

Genetic control of number of midbrain dopaminergic neurons in inbred strains of mice: Relationship to size and neuronal density of the striatum

(immunocytochemistry/tyrosine hydroxylase/choline acetyltransferase)

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ABSTRACT The activity of tyrosine hydroxylase [TyrHase; tyrosine-3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] is 20% less in whole midbrain of CBA/J mice than BALB/cJ mice and is paralleled by a comparable difference in the number of dopaminergic neurons in which the enzyme can be detected immunocytochemically. The strain-dependent difference in numbers of TyrHase-containing neurons and of TyrHase activity is not homogeneous in the midbrain but is restricted (along the rostral-caudal axis) to the medial one-third, where almost 2-fold variations are found. The volume of the striatum, a major projection field of midbrain dopamine neurons, is 20% smaller in CBA/J than in BALB/cJ mice; the difference is regional and is concentrated in the caudal half. Because the packing density of intrinsic neurons of the striatum is similar in both strains, CBA/J mice contain 20% fewer neurons than do BALB/cJ mice. The activities of TyrHase and of choline acetyltransferase (ChoAcTase; acetyl-CoA:choline-O-acetyltransferase, EC 2.3.1.6) in the whole striatum of CBA/J mice are less than in BALB/cJ. The strain-dependent differences in midbrain TyrHase activity are due to variations in the number of dopamine neurons and directly correlate with differences in the number of striatal cholinergic neurons. There is genetic control of the number of neurons of a neurochemically specific class in the mammalian brain.

It is now established that the content of several neurotransmitters or the activities of enzymes that subserve their biosynthesis may vary within the brains of different strains of mammals (1–11). The neurochemical differences which, in brain, may be regionally selective (4, 6–10) are presumably under genetic control (5, 9). The genetic variations in the content or biosynthetic capacity of central neurotransmitters have been of particular interest to behavioral biologists. They raise the possibility that regional variations in transmitter function may represent the neurochemical basis for genetically controlled differences in behavior (11), responses to psychoactive drugs, and, in man, vulnerability to some emotional diseases.

At present little is known of the cellular and biochemical mechanisms that may account for the strain-dependent variations in the content of the transmitters or their biosynthetic enzymes. Several years ago this laboratory sought to establish the mechanism that accounts for the differences in the activity of the catecholamine biosynthetic enzyme, tyrosine hydroxylase [TyrHase; tyrosine-3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2], observed in the whole brain of different inbred strains of mice (2). In an initial investigation we found that the differences in brain TyrHase activity between two inbred mouse strains, BALB/cJ and CBA/J, were restricted to regions

containing cell bodies or terminals of dopaminergic but not noradrenergic neurons and reflected differences in the number of TyrHase enzyme molecules (7, 10). By sampling the number of midbrain neurons in which TyrHase could be detected immunocytochemically, it appeared that variations in TyrHase activity could be attributed to differences in the number of neurons in which the enzyme was contained. The study suggested a genetic control over the number of neurons of a particular neurochemical class in brain.

In the present study we have attempted, by an extensive morphometric analysis of the major TyrHase containing systems in the brain—those of the ventral midbrain, including dopamine neurons of the A8–A10 groups (12–14)—to determine whether the strain-dependent difference in the number of TyrHase-containing cells is (a) uniformly distributed throughout the midbrain and (b) reflected in differences in the neuronal organization and neurochemical composition of a target area to which these cells project, the striatum.

METHODS

Animals. Six-week-old mice of the BALB/cJ and CBA/J strains were purchased from The Jackson Laboratory (Bar Harbor, ME). Upon arrival they were housed five per cage under a 12-hr light/dark cycle in a thermostatically controlled room with water and laboratory chow available ad libitum.

Biochemical Assays. Mice were sacrificed by decapitation, and the brains were removed over ice. The entire midbrain containing cell bodies of dopaminergic neurons of the A8, A9 (substantia nigra), and A10 groups (12–14) comprising the ventral mesencephalic dopaminergic system was removed *en bloc*. To assay TyrHase activity in selected portions of the midbrain, the whole brain was frozen on dry ice and 400- μ m-thick sections through the middle of the midbrain were cut on a cryostat microtome (15). The sections were placed on a glass plate on dry ice. Under a dissecting microscope, tissue was removed with a 0.5-mm micropunch (16) from either the lateral (A9) or medial (A10) midbrain and assayed immediately.

Tissues were homogenized in 10–40 vol of 5 mM potassium phosphate buffer (pH 6.5) containing 0.2% Triton X-100 and centrifuged at 6000 $\times g$ for 10 min. All enzymes were assayed in aliquots of the supernatant except micropunches which were homogenized in 100 μ l of buffer and assayed as aliquots of homogenate. In these samples, protein determinations were performed by the method of Lowry *et al.* (17) on 25- μ l aliquots of homogenate.

TyrHase activity was measured by a modification (18) of the method of Coyle (19). The final concentration of tyrosine in the reaction mixture was 0.2 mM for the substantia nigra and stri-

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Abbreviations: TyrHase, tyrosine hydroxylase; ChoAcTase, choline acetyltransferase.

atum. Choline acetyltransferase (ChoAcTase; acetyl-CoA: choline-O-acetyltransferase, EC 2.3.1.6) activity was measured by an adaptation of the method of Schrier and Shuster (20) in which the final concentration of acetyl-CoA in the reaction mixture was 0.2 mM.

Histochemical Procedures. Mice were anesthetized with 0.2 ml of Equithesin (Jensen-Salsbery Laboratories, Kansas City, MO) and perfused through the heart for 5–7 min with 50–75 ml of a 4% buffered formaldehyde solution (pH 7.2). The brains were removed and postfixed for 30 min in picric acid/formalin, washed overnight in phosphate-buffered saline (pH 7.2), dehydrated in graded alcohols, and embedded in paraffin. The brains were serially sectioned at 10 μ m in the coronal plane. Sections through the head and body of the striatum were stained with cresyl violet. The remaining sections, from the mammillary bodies to the posterior part of the superior vestibular nucleus, were saved for immunocytochemical study. Linear shrinkage of brain was less than 8% and equal for both strains.

Sections through the region of the midbrain, which incorporated the entire extent of neurons of the A8, A9, and A10 groups, were stained with specific antibodies prepared against bovine adrenal TyrHase. The procedure for preparation of antibodies to TyrHase and the criteria used to judge its specificity have been described (21). These antibodies reacted with equal affinity against TyrHase in the brains of mice of either the BALB/cJ or CBA/J strain when examined by gel diffusion or immunoelectrophoresis and equally inhibited TyrHase activity in homogenates of brains from both strains (unpublished data).

Tissues were stained immunocytochemically by the soluble peroxidase-antiperoxidase (PAP) method of Sternberger (22) as described in detail elsewhere (23).

Morphometry. Number of neurons containing TyrHase. Coronal sections stained immunocytochemically for TyrHase were sampled at intervals of 50–70 μ m. All stained neurons were examined but only those containing a clearly delineated nucleolus and cytoplasm stained for TyrHase were counted. Spurious counts, resulting from split nucleoli, were corrected

for by the method of Abercrombie (24) using the formula $N = n[t/(t + d)]$ in which N is the actual number of nucleoli, n is the number of nucleoli counted, t is the section thickness, and d is the diameter of the nucleolus (and equal to 5).

The number of neurons in the A9/A10 area was then calculated by using the formula of Königsmark (25): $N_t = N_s \cdot (S_t/S_s)$ in which N_s is the number of neurons counted, S_t is the total number of sections in the brain region, and S_s is the number of sections sampled.

Size and neuronal density of the striatum. Every 10th cresyl violet-stained striatal section (i.e., one section in 100 μ m) was examined by projecting it onto white paper at $\times 40$ magnification. The area of the striatum in each section was measured planimetrically and expressed in mm^2 . The volume of each 100 μ m of the striatum was calculated by multiplying the area in single 10- μ m samples by 10. Summing the volumes for each 100- μ m block provided an approximation of the volume (in mm^3) of the head and body of the striatum.

The density of neurons in the striatum was determined in the same sections in which the size of the nucleus was estimated. To control for regional variation in neuronal packing, cells were counted at five comparable locations in the striatum in both strains. At each of the five levels, a random field was examined. All neurons $< 18 \mu$ m in their longest dimension and falling within the confines of an eyepiece reticule (viewed at $\times 500$) were counted. A correction factor for cell fragmentation was applied in the same manner as described above for split nucleoli. At least 1000 neurons were counted in each animal. Because the area under the reticule and the section thickness were known, the average number of neurons per mm^3 of striatum

Table 1. Neuronal number and enzyme activity in ventral midbrain of two mouse strains

	BALB/cJ	CBA/J	CBA/ BALB	P
Ventral midbrain				
TyrHase activity, nmol DOPA/ hr/VM	2.13 \pm 0.08 (20)	1.66 \pm 0.04 (19)	0.78	<0.001
No. of neurons	7849 \pm 487 (6)	6223 \pm 151 (5)	0.79	<0.05
TyrHase activity/ neuron, (nmol DOPA/hr/ neuron) $\times 10^4$	2.71	2.67	0.98	NS
A9 area				
TyrHase activity, nmol DOPA/ hr/mg protein	6.28 \pm 0.89 (6)	3.60 \pm 0.47 (6)	0.57	<0.05
A10 area				
TyrHase activity, nmol DOPA/ hr/mg protein	17.76 \pm 1.89 (5)	9.96 \pm 1.01 (6)	0.56	<0.01

Results are shown as mean \pm SEM; number of animals given in parentheses.

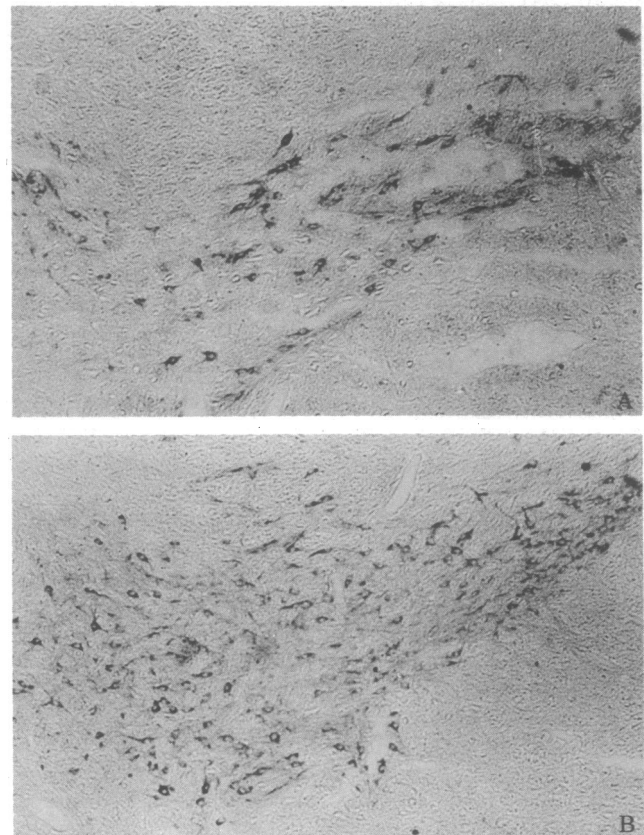


FIG. 1. Immunohistochemical localization of TyrHase-containing neurons in the same region of the ventral tegmentum of CBA/J strain (A) and BALB/cJ strain (B), demonstrating the lower density of neurons in the former. Sections are in the coronal plane at the most anterior pole of the interpeduncular nucleus; left is medial. ($\times 70$.)

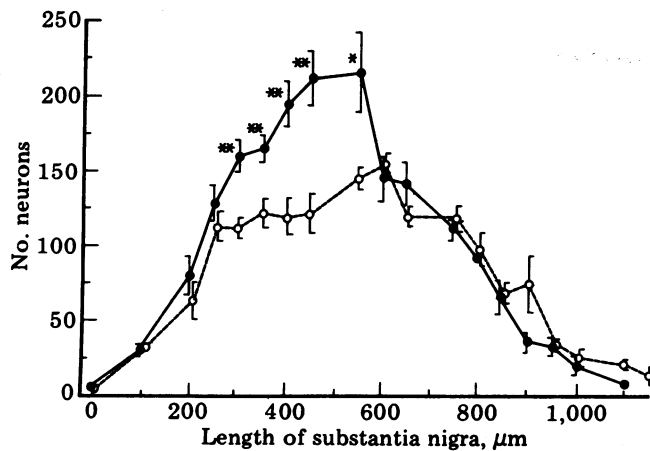


FIG. 2. Distribution of TyrHase containing neurons in the ventral tegmentum, demonstrating that strain-dependent differences in the number of dopamine neurons are topographically restricted to the region 200–600 μm from the most rostral section (0 μm) containing stained neurons. Results are shown as mean \pm SEM. ●, BALB/cJ; ○, CBA/J. * $P < 0.05$; ** $P < 0.01$.

could be calculated for each animal by multiplying the number of neurons/ mm^2 by the section's thickness to generate a packing density (expressed as neurons/ mm^3). The total number of neurons in the striatum of each mouse was calculated by multiplying the neuronal density (neurons/ mm^3) for that animal by the total volume of its striatum (mm^3).

RESULTS

TyrHase Activity and Regional Distribution of Dopamine Neurons in Ventral Midbrain. TyrHase activity in the mid-brain differed significantly between mice of the CBA/J and BALB/cJ strains (Table 1). TyrHase activity in CBA/J mice was approximately 80% that of BALB/cJ mice.

The strain-dependent differences of TyrHase activity within the whole midbrain was paralleled by a comparable variation in the total number of neurons in which the enzyme could be detected immunocytochemically. These neurons correspond in their distribution to those of the ventral dopaminergic systems of mesencephalon including the A10, nigrostriatal (A9), and caudal A8 groups (12–14). The differences in density of the TH-containing neurons in the midbrain of BALB/cJ and CBA/J mice were obvious qualitatively by inspection of comparable sections through the middle of this region (Fig. 1). Quantitatively, mice of CBA/J strain had 20% fewer neurons containing TyrHase than did BALB/cJ mice (Table 1). However, the calculated TyrHase activity per neuron did not differ between strains.

The difference in numbers of TyrHase neurons between strains was not uniformly distributed throughout the midbrain but varied with respect to the rostro-caudal gradient (Fig. 2). The differences were only present in the more rostral portion

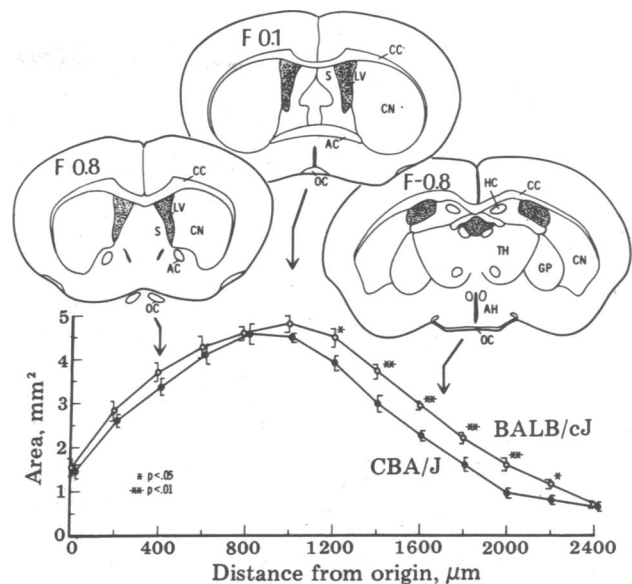


FIG. 3. Cross-sectional areas of the striatum of BALB/cJ and CBA/J mice, demonstrating that the volume difference was restricted to the posterior head and the body of the striatum. Measurements commenced where the striatum could first be visualized in the cresyl violet-stained sections. The F values for the representative sections were taken from the stereotaxic atlas of Slotnick and Leonard (15). AC, anterior commissure; AH, anterior hypothalamus; CC, corpus callosum; CN, striatum; GP, globus pallidus; HC, hippocampus; LV, lateral ventricle; OC, optic chiasm; S, septum; TH, thalamus. Results are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$.

of the midbrain with CBA/J mice having approximately 60% of the number of neurons found in the same region in BALB/cJ mice. Both A9 and A10 regions contributed to the observed strain differences in the number of midbrain neurons. The regional difference in numbers of TyrHase-containing neurons in this portion of the midbrain was paralleled by differences of a comparable magnitude in the activity of TyrHase assayed in a small punch sample through the region (Table 1). TyrHase activity in CBA/J mice in either region was approximately 57–58% of BALB/cJ, a value paralleling the differences in cell numbers.

Size, Neuronal Density, and Enzyme Activity in Striatum. The strain-dependent differences in the number of dopaminergic neurons in the midbrain were paralleled by differences in the size of the striatum (Table 2). The volume of the whole striatum was smaller in CBA/J mice than in BALB/cJ mice. The difference in striatal size was topographically selective and restricted to the caudal half of the body of the nucleus (Fig. 3). Here the differences were of a larger magnitude than for the whole striatum.

On the other hand, the packing density of neurons—i.e., the number of neurons per unit volume within the striatum—did not differ within any region (Table 1). Thus, the total number of neurons per striatum was less in CBA/J mice than in BALB/cJ mice.

Table 2. Volume, neuronal number, and enzyme activity in striatum of two mouse strains

	BALB/cJ	CBA/J	CBA/BALB	P
Volume, mm^3	7.86 \pm 0.26 (6)	6.61 \pm 0.28 (6)	0.84	<0.05
Neuronal density, no./ mm^3	90,593 \pm 5,901 (6)	89,722 \pm 5,164 (6)	0.99	NS
Total no. neurons	710,409 \pm 40,789 (5)	598,392 \pm 21,264 (5)	0.84	<0.05
TyrHase/g, nmol DOPA/hr/g	734.4 \pm 25 (26)	601.9 \pm 3 (26)	0.82	<0.001
TyrHase/striatum, nmol DOPA/hr	5800 \pm 200 (26)	4000 \pm 120 (26)	0.69	—
ChoAcTase/g, nmol acetylcholine/hr/g S	17.6 \pm 0.92 (26)	18.03 \pm 0.96 (22)	1.02	NS
ChoAcTase/striatum, nmol acetylcholine/hr	138.3 \pm 7.23 (26)	119.2 \pm 6.34 (22)	0.86	—

Results are shown as mean \pm SEM; number of animals given in parentheses.

The activities of TyrHase and of ChoAcTase, a marker of cholinergic neurons, were measured in the striatum of both strains. The results were expressed (Table 2) per unit of striatum (i.e., TyrHase activity/g of striatum) and as enzyme activity per striatum. Enzyme activity per unit weight is a reflection of enzyme activity with respect to neuronal density; activity per total volume is a measure of enzyme activity for the total number of neurons.

TyrHase activity was greater in BALB/cJ than in CBA/J mice when expressed both ways (Table 2). ChoAcTase activity, on the other hand, did not differ between the two strains when expressed per unit weight; however, it was greater in the whole striatum of BALB/cJ mice.

DISCUSSION

The present study confirms and extends our earlier demonstration (7, 10) that the activity of the catecholamine-synthesizing enzyme TyrHase differs in the midbrain of two inbred strains of mice. TyrHase activity is approximately 20% less in CBA/J mice than in BALB/cJ mice. The difference in enzyme activity, attributable to variations in the amount of enzyme protein (7), is paralleled by variations in the number of neurons in which the enzyme can be detected immunocytochemically. The distribution of the TyrHase-containing neurons corresponds exactly with that of the dopaminergic systems of the ventral midbrain (12–14).

By detailed morphometric analysis in which neurons containing the enzyme were counted along the rostral-caudal axis of the midbrain, we have discovered that the *total* number of stained neurons in the region, like enzyme activity, is lower in CBA/J mice. The strain-dependent variation in the numbers of dopamine neurons in the ventral midbrain, however, is not uniformly distributed. Rather, it is only detectable in the rostral portion of the middle third of the midbrain, equally involving medial and lateral portions of the mesencephalic dopamine systems. This finding indicates that there is a topographic organization with respect to the variability of cell numbers.

Despite the regional variation in the differences in the number of TyrHase-containing cells, there is extremely close correspondence between the number of such neurons and enzyme activity, as assessed either in the whole midbrain or in the regions in which the strain differences are greatest. Indeed, the close relationship between enzyme activity and cell number is emphasized by the fact that, when the average TyrHase activity per neuron is calculated for the whole midbrain, in each strain the values are identical. Because, on the average, midbrain dopamine neurons of both strains appear to contain comparable amounts of enzyme protein, the mechanism accounting for the strain-dependent differences of midbrain TyrHase activity between BALB/cJ and CBA/J mice must represent a difference in the number of dopaminergic neurons.

The cellular mechanism(s) that accounts for the fewer number of TyrHase-containing neurons in the midbrain of CBA/J mice is not known. Three potential mechanisms should be considered. First, differences might represent variations between strains in the antigenicity of the enzyme and, hence, affinity for the specific antibody. This possibility seems most unlikely because: (i) the immunochemical or catalytic properties of TyrHase in the brains of these strains did not differ; (ii) the strain differences in TyrHase activity did not involve all neuronal systems which contain TyrHase (there are no differences in the activity of TyrHase or in the number of cells containing the enzyme in the noradrenergic neurons of the nucleus locus ceruleus (10)); (iii) it seems most improbable that the differences in immunogenetic characteristics of the enzyme

would be restricted to a subpopulation of midbrain dopaminergic cells that cannot be distinguished from each by any obvious neuroanatomical or morphological criteria.

A second possibility is that some midbrain dopaminergic neurons of CBA/J mice either produce too little TyrHase enzyme protein to be detectable immunocytochemically or biochemically or, in fact, fail to express the enzyme at all. This possibility, difficult to prove, seems improbable because it also implies the existence of a subpopulation of dopaminergic neurons differing only in their capacity to express TyrHase.

The third possibility, which we favor, is that there are indeed 20% fewer dopaminergic neurons in the CBA/J than in the BALB/cJ mice. The relative reduction of these dopaminergic neurons could be due either to fewer of these neurons being produced during the prenatal period in which they differentiate or that, once formed, fewer survive.

This study has demonstrated that the variations in number of dopaminergic neurons in the brainstem of the two mouse strains do not exist in isolation but are associated with differences in the organization of one brain region to which some of these neurons project: the striatum. In numerous species the striatum is heavily innervated by dopaminergic neurons of the lateral midbrain group (the A9 group) via the nigrostriatal tract (12–14). The smaller number of dopaminergic neurons in CBA/J mice was associated with a proportionately smaller volume of the head and body of the striatum compared with the same regions in BALB/cJ mice. This difference in striatal size is restricted to its caudal half. Because the packing density of neurons did not differ in any portion of the striatum, the difference in striatal volume must reflect variations in the total number of neurons, largely distributed within the caudal half.

The fact that the nigrostriatal projection upon the striatum is topographically organized (14) raises the question of whether there is a topographic relationship between the striatal and mesencephalic areas in which strain-dependent differences in cell number are concentrated in mouse. It also raises the important question of whether the relationship is casual—i.e., variation in target organ neural number dictates survival of innervating neurons or vice versa. If one extrapolates from the data in rat, there appears to be, in general, a correspondence between the two regions where cell differences are concentrated. However, intimate morphologic relationships cannot be proved without a detailed neuroanatomical analysis using retrograde and orthograde tracer techniques.

The finding that TyrHase activity is greater in the striatum of BALB/cJ mice than in CBA/J mice when expressed either by unit weight or for the total striatum (26) strongly suggests that the striatal neurons are much more densely innervated by dopaminergic terminals in the BALB/cJ mice. The fact that ChoAcTase activity, a biochemical marker of the primarily intrinsic cholinergic neurons and their processes (27, 28), did not differ between the two strains when expressed per unit weight but was greater in the BALB/cJ mice for the whole striatum would be consistent with the interpretation that some of the more plentiful neurons in the striatum of that strain are cholinergic.

The demonstration of variations in the number of midbrain dopaminergic neurons regionally in the brain of two inbred strains poses the question of whether variations in numbers of neurons of a particular neurochemical class is a generalized mechanism which might underly the genetically determined differences in the content of other classes of neurotransmitters or their biosynthetic enzymes in mammalian brains (11). It also raises the issue as to whether variations in the number of neurons containing a specific neurotransmitter will be reflected in

differences in behavior, either drug-induced or spontaneous, whose expression depends upon the integrity of these systems. That indeed such may be the case is suggested by our recent evidence that BALB/cJ mice more fully express behaviors that depend upon the release of dopamine upon postsynaptic receptors (10).

Finally, questions are posed as to the relevance of these findings to man. Could variations of the number of dopaminergic neurons represent the genetic element of diseases such as schizophrenia or manic-depressive illness in which catecholamine neurons are believed to be involved (29)? Could variations in cell number provide a margin of safety with respect to expression of symptoms in diseases in which a particular neurochemical class of neurons degenerate (30) (e.g., Parkinson disease)? Could the subtle individual variations in behaviors and responsivity to drugs be explained by differences in cell number?

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