight and food intake found in DKO mice as well as other compound mutants (Figs. 1A and 2A and data not shown) appears a direct outcome of concomitant ablation of DA signaling via D1r and D2r. Notably, loss of a single D2r allele severely compromises the feeding phenotype of D1r−/−. Thus, the contribution of the D2 receptors in feeding is unmasked by the absence of D1r, similar to what we observed in the GI system.

**Aphagia Is Independent of Reduced Activity.** The coincidence of aphagia and gut failure in DKO and in D1r−/−:D2r−/− mice raises the question of whether the cessation of food intake is primarily dependent on motivational, motor, or GI dysfunctions. Loss of motivation and motor impairment in DKO mice, which is not observed in either D1r−/− or D2r−/− mice, might compensate for loss of protective effects (41, 44, 45). This expression suggests that loss of motivation toward food intake by itself cannot account for the lethal phenotype.

Similarly, the growth of DKO mice ceased before their hypoactivity became evident, and litter size had no effect on the longevity of the animals. In addition, severe hypophagia was also observed in D1r−/−:D2r−/− mice, whose motor activity was not so severely depressed as to prevent them from reaching food and water in the cage (Fig. 5A). This finding suggests that reduced activity is unlikely to be responsible for initiating the reduction of food intake and the cascade of events leading to death of DKO or D1r−/−:D2r−/− mice. In agreement, aphagia in DA-deficient mice is independent of akinesia, and the feeding behavior can be restored by striatal production of DA by virally mediated TH expression (52, 53).

DA also modulates water and electrolyte transport in the GI tract (54). A reduction in net fluid intake as well as nutritional supply secondary to aphagia is likely to play a direct role in the death of D1r−/−:D2r−/− (and probably also DKO) mice, because they will not develop GI ulcerations as long as they are fed hydrated food. Nutritional deficiency secondary to hypophagia during early postnatal development might partly contribute to the intestinal atrophy (55–58).

The hypothalamic neuropeptides, NPY, AGRP, and orexin typically fluctuate in the same direction to regulate food intake and their expression patterns reflect the individual hunger/ satiety state (26, 27). Our results suggest that, in the absence of D1r- and D2r-mediated signaling, the expression of these neuropeptides can go in opposite directions, thereby contributing, together with GI dysregulations, to abnormal feeding habits in DKO mice (Fig. 2B). However, it is presently unclear to what extent this dysregulation might contribute to alteration of food intake in DKO mice. Interestingly, in DA-deficient mice, hyperphagia is no longer induced by leptin deficiency (59).

Thus, the hypophagia observed in DKO and D1r−/−:D2r−/− mice does not appear to be principally induced either by motivational or motor impairment. It cannot be ruled out that minor alteration (undetected in our analyses) of central DA-mediated functions, together with major structural and functional abnormalities in the development and function of the GI tract, might participate in the development of the lethal phenotype of DKO mutants.

**Motor Behavior of D1r/D2r Compound Mutants.** The motor activity of D1r−/− was reduced in the home cage and rotarod tests (Fig. 5A and D), whereas it was increased in the open-field test with respect to WT mice and independent of the presence of either one or two functional D2r alleles (Fig. 5B). These results apparently diverge from those previously reported on the same line of D1r−/− (3). However, variables in the behavioral setting, for example, novelty, width, brightness of the environment, and time of testing in the circadian cycle, very likely affect the motor phenotype of D1r−/− (10). Thus, the motor phenotype of D1r−/− cannot be simply described as hypo- or hyperactivity, but it is...
very much dependent on the experimental conditions or behavioral topographies used (60).

D2<sup>−/−</sup>—simple mutants showed deficits in all motor tasks, in agreement with previous data (4). In sharp contrast to the G1 and feeding phenotypes, the analysis of compound mutants revealed that absence of D1 dominates the phenotype of D2 heterozygotes, or D1 deficiency alters the D2 control of motor function, in which these two receptor systems are otherwise functionally linked (17). Future analyses will help elucidate D1–D2 receptor interactions and unravel the participation of each D2 receptor isoform in these processes. Our study illustrates the cooperative/synergistic activities of D1 and D2 receptors in DA-mediated functions, indicating the relevance of each receptor in particular tasks. It also highlights the importance of an in-depth knowledge of D1–D2 interactions in the pharmacological treatment of DA dysfunctions in humans.

We thank Dr. Hiroshi Kase for support and helpful discussions, and R. Latzting and E. Erbs for technical assistance. This work was supported by grants from Kyowa Hakko Kogyo Co., Ltd., Hoechst-Marrion-Russell, Institut National de la Sante et de la Recherche Medeciale, Centre National de la Recherche Scientifique, Université Louis Pasteur, and Fondation de France (to E.B.).